Osmoadaptation Strategies in Halophilic Bacteria

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Date: April 2023

DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation entitled, **"Osmoadaptation Strategies in Halophilic Bactria"** is based on the results of investigations carried out by me in the Marine Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision 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 or experimental or otherfindings given the dissertation.

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This is to certify that the dissertation **"Osmoadaptation Strategies in Halophilic Bactria"** is a bonafide work carried out by **Ms. Mayuri Brijbhushan Nirmala Singh** under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of Master's in the Discipline Marine Biotechnology at the School of Biological Sciences and Biotechnology, Goa University.

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Abbreviations

°C	Degree Celsius
mg	Microgram
g	Gram
µl	Microlitre
ml	Millilire
µM	Micromolar
mM	Millimolar
M	Molar
nm	Nanometer
NA	Nutrient Agar
NB	Nutrient Broth
No.	Number
NaCl	Sodium Chloride
%	Percentage
rpm	Rotation per Minute
NBT	Nitrobluetetrazolium
NADH	Nicotinamide Adenine Dinucleotide Hydrogen
DPPH	2,2-diphenyl-2-picryl hydrazyl
PMS	Phenazine Methosulphate
O.D.	Optical Density
PBS	Phosphate Buffer Saline
TE	Tris-EDTA Buffer
NMR	Nuclear Magnetic Resonance
ROS	Reactive Oxygen Species
EPS	Exopolysaccharide

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CHAPTER 01:

INTRODUCTION

From micrometers to several meters, there are many different types of living forms in the world. The bacterial communities exhibit the capacity to thrive in a variety of environments Extremophiles are organisms that live under constant environmental conditions that can be termed extreme in relation to the physicochemical aspects of the environment that human cells normally experience. The following table summarizes the classification of extremophiles:

EXTREMOPHILES	PARAMETERS	OPTIMUM
Sacchrophiles	High sugar content	6-92 ng/ul
Halophiles	High salt content	2-5 M NaCl
Thermophiles	Hot springs	Between 60-80°C
Psychrophiles	Cold environment	15°C or lower
Barophiles	extreme pressure	>0.1M Pa
Acidophilic	Acidic pH	pH 1-5
Alkaliphilic	Basic pH	pH above 9
Xerophilic	Dry conditions	Low water
Radiophilic	High radiation	-

Table No. 01: Extremophilic organisms with their optimum requirements (Gupta et al., 2014).

Bacteria and archaeal species that have modified cellular machinery to reside in surroundings with high salt content are termed halophiles. Hence, they were given the identities halobacteria and haloarchaea, respectively. Halophilic bacteria strictly require salt for growth, 1.5 to 5 M, whereas halotolerant bacteria survive with or without salt. The concentration of salt is a significant consideration used to classify halophilic bacteria (Shivanand & Mugeraya, 2011).

A bacterial membrane is made up of lipids that are permeable to water and some hydrophobic molecules. Due to this, in a high salt environment, the bacterium must maintain iso-osmotic conditions. Halophilic bacterium exhibits two distinct mechanisms to maintain slight hyperosmotic behavior of a cell i.e, “salt in cytoplasm" and “accumulation of compatible solutes” (Zajc et al., 2014).

In salt in cytoplasm strategies, organisms keep a low concentration of Na^+ while a high concentration of K^+ and Cl^- inside a cell. (Oren, 2002). In the second mechanism, bacteria accumulate numerous organic solutes such as polyols, sugars, and amino acids to eliminate salt and maintain osmotic balance (Motta et al., 2004).

	Halophilic bacteria	**Halotolerant Bacteria**
Concentration of salt	1.5 to 5 M NaCl	0 to < 1 M NaCl
Ecosystem	Salty mud, salty soil, salty food	Salt marshes, salt springs, coastal dunes
Survival mechanism	Salt in strategies	Accumulation of compatible solutes
Transporters	K+ uptake uniporter, Na+/H+ antiporter	K+(Na+) efflux antiporter, H+ exporter P-type ATPase
examples	Slight halophilic: - *Erythrobacter flavus* Moderate halophilic: - H*alomonas*, *Chromohalobacter*, Extreme halophilic: - *Thermus thermophiles*, *Ruberobacter radiotolerans*	*Halobacterium salinarum*, *Debaryomyces hansenii*, *Halococcus hamelinensis*

Table No. 02: Comparative study of halophilic and halotolerant bacteria (Gunde-Cimerman et al., 2018).

CHAPTER 02:

AIM AND OBJECTIVES

Aim and Objective:

Aim:

To explore osmoadaptation measures and survival mechanisms in halophilic bacteria.

Objective:

1. To study salt's impact on the growth of halophilic bacteria.
2. To examine the possible effect of salt on the pigmentation of the isolate.
3. To study the osmoadaptation mechanism of halophilic bacteria.

CHAPTER 03:

REVIEW OF LITERATURE

All over the planet, there are many unique species of life. These living forms are categorized as bacteria, archaea, and eukaryotes first. Both bacteria and archaea are single-celled microscopic organisms and correspond to the prokaryote group. Prokaryotes can withstand a wide range of environmental factors, such as those attributed to nutrition, temperature, vacuum, pressure, salinity, pH, and radiation. The characterization of organisms as thermophiles, barophiles, halophiles, alkalophilic/acidophiles, radiophiles, etc., is derived from this requirement.

We can witness drastic changes in the environment, such as an increase in temperature, salinity, and pH of seawater, as a consequence of global warming and anthropogenic factors. Extremophiles are microbes that can adapt to this environment. Halophilic organisms that are specially adapted to a hypersaline environment are one of them (Thombre et al., 2016).

3.1 Extreme environment:

Evolution has caused drastic changes in environmental factors over the last few decades. Temperatures in the earth's crust or deep oceans, extreme acidic/basic conditions of hydrothermal vents, frozen seas, and extreme pressure are all examples of extreme conditions for humans. Extremophiles are microorganisms that can survive in this environment. Archaea and cyanobacteria are the most adapted prokaryotes to extreme environments. Extremophiles are classified as thermophiles for their ability to withstand high temperatures, halophiles for their affinity for salt, barophiles for their ability to withstand extreme pressure, and many more (Rampelotto, 2013).

3.2 Hypersaline Environment:

The primary cause of a hypersaline environment is the evaporation-induced gradient of salinity in seawater. In this type of scenario, halophiles necessitate a salt concentration of 0.5 to 1.5 M, while halotolerant individuals can survive without salt. (Larsen, 1986) Based on ionic concentration, the hypersaline environments are split into two categories: thalassohaline (ionic concentrationcomparable to that of the sea) and athalasohaline (ionic concentration greatly varies from seawater) (Cui & Dyall-Smith, 2021). A hypersaline lake is developed when sea seepage occurs occasionally through small inlets. The two hypersaline lakes that have been the subject of the most study are the Dead Sea in the Middle East and the Great Salt Lake in Western America. The dead sea has a high concentration of magnesium ions, whereas the Great Salt Lake exhibits an ionic concentration similar to seawater (Kerkar, n.d.).

Halophilic organisms survive in high salt concentrations. There are three types of halophiles according to salt concentration for their growth, slight halophiles (0.2-0.85 M or 1-5 % NaCl), moderate halophiles (0.85-3.4 M or 5-20% NaCl), and extreme halophiles (3.4-5.1 M or 20-30% NaCl). Halophiles show adaptation to high salinity, low oxygen level, high temperature, and alkaline pH and for this, it applies two specific mechanisms i.e., “accumulation of compatible solutes” and “salt-in strategy” (Gregory & Boyd, 2021).

In the case of compatible solute halophilic organisms accumulate small and highly soluble amino acids like glycine, betaine, glutamine, ectoine,hydroxyectoine, glutamate, and proline, polyols like glycerol, sorbitol, and mannitol, sugars like sucrose and trehalose. (Empadinhas & Da Costa, 2008) In the case of salt-in strategies, cells will absorb a high percentage of KCl to maintain an isotonic

environment for the bacterial cell. Moderate halophiles like *Proteobacteria and Firmicutes* use compatible solutes for their survival in high salt conditions. While in extreme halophiles like *Thermus thermophiles, Ruberobacter radiotolerans* uses salt-in strategies to withstand high osmotic stress. *Thermus thermophilus, Ruberobacter radiotolerans* also contains carotenoids which help in membrane stabilization (Salma et al.,2020.).

3.3 Compatible solutes as osmolyte:

To maintain the stability of the protein's hydration shell, compatible solutes are efficient water structure formers that are probably kept away from the protein. A minimum iso-osmotic interaction between the extracellular environment and the intracellular environment is therefore required. Compared to organisms that employ the "salt-in" method, microorganisms that use organic solutes are often more adaptable and can more easily readjust to dilution stress or significant changes in salinity (Gunde-Cimerman et al., 2018).

The accumulation of compatible solutes is also known as the salt-out strategy, and it is a very energetic process. The external salinity determines the sizeof the cytoplasmic pool for compatible solutes, which can accumulate to extremely high intracellular levels. Organic molecules such as e trehalose, proline, glycine betaine, ectoine/5-hydroxyectoine, glucosylglycerol, and dimethylsulfoniopropionate are all compatible solutes (León et al., 2018).

3.3.1. Amino acid as compatible solute:

1. Ectoine:

Halophiles and Halotolerant bacteria consist of Ectoine (1,4,5,6-tetrahydro-2-mthyl-4-pyrimidincarboxylic acid) to maintain osmotic balance. Ectoine was first discovered in *Ectothiorhodospira halochloris,* a Sulphur reducing haloalkaliphile. Ectoine molecule expression occurs by gene ect ABC, which forms enzymes Ect ABC present in all halophilic organisms (Shivanand & Mugeraya, 2011).

Ectoine is produced from the natural amino acid aspartate, which is then changed into aspartylposphate by the enzyme aspartokinase. Later, L-aspartate-semialdehyde is replaced by aspartate-β-semialdehyde dehydrogenase. Then L-aspartate-β-semialdehyde is converted into 2,4-diaminobutyrate by enzyme L-diaminobutyric acid transaminase form by Ect B, then it is converted into Y - acetyl-2,4-diaminobutyrate by enzyme l-diaminobutyric acid acetyl transferase coded Ect A, finally, it is converted into ectoine by enzyme Ect C formed by, ectoine synthase (Crowley, 2017) (Peters et al., 2006).

2. Hydroxyectoine:

With the aid of the enzyme ectoine hydroxylase, which is encoded by the gene ect D (thpD), a solute can be produced from ectoine. The conversion of N-acetyldiaminobutyric acid to hydroxyectoine is a different process. The temperature has a significant impact on enzyme activity, which is why it is also evident in mutant conditions. When an organism is cultured in a solution of 0.5 to 2.5 M NaCl, it produces a buildup of hydroxyectoine that rises in the exponential part of the growth curve before stabilizing in the stationary phase. Using the high-performance liquid chromatography technology, the concentration of hydroxyectoine in the stationary

phase was calculated to be 4.4 mol mg of protein (García-Estepa et al., 2006).

3. Betaine:

Betaine is an osmolyte found in various halotolerant and moderately halophilic organisms. It is also defined as glycine betaine (N, N, N, -trimethyl glycine) (Shivanand & Mugeraya, 2011).

Glycine acts as a precursor for betaine production inside a cell. Glycine is converted into sarcosine by GNMT (glycine N-methyltransferase) and later it changes into dimethylglycine by GSMT (glycine sarcosine N-methyltransferase). After this final product glycine betaine is formed by SDMT (sarcosine dimethylglycine N-methyltransferase). Detection of betaine biosynthesis's observed using liquid chromatography-mass spectroscopy and it is found thatbetaine gave 118 molecular weights with a retention time of 2.8-2.9 min. Confirmation of betaine presence in an organism is observed using the detection of genes Mpgsmt and Mpsdmt (Lai et al., 2014).

The EMP pathway converts glucose, a carbon source in prokaryotes, into pyruvate. 3-phosphoglycerate dehydrogenase in this pathway converts 3-phosphoglycerate, one of the intermediate product of glucose metabolism, into serine. The enzymes phophotidylserine decarboxylase and phosphoethanolamine N-methyltransferase then converts serine to choline via ethanolamine. Finally, using the enzyme choline dehydrogenase, choline is converted into betaine aldehyde, and betaine is formed using aldehyde dehydrogenase (Yang et al., 2022).

4. Glutamate/Glutamine:

For the conversion of 2-oxogluterate to glutamate throughout the biosynthesis process, glutamate dehydrogenase is used. Similar in that glutamate synthase is engaged in converting glutamine and 2-oxogluterate into glutamate. The ability of glutamate synthetase to reversibly convert glutamine to gamma-glutamyl hydroxamate is demonstrated. For halotolerant and halophilic bacteria to survive under salt stress conditions, all three enzymes exhibit significant enzyme activity when chloride ions are present (Saum et al., 2006).

5. Proline:

Proline, a naturally occurring amino acid, also functions as a suitable solute at high salinity. Proline is substituted for glutamate/glutamine and the salt concentration is raised for osmoregulation. Proline is biosynthesized by bacteria using enzymes encoded by three nearby genes as an organic solute (Saum & Müller, 2007). With the aid of the enzyme pyrroline-5-carboxylate dehydrogenase, glutamine is first transformed into pyrroline-5-carboxylate, which is subsequently transformed into the ultimate product, L-proline, by the enzyme proline dehydrogenase (Trovato et al., 2008).

3.3.2. Sugars as compatible solute:

1. Glucosylgycerol:

The principle compatible solute in cyanobacteria and purple-non-sulfur bacteria, [2-O-(a-D-glucopyranosyl)-glycerol (glucosylglycerol), is synthesized by several *Pseudomonas* species.

The enzyme glucosylglycerol-3-phosphatase catalyzes the conversion of ADP-glucose and glycerol-3-phosphate into intermediate glucosylglycerol-3-phospate for glucosylglycerol synthesis (Mikkat\ et al., 2000).

The accumulation of glucosyl glycerol is the sole cause of an organisms' high salt tolerance. In conditions of salt stress, glucosyl glycerol inhibits cell division in addition to serving as osmolytes. According to the research, there's a chance that glucosyl glycerol synthesis and accumulation will be inhibited by glycine betaine accumulation (Roder et al., 2005).

2. Trehalose

With an increase in salinity, trehalose builds up in bacterial cells. But this ability is limited to only halotolerant species as in strict halophiles due to high salinity, high subcellular trehalose causes enzymatic inhibitions. The low growth rate is further a result of that (Welsh et al., 2006).

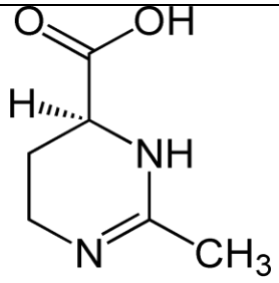
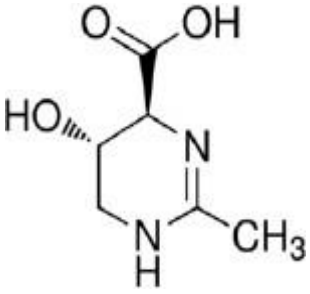
OtsA and OtsB, which are activated by high osmolarity and encode the enzymes trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase respectively, are essential for trehalose production. Both genes are most actively transcribed at moderate salinity. This leads to the reporting of osmoregulated gene expression (Hengge-Aronis et al., 1991).

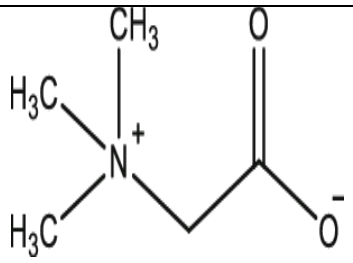
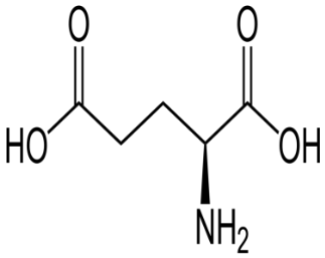
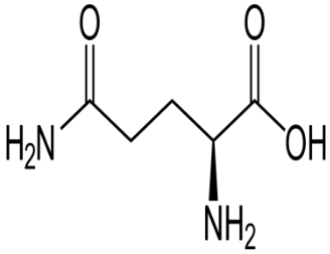
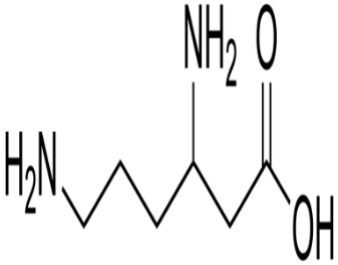
(Welsh et al., 2006) discovered that in the absence of glycine betaine, trehalose acts as an intracellular organic osmolyte. Trehalose keeps the osmolarity of the cell constant and is measured using the Anthrone reagent. Cells were lysed, and intracellular components were removed. After that, the extract is combined with anthrone reagent made with concentrated H_2SO_4. After incubation, absorbance measurements are made for each reaction at 630 nm. A standard calibration plot made of trehalose ranging from 0 to 50 μg/ml was used to determine the trehalose

concentration.

3. Sucrose:

Sucrose is one of the main compatible solutes used by cyanobacteria in low-salt environments. (Kirsch et al., 2019) The enzyme that controls sucrose production is designated Sps. NaCl stimulates sps activity. Sps gene transcript is involved in the generation of sucrose from D-fructose 6-phosphate and uridine diphosphate glucose (UDP-glucose) (Huber & Huber, 1992).

Classification	Compatible solutes	Structure	Organism
Zwitter ionic solutes	Ectoine		*Halomonas elongate; Halomonas variabilis; Actinopolyspora sp.*
	Hydroxyectoine		*Halomonas elongata; Nocardiopsis halophila;*

	Glycine Betaine		*Actinopolyspora halophila; Halorhodospira halochloris; Methanohalophilus portulcalensis FDF1*
	Glutamate		*Methanothermoccus thermolithotrophicus; Nocardiopsis halophila*
	Glutamine		*Methanohalophilus portucalensis FDF1*
	β –lysine		*Methanothermoccus thermolithotrophicus; Methanosarcina mazei Gö1*

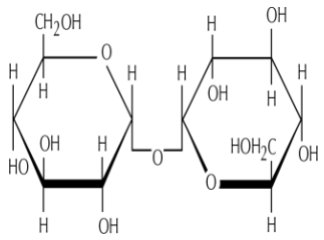
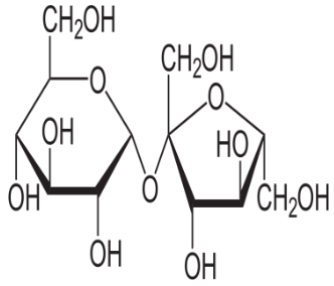
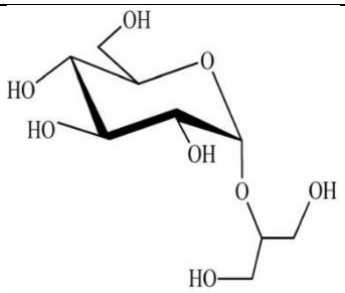
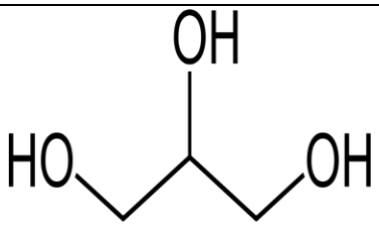
Uncharged solutes	Trehalose		*Desulfovibrio halophilus; Rhodothermus obamensis; Natrialba magadii*
	Sucrose		*cyanobacteria; proteobacteria*
	glycosylglycerol		*Synechococcus; Pseudomonas mendocina; Microcystis firma*
	Glycerol		*Hortaea werneckii; Chlorophyceae: Dunaliella parva; D. salina; D. viridis*

Table No. 2: Types of compatible solutes and possessing organism (*Chapter 2 Review of Literature 2.1 Extremophiles*, n.d.; Roberts, 2005).

Nuclear magnetic resonance spectroscopy with 1H and 13C was used in a study by (Nagata et al.,1996) to analyze a compatible solute. This was accomplished by using 80% ethanol to lyse bacterial strains, and the lysate that resulted was collected. Later, the components of the combination, including all suitable solutes and intracellular materials, were extracted by evaporating the mixture. After being dissolved in milli-Q water, the dissolved pellet is dried. The signals for different aminoacids, including ectoine (EC), glutamate (GLU), glycine betaine (GB), hydroxyproline (HP), TMAH, -aminobutyrate (GABA), trehalose (TRE), and proline (PRO), which also function as compatible solutes, were afterward obtained from fridge-dried contents.

3.4 Salt in cytoplasm strategy:

Na+/K+ ions:

In a hypersaline environment to avoid losing water, many halophilic and halotolerant microorganisms accumulate high concentrations of solutes. The KCl concentration that halobacteria accumulate is equivalent to the external NaCl concentration. As a result of the iso-osmotic equilibrium, the cell volume is conserved (Dassarma & Arora, n.d.).

When the osmolarity of the medium is raised, the turgor pressure on the cell membrane is decreased. As a result, the cells contract and begin to take up potassium. Potassium ion accumulation is combined with glutamic acid accumulation. This establishes a link between positively charged potassium ions and negatively charged glutamic acid. A sufficient intracellular potassium concentration is required for the active transport of different substrates. High intracellular concentrations are required to keep a cell's metabolism runningsmoothly under osmotic stress (Imhoff, 1986).

3.5 Oxidative stress:

Osmotic stress in organisms caused by the salt causes the biomolecules to peroxide, resulting in the formation of reactive oxidative species. Antioxidant enzyme activity arises out of oxidative stress. The protective mechanism against reactive oxygen species is antioxidant capacity. With salinity-induced ROS species, a considerable rise in superoxide scavengers is seen. High salinities are associated with a rise in reactive oxygen species, which indicates reduced antioxidant activity (Zhou et al., 2020).

3.6 Antioxidants:

Pigment-producing bacteria are found in a wide variety of conditions, including soil, salty water, and fermented foods. Because they are both stable and simple to grow, these pigments are of interest for research. (Sricharoen et al., n.d.) The type of bacterium and their incubation time influence pigment production (Vora et al., n.d.).

As a result of constant exposure to external stresses like desiccation, freezing, ultraviolet radiation, and alteration in heavy metals, coastal life forms like seaweeds and the bacteria that live there generate and pile up Reactive Oxygen Species (ROS) over time (Pawar et al., 2015).

In halophilic archaea, the hydrophobic chain namely carotenoids (lycopene and β-carotene) and xanthophylls (canthaxanthin, astaxanthin) differentiate based on the absence and presence of oxygen molecules respectively. This pigment causing molecules are free radical scavengers of both Reactive Oxygen Species and Reactive Nitrogen Species (Waditee-Sirisattha et al., 2016).

Antioxidants are reducing substances that limit oxidative damage to biological structures by giving and passivating free radicals electrons. Free radicals

are intimately related to oxidative damage. Marine invertebrates like sponges produce antioxidants against free radicals-mediated toxicity. In phenazine, methosulphate-nicotinamide adenine dinucleotide (PMS NADH) systems, superoxide radicals were produced by the oxidation of NADH and measured by the reduction of nitrobluetetrazolium (NBT). (Velho-Pereira et al., n.d.) In order to begin the reaction, an equal volume of 0.1 ml of extracted EPS solution, 156 M NBT, and 468 M NADH are added. Antioxidant activity is clearly visible as a result of this increase in concentration. At 90% concentration, the greatest amount of scavenging activity is visible. The reaction is examined for analysis at 560 nm. (Kodali & Sen, 2008) Scavenging activity is identified using the following formula:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

According to research by (Vora et al., n.d.-b), pigments exhibit antioxidant activity, which means that they can reduce DPPH by giving hydrogen or electrons. The consequence is a purple to yellow colour change in the DPPH. For this work, pigment extract is mixed with 0.05mM of DPPH that has been dissolved in methanol. Once the mixture has been incubated for 20 to 60 minutes at room temperature, a spectrophotometer is used to detect the reaction's absorbance at 517 nm. Once absorbance has been determined using the formula below, % scavenging activity is computed.

Scavenging activity (%) = Absorbance of control – Absorbance of test X 100

Absorbance of control

3.7 Effect of high salinity on the pigmentation of bacteria:

Bacterial pigmentation in halophiles varies with shifts in salt content and incubation time. Additionally, pigments have antifungal, antibacterial, and antiprotozoal properties. Pigment aids bacterial growth regardless of relatively low nutrient supply and climatic circumstances (Vora et al., n.d.).

3.8 Application of Compatible Solute:

The majority of halophilic bacteria and eukaryotes build high solute concentrations within the cytoplasm. These osmolytes are typically sugars and polyols, such as sucrose, trehalose, and glycerol, which have no net charge at physiological pH and don't interfere with metabolic activities. Other examples of these osmolytes include amino acids, such as glycine-betaine and ectoine. Additionally, all suitable substances demonstrate a variety of industrial uses that are beneficial to humanity (P. Dassarma et al., 2010). All of the applications are listed below:

- Ectoine is used in various cosmetic products and also as a stabiliser in a polymerase chain reaction.
- Glycine betaine acts as a feed additive, anti-inflammatory drug, and anti-coagulant.

- Proline is used in medicines for skin healing.
- Glutamine is used as food additive and enhancing flavor of foods.
- Trehalose is widely used in biopharmaceutical, food, and cosmetic industries. It is also used as cryoprotectant.
- Acetylcholine is used as blood pressure regulator.

The literature review mentioned above explains the numerous mechanisms by which halophilic and halotolerant microorganisms adapt to different salinities. To maintain osmolarity, these bacteria primarily take a variety of non-ionic solutes. Due to the fact that these solutes are intracellular components and accumulate within the confines of the minimal nutritional requirement, this field of study has emerged. Additionally, this solution offers a number of industrial, medicinal, and culinary enhancement applications. Consequently, here research interests are on isolating and growing organisms that produce a variety of compatible solutes.

CHAPTER 04:

MATERIALS AND METHODS

4.1 Growth condition of bacterial isolate:

According to previous research, the isolate PSDM 20 was grown on Nutrient Agar (NA) (Himedia, Mumbai, India) supplemented with 8% NaCl. Sub-cultured and purified isolated colonies were used in subsequent experiments.

4.2 Preparation of bacterial cell pellet:

An isolated colony from a Nutrient Agar plate was inoculated in 50 ml of Nutrient broth supplemented with 8% NaCl, then incubated at 28°C for 48 hours at 120 rpm to prepare the inoculum.

One percent of the inoculum was inoculated into Nutrient broth supplemented withfive different NaCl concentrations (0.5%, 2%, 8%, 12%, and 16% NaCl) and one without NaCl incubated at 28°C for 48 hours at 120 rpm. The cell pellet was harvested by centrifugation at 10,000 rpm for 10 minutes. The cell pellet was washed with a respective concentration of NaCl solution before being used in further experiments.

4.3 Dry weight determination of bacterial culture:

One percent of the inoculum was added to nutrient broth supplemented with five different concentrations of sodium chloride (0.5%, 2%, 8%, 12%, and 16% NaCl) and one without NaCl, and incubated at 28°C for 48 hours at 120 rpm. The cell pellet was obtained by centrifugation at 10,000 rpm for 10 minutes. The cell pellet was washed with a solution of respective NaCl concentration. The obtained cell pellet was re-suspended in 0.85% saline, and the suspension was transferred to

the pre-weighed aluminum foil coil cups. All cups were dried at 80°C for 72 hours. Cups were weighed after drying to determine the amount of bacterial biomass at various NaCl concentrations.

4.4 Pigment analysis:

The isolate was grown at various NaCl concentrations, followed by pigment extraction using methanol to study how salinity affected the level of bacterial pigmentation.

By centrifuging the 50 mL culture broth, the bacterial cells were pelleted. The pellet was cleaned with phosphate-buffered saline. To extract pigment, 5 ml of methanol (HPLC grade) was added to the cell pellet, followed by 2 hours of vigorous vortexing until the cells were completely bleached. The pigment-containing supernatant was separated from the test tubes by centrifugation at 10,000 rpm. A 00.22-micron filter was used to sterilize the pigment's methanolic extract. Further UV-Visible Spectroscopic analysis was performed using the obtained crude pigment extract.

4.5 Radical scavenging assay:

As described in 4, the methanolic pigment extract was obtained after growing in NB with varying NaCl concentrations. Using the 2,2-diphenyl-2-picryl hydrazyl (DPPH) assay, the radical scavenging experiment of the methanolic pigment extract was performed as reported by (Velho-Pereira et al., n.d.).

The methanolic pigment extracts were taken in the test tube (1. 5 ml) and were

combined with 1.5 mL of a 0.1 mM DPPH solution in 95% ethanol. The mixture was incubated in the dark for 30 minutes at 28°C room temperature. L-ascorbic acid was used as a positive control. The reaction mixture absorbance was determined at 517 nm on a UV-VIS spectrophotometer UV2080TS. The ability to scavenge radicals were demonstrated by a color change of the reagents from purpleto yellow. The antioxidant % was calculated using the formula below:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

4.6 Superoxide anion free radical scavenging assay:

The superoxide anion free radical scavenging assay of the pigment extract obtained from the isolate grown at varying NaCl concentrations was performed.

Nicotinamide adenine dinucleotide from the methanolic/cellular pigment extract is oxidized using phenazine methosulphate-nicotinamide adenine dinucleotide (PMS-NADH). The reaction produces superoxide anion, which is then quantified by the reduction of nitro blue tetrazolium (NBT).

The cells were pelleted by centrifuging the 50 mL culture broth. The harvested cell pellet was washed with phosphate-buffered saline. Five mL of HPLC-grade methanol was added to the cell pellet, followed by vigorous vortexing of the cells for two hours was done to bleach the cells completely. The supernatant containing methanolic pigment extract was separated by centrifugationand filter sterilized using a 0.22-micron filter.

In a test tube, 1 ml of the crude methanolic extract was combined with 1 ml of 50 mM nitroblutetrazolium (NBT) and 1 ml of 78 mM nicotinamide adenine dinucleotide. NBT and NAD were prepared in 16 mM Tris-HCL solution. One ml of 10 microMole phenazine methosulphate was added to initiate the reaction. The absorbance of the reaction mixture was recorded using UV-VIS spectrophotometer UV2080TS at 560 nm after 5 minutes at 28°C. The following formula was used to determine the scavenging activity percentage:

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

4.7 Estimation of Trehalose as a compatible Solute:

The trehalose was estimated using the Anthrone Method described by (Welsh et al., 20). Isolate was grown in nutrient broth containing varying concentrations of NaCl. The test flasks were incubated for 48 hours at 28 °C at 120 rpm. The cell pellet was harvested by centrifugation for 10 minutes at 10,000 rpm. The cell pellet was first rinsed with the respective NaCl concentration, followed by PBS wash. After adding 1 mg/ml of Lysozyme, the mixture was maintained at 37°C for 30 minutes with intermittent mixing after every 10 minutes. The mixture was centrifuged at 10,000 rpm. The obtained supernatant was collected in a fresh tube and mixed with 3 mL of 80% ethanol, and vortexed for 10-15 minutes. The mixture was centrifuged further, followed by evaporation of the supernatant at 70°C. The residual pellet was dissolved in 5 ml of distilled water.

One mL of aliquot was transferred into a fresh tube, followed by adding 2 mL 0.2% Anthrone reagent prepared in concentrated H_2SO_4. The reaction mixture was incubated for 15 minutes in a water bath maintained at 95 to 98°C, followed by 5 minutes of incubation in ice. The absorbance was recorded at 630 nm using UV-VIS spectrophotometer UV2080TS. Trehalose concentrations were determined by referring to a standard curve prepared using trehalose dihydrate ranging from 10 to 100 μg/mL.

4.8 Nuclear magnetic resonance analysis of osmolytes:

Cells were grown at higher salinity i.e. NB with 8, 12, and 16% NaCl were used for NMR studies. Briefly, the isolate was grown in NB supplemented with 8, 12, and 16% NaCl and incubated at 28°C for 48 hours at 120 rpm. The cell pellet was harvested by centrifugation at 10,000 rpm for 10 minutes followed by two times washing of the cell pellet with respective NaCl solution. The cell pellet was further washed with PBS.

1 ml of lysozyme (1 mg/ml) was added to the obtained cell pellet and incubated for 30 minutes at 37°C. The reaction mixture was centrifuged followed by the addition of 80% ethanol to the supernatant. Using a vortex mixer, the contents were mixed. Centrifugation of the mixture was carried out at 10000 rpm for 10 minutes followed by evaporation of the supernatant at 70 °C. The residual pellet obtained was dissolved in sterile distilled water followed by lyophilization. H_2O-D_2O was added to the freeze-dried powder and analyzed using nuclear magnetic resonance spectroscopy (NMR) Bruker Avance Neo 500 MHz Solid state NMR for 1H and 13C.

NMR graphs were evaluated and compared with standards using a software program called Bruker Top Spin 4.2.0 in order to determine the samples.

CHAPTER 05:

RESULTS AND DISCUSSION

5.1 Effect of salinity on bacterial growth:

In order to study the effect of NaCl on the growth of bacteria, the dry weight of organisms growing at varying salt concentrations was determined. Bacterial biomass was the highest (98.87 mg) after 48 hours of incubation after growing in a medium that contained NB supplemented with 2% NaCl. The minimal dry weight (25 mg) in 48 hours was seen in a medium devoid of NaCl.

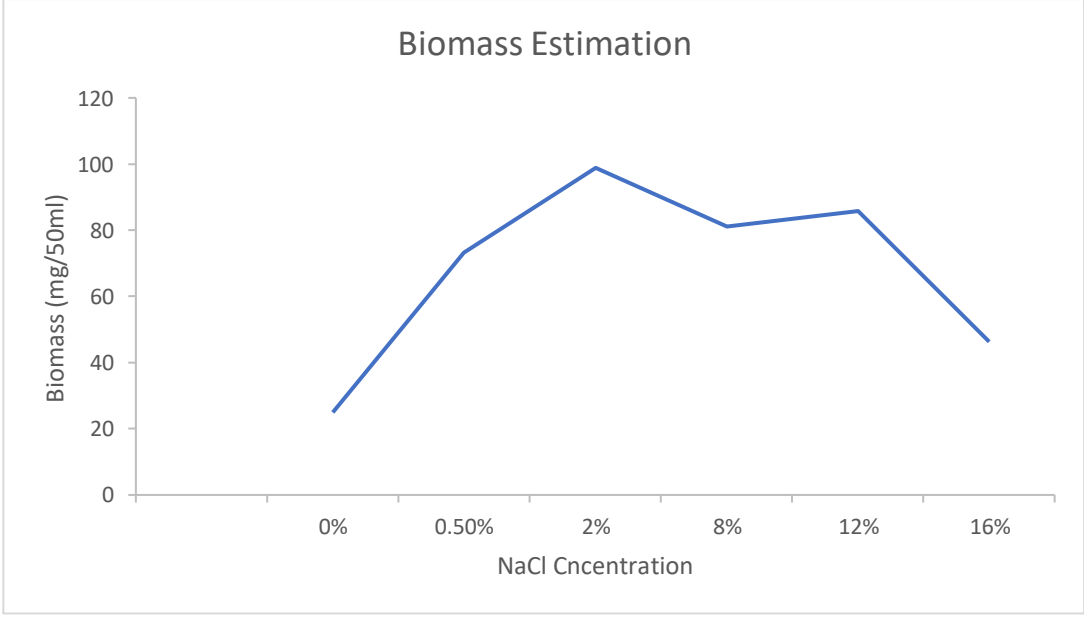


Figure No. 01: Effect of varying salinity on the growth of the isolate

The strain PSDM 20 showed growth over a broad range of salinities, as indicatedin the figure above suggesting the ability of bacteria to adapt to variations insalinity.

5.2 Pigment analysis:

In this investigation of the effect of salinity on the pigmentation of the bacteria, the highest growth was shown when the isolate was grown in media

supplemented with 2% NaCl out of all NaCl concentrations. The bacteria cultured on the same media showed the most pigmentation, with absorption maxima of about 450nm indicating carotenoid. Bacteria cultured in NB with no NaCl,0.5% NaCl8% NaCl, 12% NaCl, and 16% NaCl, on the other hand, showed a steady drop in pigmentation.

A study carried out by (Baxter R,1960) found that bacteria's pigmentation changes when they are cultivated in high salinity under ideal conditions, including temperature, agitation, and medium nutrients. The results support this. In a similar manner, according to research by (Khanafari A et al. n.d.), adding a certain concentration of salt to the mixture speeds up generation times while also increasing the strain's growth and pigment synthesis.

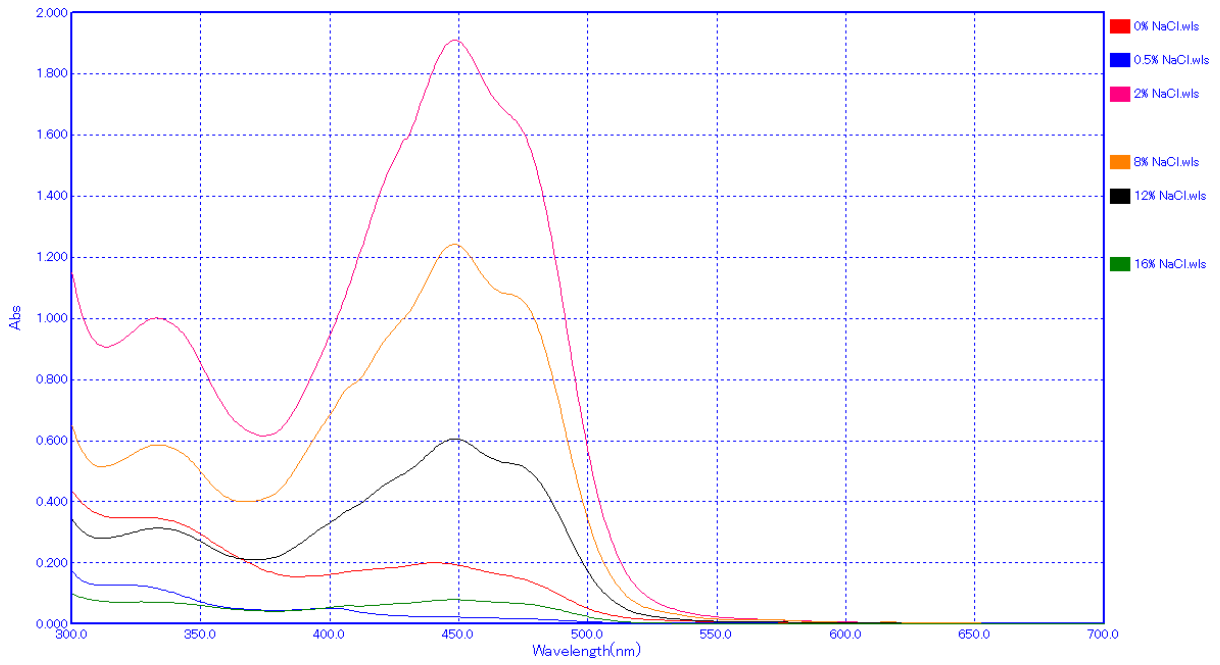


Figure No. 02: UV-Visible spectrum of pigments obtained from isolate grown at varying salinity.

5.3 Radical scavenging assay:

The production of antioxidant scavengers against ROS by a wide variety of microorganisms has been observed. Because oxygen is less soluble in water under severe salt stress, oxygen molecules combine to create ROS.

With the aid of 2, 2-diphenyl-2-picryl hydrazyl (DPPH), the pigmented methanolic bacterial extract was studied for its capacity to scavenge free radicals. The proportion of antioxidant activity was higher when the isolate was grown at 0.5% NaCl concentration (62.33%) compared to when grown at 12% NaCl concentration (7.59%). The antioxidant activity was found to be 25.20%, 23.58%, 43.90%, and 30.62%, respectively, for other concentrations of 2% NaCl, 8% NaCl, 16% NaCl, and without NaCl.

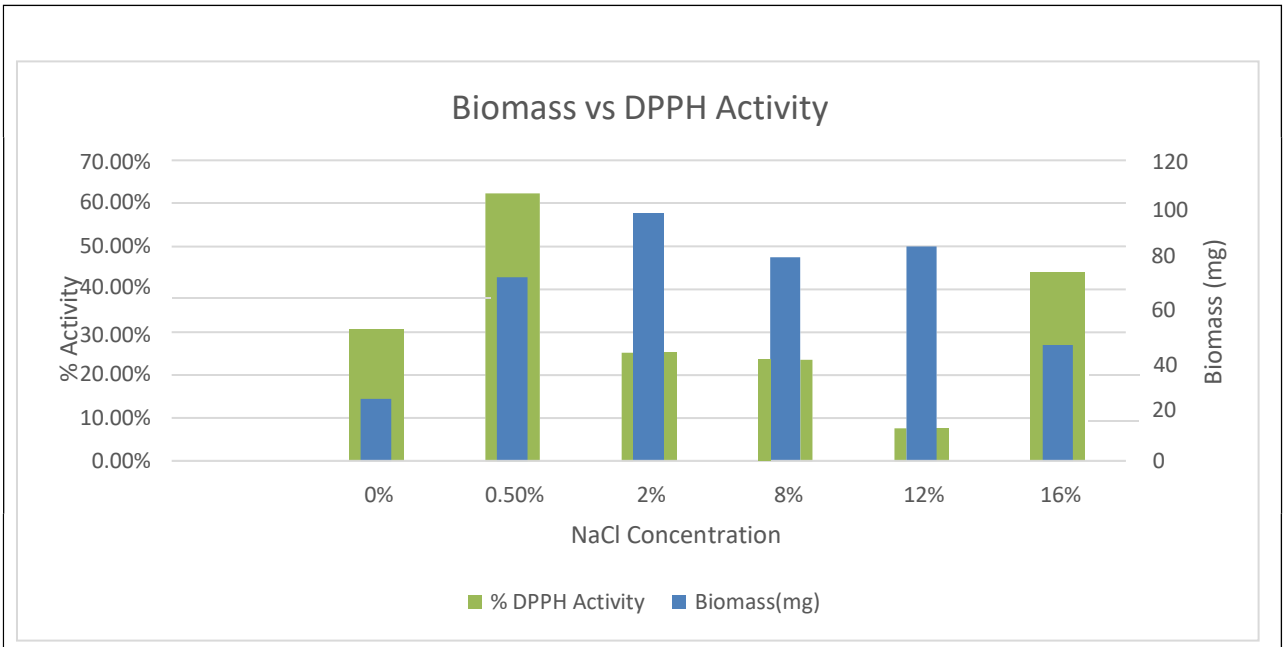


Figure No. 03: Radical scavenging activity.

5.4 Superoxide free radical scavenging assay:

To scavenge free radical species produced by high salinity, a superoxide free radical test is used. Bacteria create many scavengers, which are examined in this methodology, to prevent free radicals from damaging their cells. For a strain, PSDM 20, the ability to scavenge superoxide free radicals varies with varying NaCl concentrations. Organisms living in Nutrient broth with and without 2% NaCl exhibit approximately equal scavenging activity, with scavenging activities of 17% and 17.4%. However, with respective levels of 41.8% and 61.4% in the medium supplemented with 12% and 16% NaCl, the activity was higher. The other two concentrations were found to have scavenging activity of 8.8% and 30% for 0.5% and 8% NaCl concentrations, respectively. Through this study, an isolate with superoxide anion scavenging abilities has been found.

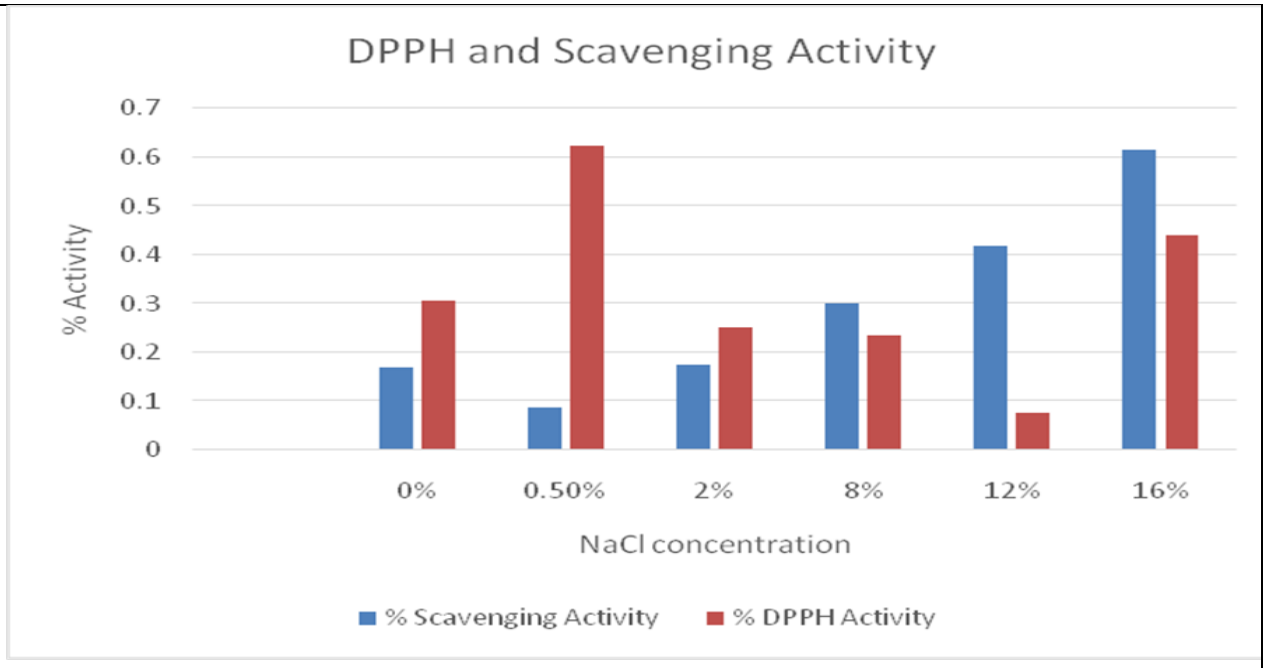


Figure No. 04: Comparison between organisms growing at different concentrations and the scavenging ability of organisms.

The presence of flavonoids and polyphenols contributes to the free radical scavenging activity. Maximum activity is displayed by the alcoholic extract (Lalhminghlui & Jagetia, 2018). Here For carotenoids and flavenoids, the overall scavenging effect is the combined quenching effect of superoxide free radical scavenging assay, as indicated in (Pawar et al., 2015).

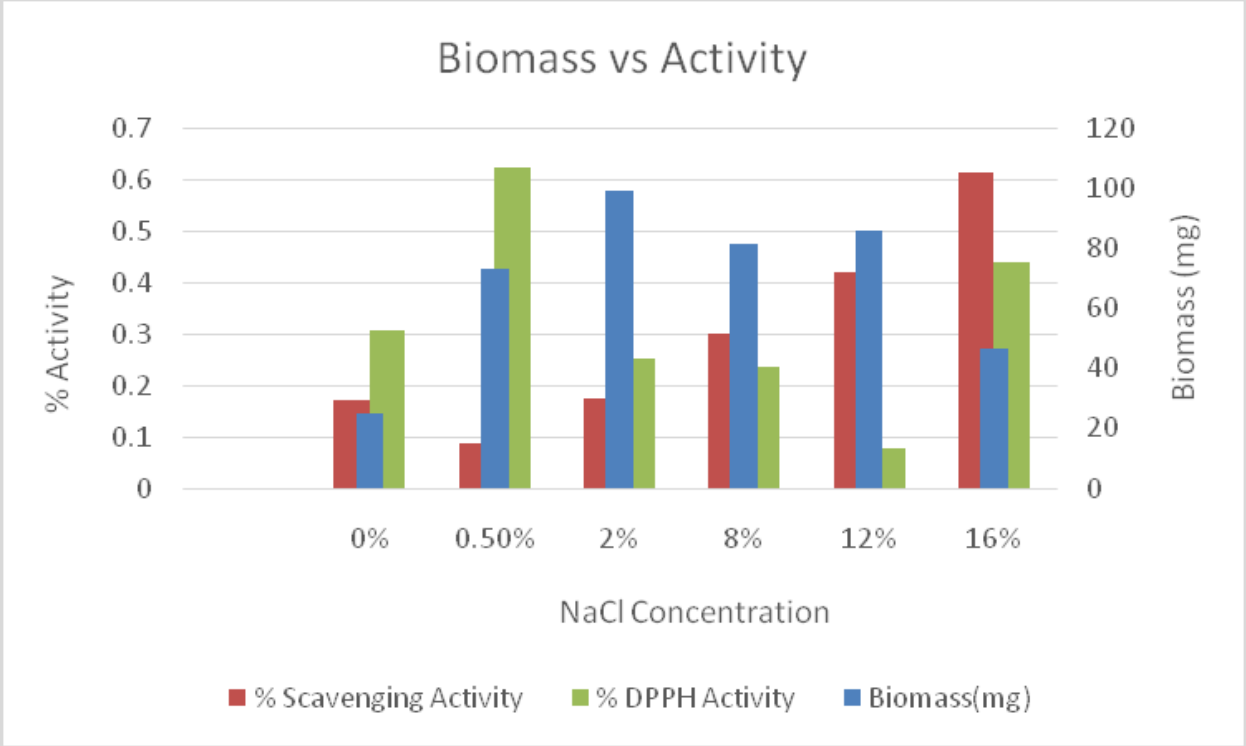


Figure No. 05: Comparative study of Scavenging activity and biomass of Bacteria.

5.5 Estimation of Trehalose as a compatible solute:

Halophilic bacteria are known to accumulate the disaccharide trehalose molecule among sugar as a compatible solute. The given strain displayed higher intracellular trehalose accumulation on medium supplemented with 2% and 8% NaCl, i.e., 0.11mg/ml and 0.10 mg/ml respectively. Trehalose concentrations observed at 0.5%, 12%, and 16% NaCl are found to be 63 µg/ml, 95 µg/ml, and 62

µg/ml, respectively, while trehalose concentrations in medium devoid of salt are 72 µg/ml.

In salt stress instances and when glycine betaine is absent, trehalose is a compatible solute. Trehalose thus is known to participate in the osmoregulatory function of both halophilic and halotolerant organisms.

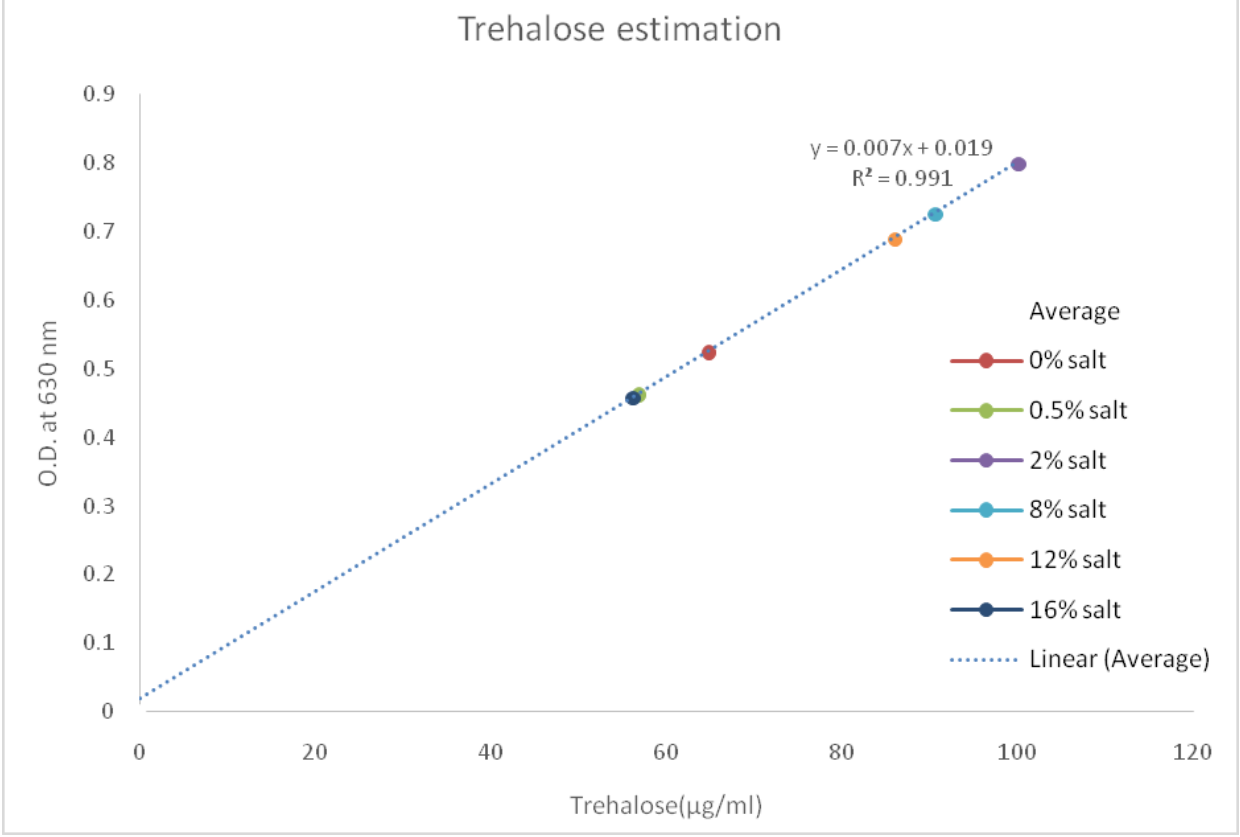


Figure No. 06: Trehalose estimation at different salt concentrations.

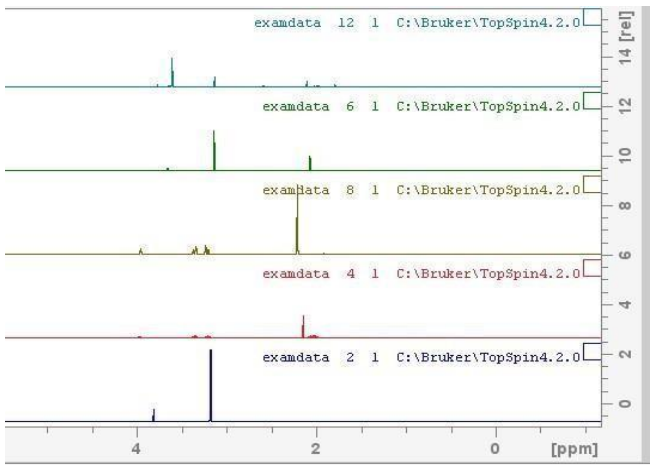
According to (Galinski & Herzog, 1990), when organisms are placed in nitrogen-depleted environments, they can no longer generate amino acids as suitable solutes, primarily betaine. As a result, trehalose biosynthesis, an alternate solute, is produced. Additionally, it has been discovered that bacteria's ability to

recycle nitrogen stimulates the production of non-nitrogenous solutes, trehalose, which have been measured at 100 mg/g of wet bacterial weight.

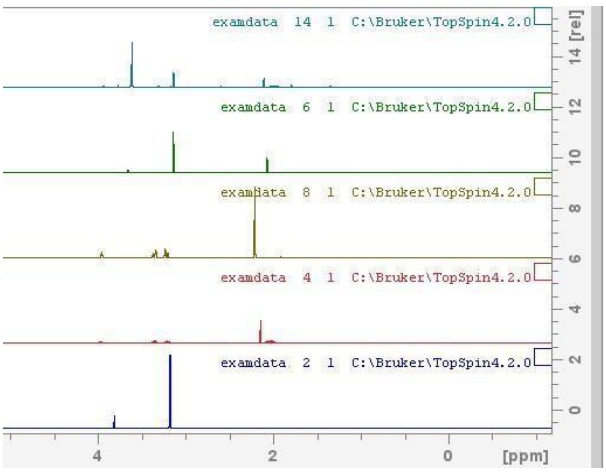
5.6 Nuclear Magnetic Resonance Spectroscopy Analysis:

For isolate cultivated in NB supplemented with 8%, 12%, and 16% NaCl, nuclear magnetic resonance spectroscopy, 1H, and 13C, was conducted. Additionally, reference standards such as betaine, ectoine, hydroxy ectoine, and acetylcholine were also evaluated for comparison investigations. The chemical shifts (ppm) and multiplicities of the structural pieces found in the 1 H and 13C NMR spectra correlate to the compatible solutes found in extracts of isolates grown on various NaCl concentrations.

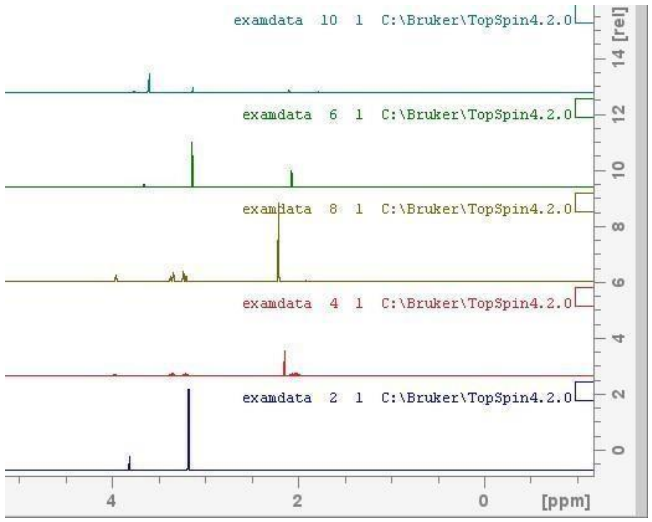
As depicted in Figure No. 07, graphs (A), (B), and (C), isolates cultivated on 8%, 12%, and 16% NaCl produced solutes that were equivalent to the reference standards acetylcholine, betaine, and ectoine. The signals change among medium concentrations is shown in Graph (D). Osmolyte signals are higher when the extract is obtained from 12% NaCl, whereas it is lowest when 16% NaCl is used. The intermediate osmolyte content was achieved at 8% NaCl.



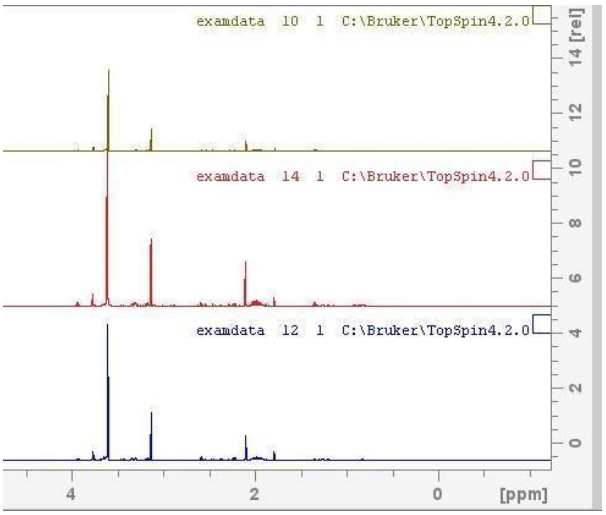
A



B



C



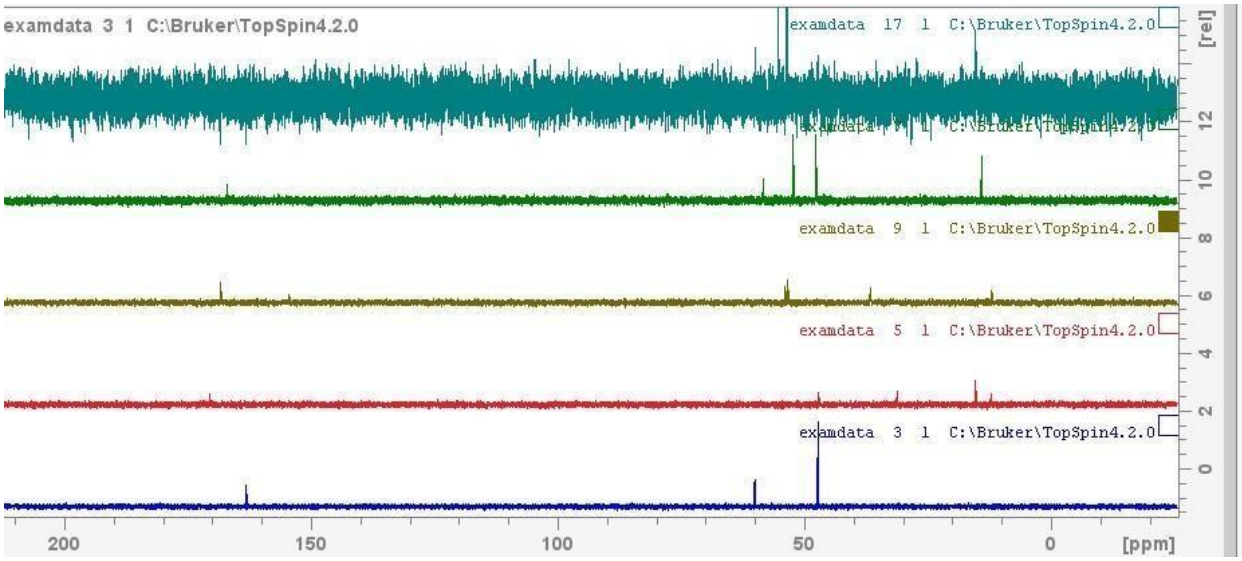
D

Figure No. 07: Nuclear Magnetic resonance spectroscopy scans,1H for samples and reference standards

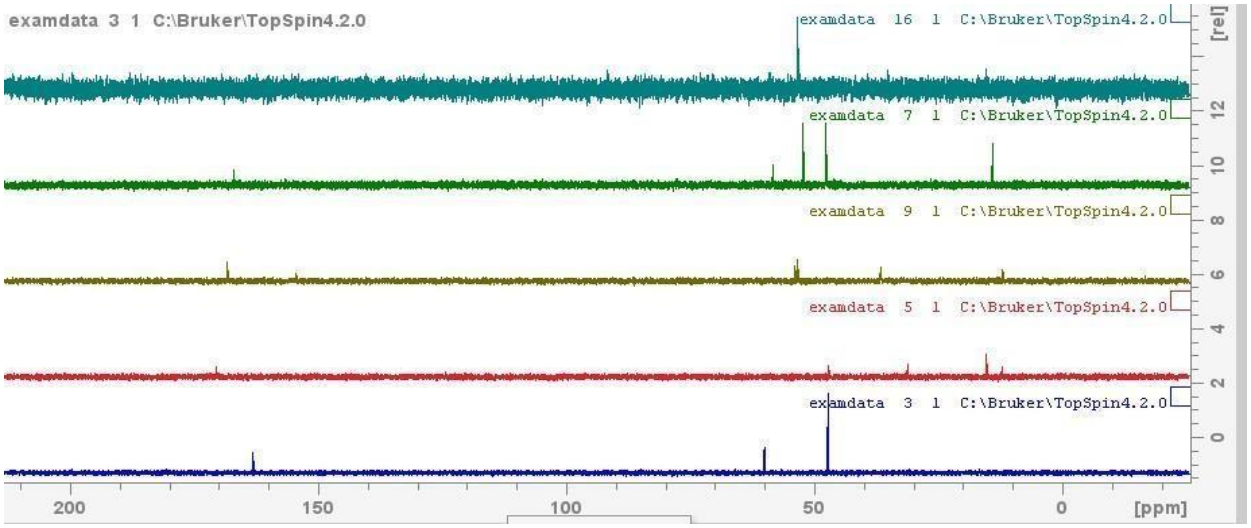
A to C: Betaine Ectoine Hydroxyectoine Acetylcoline

A: Isolate grown in 8% NaClB: Isolate grown in 12% NaCl C: Isolate grown in 16% NaCl

D: Isolate grown in 8% NaCl Isolate grown in 12% NaCl Isolate grown in 16% NaCl



A

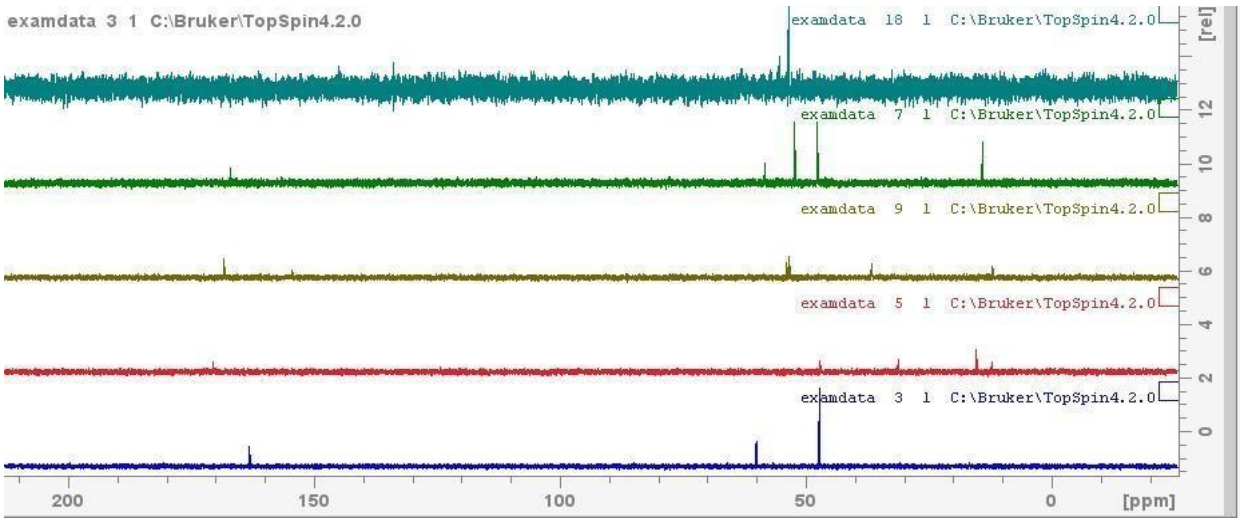


B

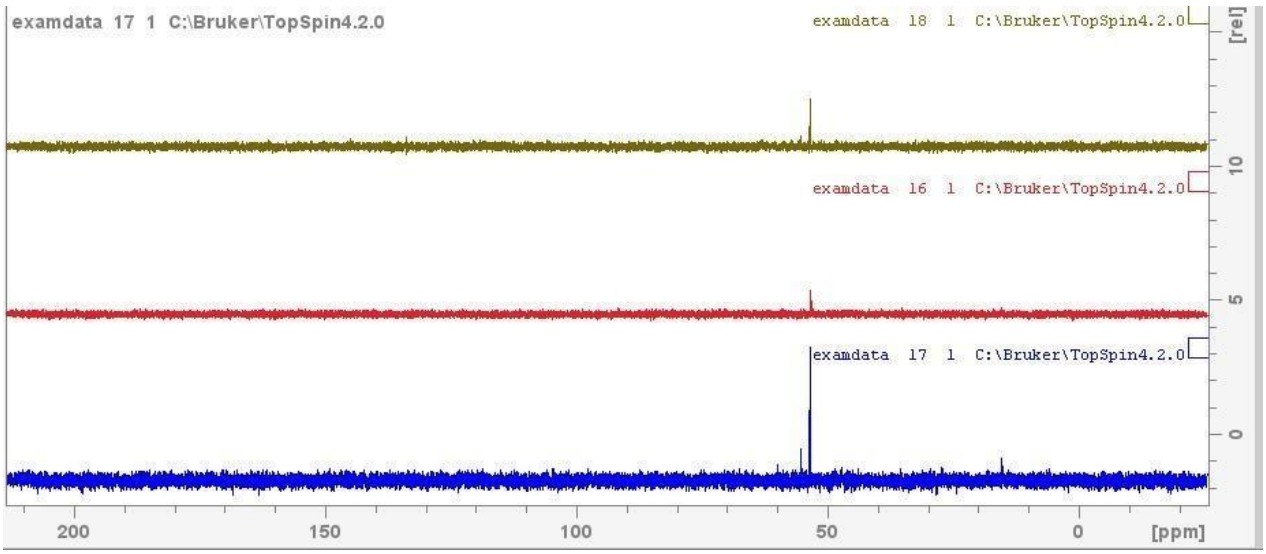
Figure No. 8.1: Nuclear Magnetic resonance spectroscopy Scans,13C for samples and reference standards

A to B: Betaine Ectoine Hydroxyectoine Acetylcoline

A: Isolate grown in 8% NaCl B: Isolate grown in 12% NaCl



C



D

Figure No. 8.2: Nuclear Magnetic resonance spectroscopy Scans,13C for samples and reference standards

C: Betaine Ectoine Hydroxyectoine Acetylcholine Isolate grown in 16% NaCl

D: Isolate grown in 8% NaCl Isolate grown in 12% NaCl Isolate grown in 16% NaCl

Studying 13C NMR for osmolytes in Figures No. 8.1 and 8.2 aids in adapting to osmotic stress conditions. Comparative similarity among isolates cultivated in 8%, 12%, and 16% NaCl and with Acetylcholine, Betaine, and Ectoine is shown in Graphs (A), (B), and (C). As demonstrated in graph (D), the 13C NMR scan shows more resemblance to acetylcholine. Signals for Acetylcholine are higher in isolates grown in 8% NaCl than in those grown in 12% and 16% NaCl.

According to the research of (Avendao et al., 2015), the sample's salinity, temperature, and pH produce a change in signal position when compared to the reference standards. As a result, it is concluded that the isolate may include intracellular concentrations of betaine, acetylcholine, and ectoine for osmoregulation mechanisms. Since hydroxy ectoine is a hydroxylated form of ectoine, it could exist as a compatible solute.

CHAPTER 06:

SUMMARY AND CONCLUSION

The current research focuses on microorganisms that have adapted to varing concentrations of salinity. When the surrounding environment is salty, the cells that are growing there may shrink as a result of hyperosmosis. Consequently, the cell is unable to carry out its metabolic duties. As a result, cells synthesize various osmolytes or accumulate amino acids and carbohydrates, which also act as osmolytes, to maintain the osmotic balance.

The accumulation of uncharged, or zwitterionic, extremely water-soluble organic solutes is the principal factor that lowers the chemical potential of the cell water. This investigation discovered that the isolate under examination largely accumulated sugar molecules in the form of trehalose and amino acids like ectoine, betaine, acetylcholine, and hydroxy ectoine. Acetylcholine serves as the intermediate product in the synthesis of betaine.

Similarly, bacteria produce carotenoid pigments that give organisms their colors. Furthermore, pigments demonstrate free radical scavenging activity in tests because high salinity limits oxygen's solubility and leads to a buildup of reactive oxygen species. It's crucial to deal with the medium because ROS causes toxicity in the cell.

As a result, this study suggests an extraction method for biologically active osmolytes that could be used for various industrial applications.

CHAPTER 07:
FUTURE PROSPECTS

Future Prospects:

This project's future research interests include the following objectives:

1. Proper characterization of bioactive molecules produced due to the stress adaptations of bacteria.
2. Deducing the role of discovered osmolytes in protection against stress.
3. Applications of compatible solutes.

CHAPTER 08:

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APPENDIX

MEDIA

1. Nutrient Agar (pH 7.4)

INGREDIENTS	COMPOSITION
Peptone	5 grams
Sodium Chloride	5 grams
Yeast Extract	2 grams
Agar Powder	15 grams
Distilled Water	1 litre

2. Nutrient Broth (pH 7.4)

INGREDIENTS	COMPOSITION
Peptone	5 grams
Sodium Chloride	5 grams
Yeast Extract	2 grams
Beef Extract	1 gram
Distilled water	1 litre

BUFFER

1. Phosphate Buffer Saline (pH 7.4)

INGREDIENTS	COMPOSITION
Sodium Chloride	8 grams
Potassium Chloride	0.2 gram
Sodium Phosphate Dibasic	1.44 gram
Potassium Phosphate Monobasic	0.245 gram
Distilled Water	1 litre

2. **Tris-EDTA Buffer (pH 8)**

INGREDIENTS	COMPOSITION
Tris-HCl	15.759 grams
EDTA	2.92 grams
Distilled Water	1 litre

3. **Tris-HCl Buffer (pH 7)**

INGREDIENTS	COMPOSITION
Tris-HCl	121.4 gram
Distilled water	1 litre

REAGENTS

1. **Anthrone Reagent**

INGREDIENTS	COMPOSITION
Anthrone Reagent	2 grams
Conc. H_2SO_4	1 litre

2. **2,2-diphenyl-2-picrylhydrazyl reagent(0.1mM)**

INGREDIENTS	COMPOSITION
2,2-diphenyl-2-picrylhydrazyl reagent	0.394 gram
95% Ethanol	1 litre

3. **Nitrobluetetrozolium (50µM)**

INGREDIENTS	COMPOSITION
Nitrobluetetrazolium	0.0043 mg
Tris-HCL buffer	100

4. Nicotinamide adenine dinucleotide (78 µM)

INGREDIENTS	COMPOSITION
Nicotinamide adenine dinucleotide	0.0055 mg
Tris-HCL buffer	100 ml

5. Phenazine Methosulphate (10 µM)

INGREDIENTS	COMPOSITION
Phenazine Methosulphate	0.0030 mg
Distilled Water	1 litre

INSTRUMENTS

- Weighing balance
- Autoclave
- Laminar Air Flow hood
- Cooling Centrifuge (Sorvall ST 8R)
- UV-VIS Spectrophotometer (UV 2080TS)
- Lyophilyser
- Nuclear Magnetic Resonance Spectroscopy (Bruker Avance Neo 500 MHz)