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ORIGINAL RESEARCH PAPER

Isolation, Characterization and Exploring the Biotechnological Potential of Halophiles

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Abstract

Soil salinity is a major challenge for agriculture worldwide, making it difficult for crops to grow and reducing overall productivity. On the other hand, halophiles are a type of microbe that has evolved to live in very salty conditions. Soda and salty lakes are rich habitats for salt-loving microorganisms, which may be essential for crop improvement in salty soils. In addition to their usefulness in agriculture, halophiles have industrial value due to the significant enzymes they create, including as amylase, protease, and lipase.

In this study, researchers collected microbial samples from three highly saline environments: the Sambhar salt pan (27°58′N 75°55′E) and Sambhar Lake (26.9261°N 75.0962°E) in Rajasthan, as well as the Halar salt pan in Jamnagar, Gujarat (22°47′N 70°05′E). These microorganisms were tested for their ability to produce useful enzymes and support plant growth, potentially helping crops withstand salt stress. Interestingly, some of the isolates were found to produce polyhydroxybutyrate (PHB) granules—an indicator of their ability to generate bioplastics, a promising sustainable material.

To better understand these microbes, scientists conducted antibiotic sensitivity tests and used 16S rDNA amplification with specialized primers for haloarchaea. Based on initial findings, two isolates (SSP and SL) were classified as part of the Haloarchaea group, while another (JSP) belonged to the Eubacteria group. However, further genetic analysis, including genome sequencing and phylogenetic studies, will be needed for precise classification.

Researchers also studied pigmented isolates, focusing on their carotenoid content due to the strong antioxidant properties of these compounds. The antioxidant activity was measured using DPPH radical scavenging assays, with ascorbic acid as a reference. Given their ability to combat oxidative stress caused by reactive oxygen species (ROS), these microorganisms could have potential applications in medical research as well.

Overall, this study highlights the incredible versatility of halophilic archaea and bacteria. Their potential goes far beyond agriculture—they could be used for bioremediation, biofertilizers, biofuels, microbial fuel cells, halocin production, biofilm formation, and biosurfactants. This makes them valuable not just for improving soil health and crop yields but also for advancing sustainable industrial processes.

Keywords: Halophiles, Extremophiles, Isolation Techniques, Characterization, Salt-Tolerant Microorganisms, Biotechnological Applications, Enzyme Production.

Introduction

All three kingdoms of life, Archaea, Bacteria, and Eukarya, are home to halophilic bacteria, which love salt. They are grouped according to their salt tolerance and optimal growth conditions. The optimal salt concentration for extreme halophiles is between 2.5 to 5.2 M, and they flourish in extremely salty environments. Optimal growth conditions for moderate halophiles range from 0.5 to 2.5 M salt. Then there are the halotolerant microbes; they aren't strictly salt-dependent, but they can nevertheless make it in the most saline of conditions. Those organisms are said to be very halotolerant if they can thrive at salt concentrations higher than 2.5 M. Because of their incredible adaptability, these microbes are found in some of the harshest places on Earth and offer exciting potential for research and biotechnology (Crespo *et al*., 2017).

Although thermophilic extremophiles often steal the spotlight in biotechnology, halophilic microorganisms are proving to be just as valuable. Their potential spans multiple fields. First, many of the enzymes they produce can withstand high salt concentrations, making them useful for industrial processes where traditional enzymes would fail. Second, halophiles produce unique, organic

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stabilizers that help protect proteins and enzymes, which can be beneficial in various applications (Aharon, O., 2003). Third, some halophilic microbes create rare and valuable compounds that aren't found in other organisms, making them attractive for biotechnological development. In some cases, halophiles even offer advantages over non-halophilic organisms when producing certain products.

One major benefit of working with halophilic microorganisms is that high-salt environments naturally reduce the risk of contamination from other microbes, creating a more controlled setting for industrial applications. However, growing these microbes at scale presents its own challenges. Their nutritional requirements vary widely—some need rich nutrient sources like yeast extract, while others can survive on simpler nutrients. For example, most species in the Halobacteriaceae family prefer amino acids as their nitrogen source, though some can also use ammonia or nitrate. Additionally, not all halophilic species can efficiently metabolize sugars like glucose and sucrose, but certain genera, such as Haloferax and Haloarcula, have adapted to do so.

One of the most fascinating aspects of halophilic archaea is their retinal pigments, particularly those found in the Halobacteriaceae family. Scientists have been intrigued by these pigments since the discovery of bacteriorhodopsin in the early 1970s. These proteins share a unique seven-helix structure, with a retinal group chemically linked to the protein. Unlike most halophilic proteins, retinal proteins don't require salt to function, making them an unusual exception (Akolkar, A. V., 2008). Since researchers first identified retinal as the key component of the purple membrane pigment in *Halobacterium salinarum*, interest in these pigments has surged.

One groundbreaking discovery was the retinal-based proton pump, which allows these microbes to harness light energy, a trait once thought to be unique to halophilic archaea. Another key retinal protein, halorhodopsin, acts as a chloride pump, moving chloride ions into the cell when exposed to light (with a peak absorption at 578 nm). This process is crucial for maintaining the internal ionic balance needed for growth and metabolism.

Haloarchaea, a subset of halophilic archaea, mostly thrive in oxygen-rich environments, but some can survive without oxygen by using nitrate in a process called denitrification. Many of these microbes are naturally red due to their pigments, which help them withstand harsh conditions like extreme salinity, high solar radiation, low water availability, and nutrient scarcity (Bajwa, K., Bishnoi, N., Toor, M., Gupta, S., *et al*., 2018). One of their key survival strategies is accumulating high levels of potassium chloride (KCl) inside their cells, a mechanism known as the "salt-in" strategy. This allows them to function in nearly saturated salt conditions, but their proteins must be specially adapted to remain stable under these extreme conditions.

Unlike proteins from non-halophilic organisms, halophilic proteins have a higher concentration of acidic amino acids, such as glutamate and aspartate. These negatively charged residues, primarily located on the protein surface, attract water molecules, keeping the proteins hydrated and preventing them from clumping together (Bhatnagar, T., & Bhatnagar, N., 2005). However, these proteins also require counterions like potassium to maintain their structure and function. This unique adaptation makes many enzymatic processes in haloarchaea both salt-dependent and salt-tolerant. Additionally, strong hydrophobic interactions within these proteins provide further stability in high-salt environments.

The genomes of halophilic microorganisms are packed with genes related to salt resistance, giving them a competitive edge in extreme habitats. These adaptations make them a valuable source of bioactive compounds, with growing interest in their potential applications. Haloarchaea produces a variety of metabolites in response to their environment, some of which have shown promising biological activities (Cai, S., Sun, W., & Shao, Z., 2011). In particular, certain bioactive molecules from these microbes have demonstrated anticancer, antioxidant, antiproliferative, and apoptosis-inducing effects, sparking interest in their potential medical and therapeutic applications.

This study explores the biological properties of carotenoids extracted from archaea, emphasizing their potential as bioactive molecules with significant health and nutraceutical benefits.

Materials and Methods

Sampling site

The salt beds of Sambhar Salt Lake, located in Sambhar, Rajasthan, India, were selected as the site for collecting microorganisms. As the largest inland saline lake in the country, Sambhar Salt Lake (Figure 1) lies southwest of Jaipur and provides a unique environment for microbial life.

The sample from the Jamnagar salt pan was meticulously gathered from the Halar salt pans, an area known for its distinct geological characteristics (Figure 2). These salt pans are primarily notable for their high sodium carbonate content, which frequently forms unique complexes in

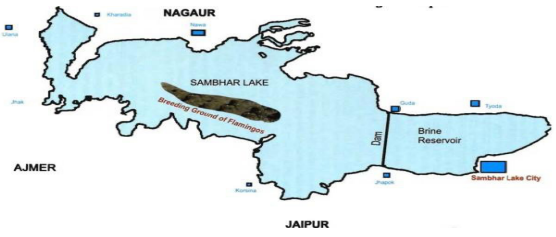


Figure 1: Sambhar Salt Lake (26°52′-27°02′N, 74°54′-75°14′E)

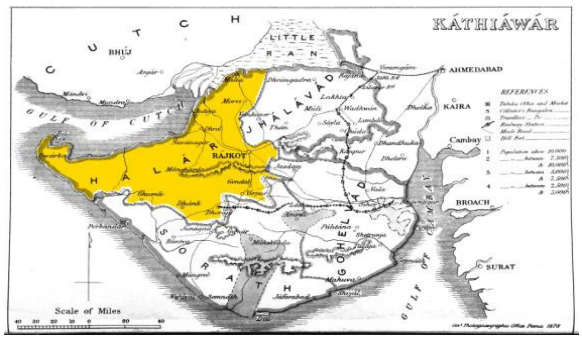


Figure 2: Halar (22°47'N, 70°05'E)

the environment. This elevated concentration of sodium carbonate gives the Halar salt pans their distinct chemical and mineral characteristics, making them a fascinating site for scientific research and exploration.

Isolation

To cultivate the microorganisms, 10 mL of the liquid culture (inoculum) was introduced into Zobell Marine Broth, a nutrient-rich medium containing a precise blend of ingredients per liter: 5 g of peptone, 1 g of yeast extract, 0.1 g of ferric citrate, and a mixture of essential salts, including 19.45 g of sodium chloride, 8.8 g of magnesium chloride, and smaller amounts of calcium chloride, potassium chloride, and other trace minerals. The final pH was adjusted to 7.6 ± 0.2 at 25°C, with a salt concentration of 20%. The flasks were then placed on a rotary shaker at 180 rpm and incubated at 37°C for 7 days.

For colony characterization, the microorganisms were streaked onto Zobell Marine Agar, which contained the same nutrient composition along with 2.5% agar and 20% NaCl. To ensure sterility, the media were autoclaved at 121°C and 15 psi for 20 minutes. Once cooled to 45 to 50°C, the 20% salt solution was mixed in, and the medium was poured into sterile petri plates. After solidifying, a small amount of culture was streaked onto the plates. These plates were wrapped in paper, sealed in plastic bags, and incubated at 37°C for 15 to 20 days, allowing isolated colonies to develop using the four-sector streaking method (DasSarma, S., & DasSarma, P., 2015). Once the colonies were fully formed, master plates were prepared and stored with parafilm wrapping.

Growth Conditions and Optimization of pH and Salt Concentration

For further growth studies, 100 mL of media containing 20% salt was prepared in 250 mL Erlenmeyer flasks. A 1% inoculum from the sample was added, and the cultures were incubated at 37°C on a rotary shaker at 180 rpm for seven days. When streaked onto Zobell Marine Agar with 20% salt, visible growth began after nine days, with isolated colonies appearing after 12 to 15 days (Figure 3). (Detkova, E. N., & Boltyanskaya, Y. V., 2007). Each streaked culture was pure, growing as a single strain.

Because these isolates originated from soda lakes and salt pans, their growth was tested at varying salt concentrations (20, 25, and 30%) and pH levels (7, 8, 9, 10, and 11) to determine optimal conditions. The growth curve and ideal conditions were analyzed spectroscopically by measuring absorbance at 600 nm.

Characterization of Microorganisms

Gram Staining and Pigmentation

Gram staining was performed using a standard method. A small loopful of culture was heat-fixed onto a clean slide, stained with crystal violet, and treated with Gram's iodine. After decolorizing with ethanol, the slide was rinsed under running water and counterstained with safranin for one minute. Once air-dried, a drop of paraffin oil was added, and the samples were examined under a 100X microscope lens. The isolates SSP and SL exhibited pigmentation (Elmahdi, S., & Smith, L. T., 2009).

Biochemical Characterization

Screening for Extracellular Enzyme Production

All three isolates were tested for their ability to produce extracellular enzymes.

- *Amylase production*

A 1% starch solution was added to Zobell Marine Agar plates containing 20% NaCl.

- *Protease production*

Skimmed milk agar plates were prepared by incorporating a 1% skimmed milk solution into plates with 20% NaCl.

- *Lipase production*

A 1% tributyrin solution was mixed into ZM agar plates with 20% NaCl.

After applying a 1% inoculum of each isolate onto the prepared plates, the presence of extracellular enzyme activity was confirmed by the appearance of clear hydrolysis zones surrounding the bacterial colonies. These zones indicated the microorganisms' ability to break down starch, proteins, or lipids.

Screening for Plant Growth-Promoting (PGP) Traits

To assess whether the isolates could support plant growth, they were tested for three key attributes: phosphate solubilization, siderophore production, and chitinase production, all at their optimal salt tolerance levels.

Phosphate Solubilization

The isolates were spot-inoculated onto Pikovskaya's agar plates containing tri-calcium phosphate. These plates were then incubated at 28°C for 7 to 12 days. A positive result was indicated by the formation of clear zones around

the colonies, showing that the microbes were effectively breaking down and solubilizing phosphate.

Siderophore Production

To determine whether the isolates could produce siderophores—compounds that help plants absorb iron—each isolate was inoculated onto Chrome Azurol S agar plates. The plates were incubated at 28°C for 48 to 72 hours, and the appearance of a yellow-orange halo around the colonies indicated positive siderophore production.

Chitinase Production

Chitin was converted into its colloidal form using a modified method from Mathivanan (1995). This involved treating non-colloidal chitin with hydrochloric acid, followed by thorough washing and desalting. The resulting colloidal chitin was incorporated into Zobell Marine (ZM) agar containing 20% NaCl. The presence of a hydrolysis zone around the bacterial colonies confirmed chitinase production, showing that the microorganisms were capable of utilizing chitin.

Screening for PHB Production

To check for the presence of polyhydroxybutyrate (PHB), a biopolymer used by bacteria as an energy reserve, the isolates were stained using Sudan Black B. A bacterial smear was first heat-fixed onto a glass slide and then stained with a 3% (w/v) Sudan Black B solution in 70% ethanol for 10 minutes. The slide was then immersed in xylene until fully decolorized. Finally, the sample was counterstained with a 5% (w/v) safranin solution for 10 seconds, rinsed with water, and left to dry. This staining method was adapted from Murray *et al*. (1994).

Antibiotic Sensitivity Testing

To evaluate antibiotic resistance, a 1% inoculum of each isolate was spread onto ZM agar plates. Small discs containing different antibiotics were placed on the surface, and the plates were incubated at 37°C for 7 to 12 days. The effectiveness of each antibiotic was determined by measuring the zone of growth inhibition around each disc. The antibiotics tested included:

- Neomycin (30 mcg)
- Rifampicin (15 mcg)
- Chloramphenicol (25 mcg)
- Streptomycin (25 mcg)
- Novobiocin (30 mcg)
- Spiramycin (30 mcg)
- Bacitracin (10 units)

Carotenoid Extraction

When the bacterial cultures reached the stationary growth phase, 50 mL of the culture was centrifuged at 6000 rpm for 20 minutes at 0°C to separate the cells from the liquid medium. The resulting cell pellet was then extracted in darkness using 100 ml of acetone containing 50 mg of the antioxidant butylhydroxytoluene (BHT). After another round of centrifugation at 6000 rpm for 10 minutes at 4°C, the acetone cell suspension was mixed with 5 mL of hexane and 5 mL of a 25% NaCl aqueous solution to facilitate phase separation. The aqueous acetone layer underwent additional hexane extractions.

Following a brief agitation period, the carotenoid-rich hexane extract was dried using a rotary vacuum evaporator, weighed, and resuspended in ethanol. The carotenoid extract was then analyzed using a spectrophotometer, scanning wavelengths between 400 and 600 nm, as described by Abbes *et al*. (2013).

DPPH Radical Scavenging Assay

To assess antioxidant activity, the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay was performed. In this method, 100 µL of the carotenoid extract was mixed with an equal volume of acetone and methanol, then combined with 950 µL of a 100 µM DPPH solution in methanol. The mixture was shaken thoroughly and allowed to react in darkness at room temperature for 30 minutes. Absorbance was then measured at 580 nm using a spectrophotometer, with an acetone/methanol (1:1, v/v) blank serving as a reference. The percentage of DPPH radical scavenging activity was calculated using a standard formula.

16S rDNA Amplification

The process of amplifying the 16S rRNA gene began with extracting total genomic DNA from the bacterial isolates, following the protocol by Wan Lam (1991). A 200 µL culture in the stationary phase was centrifuged to collect the cells, which were then lysed by vortexing with distilled water. To remove proteins and other impurities, buffer-saturated phenol (pH 8) was added, and the mixture was incubated at 65°C before centrifugation to separate the aqueous phase. The extracted DNA was then precipitated with cold ethanol, collected via centrifugation, washed with 70% ethanol, and air-dried. Finally, the DNA pellet was dissolved in distilled water and stored at -20°C.

Using specific haloarchaeal primers, the 16S rRNA gene of the isolates was successfully amplified, with *E. coli* serving as a reference control for comparison.

Primer Sequences and PCR Conditions

To amplify the 16S rDNA of the isolates, the following primers were used:

Forward Primer (F27)

5′ ATTCCGGTTGATCCTGCCGAAG 3′ (Position: 6–27 in *Haloferax volcanii*), as referenced by Gupta *et al*. (1983).

Reverse Primer (R1521)

5′ AGGAGGTGATCCAGCCGCAG 3′ (Position: 1540–1521 in *H. volcanii*), as cited by Xu *et al*. (2005).

Table 1: PCR reaction mix composition for a 20 µL reaction volume

Component	*Volume (µL)*
Sterile MilliQ Water	5
PCR Master Mix	10
Forward Primer	1
Reverse Primer	1
DNA Sample	3
Total Volume	**20**

PCR Preparation and Conditions

To prepare the PCR reaction mix, the following components were combined, as shown in Table 1.

PCR Amplification Steps

The polymerase chain reaction (PCR) was carried out under the following thermal cycling conditions:

Initial Denaturation

95°C for 3 minutes

Denaturation

94°C for 30 seconds

Annealing

72°C for 1.5 minutes

Extension

64°C for 30 seconds

Cycle Repetition

Steps 2, 3, and 4 were repeated for 30 cycles

Final Extension

72°C for 10 minutes

These conditions ensured efficient amplification of the 16S rDNA for further sequencing and analysis.

Results And Discussion

Isolation

The microbial isolation process was carried out by streaking the samples onto ZM Agar plates containing 20% NaCl. These plates were then incubated for 7 to 12 days. Initial signs of growth appeared by the 7th day, but the colonies fully covered the plate by the 12th day. Since the plates contained a high salt concentration (20%), extra care was taken in wrapping them properly. This precaution was necessary to prevent salt from precipitating and forming crystals, which could interfere with the continued growth of the isolates.

Optimization of growth conditions

To study growth optimization and growth curves, fifteen 250 mL Erlenmeyer flasks were prepared for each isolate. Since the samples were collected from the Sambhar salt pans, Sambhar Lake, and Jamnagar (Halar) salt pans, which are soda lakes with alkaline conditions, their growth was studied not only by optimizing salt concentration but also by determining their optimal pH levels. The growth of each isolate was monitored spectrophotometrically by measuring absorbance at 600 nm until the stationary phase was reached (Figures 3-5). The highest absorbance values

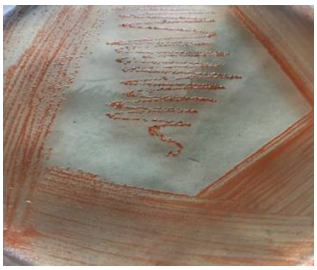


Figure 3: Growth of isolate SL on the ZM agar plate containing 20% NaCl

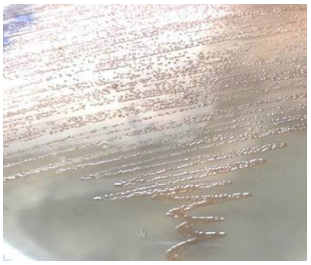


Figure 4: Growth of isolate SSP isolated on the ZM agar plate containing 20% NaCl

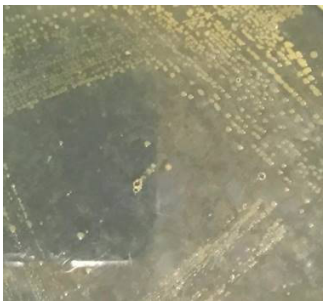


Figure 5: Growth of isolate JSP on the ZM agar plate containing 20% NaCl

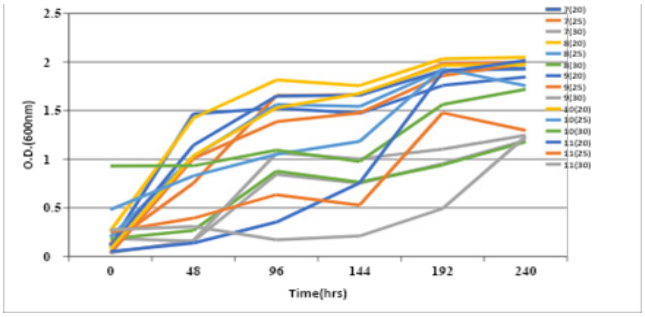
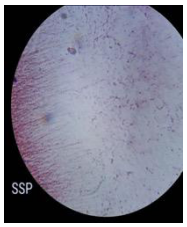
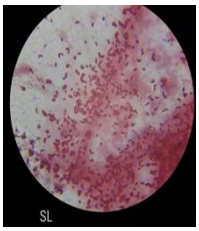
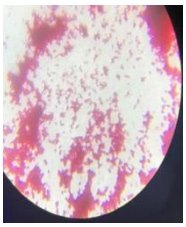
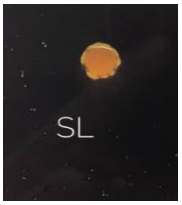
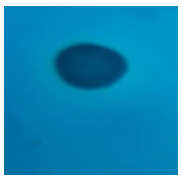
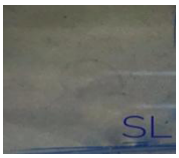
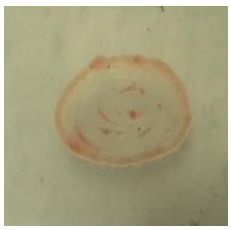
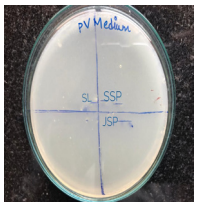
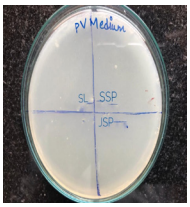
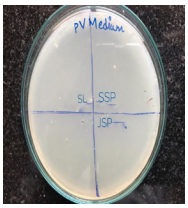
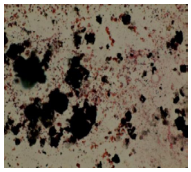
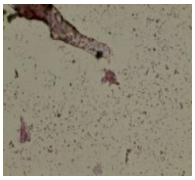
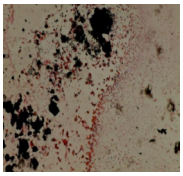


Figure 6: Growth of isolate SSP under different pH and salt condition

Table 2: Results of various tests performed

Sr. no.	*Characteristics and tests*	*SSP*	*SL*	*JSP*	*Remarks*
1.	Gram's staining				Isolate SSP is Gram positive, and isolate SL and JSP are Gram negative.
2.	Amylase production				Zone of hydrolysis was observed by SSP isolate.
3.	Protease production				Zone of hydrolysis was observed by all isolates
4.	Lipase production				Zone of hydrolysis not observed in any isolate.
5.	Siderophore production				Zone of hydrolysis not observed in any isolate.
6.	Chitinase production				Zone of hydrolysis not observed in any isolate.
7.	Phosphate solubilization				Zone of solubilization was not observed in any isolate.
8.	PHB production				PHB granules observed within the cells of SSP and JSP only.

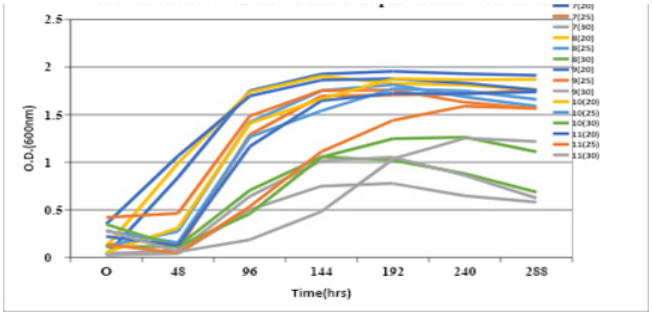


Figure 7: Growth of isolate SL under different pH and salt condition

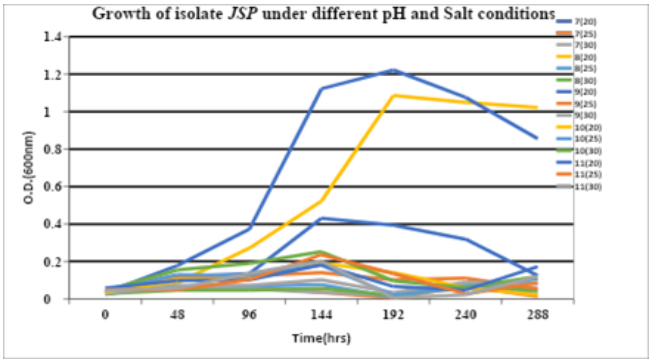


Figure 8: Growth of isolate *JSP* under different pH and salt conditions

indicated the ideal pH and salt concentration for each isolate. These optimal conditions were then used for further analysis while preparing ZM broths and ZM agar plates.

For the isolate SSP, the optimum pH is 8, and 20% IS the optimal salt concentration (Figure 6).

For the isolate SL, the optimum pH is 7, and 20% IS the optimal salt concentration (Figure 7).

For the isolate JSP, the optimum pH is 9, and 20% IS the optimal salt concentration (Figure 8).

Characterization

Gram's Staining and Pigmentation

Gram staining was performed to determine the gram nature of the isolates. Additionally, pigmentation was observed in both SSP and SL isolates when they were cultivated in broths or agar plates.

Biochemical Characterization

- *Screening for Extracellular Enzyme Production*

The isolates were tested for the production of amylase, protease, and lipase enzymes:

- Amylase production: Isolate SSP exhibited amylase activity, as indicated by a clear zone of hydrolysis after the application of iodine solution. This result confirmed the breakdown of starch by the enzyme. Since these isolates are halophilic, they primarily utilize amino acids as an energy source rather than carbohydrates.
- Protease production: All isolates tested positive for protease production. The presence of a visible hydrolysis zone after the addition of Coomassie Brilliant Blue confirmed protein degradation.
- Lipase production: None of the isolates showed lipase activity when grown on tributyrin plates, as there were no visible hydrolysis zones.

- *Screening for Plant Growth-Promoting (PGP) Attributes*

The isolates were also tested for phosphate solubilization, chitinase production, and siderophore production. However, none of these tests showed positive results:

- *Phosphate solubilization and siderophore production*

The isolates did not exhibit any growth, indicating their inability to perform these functions under the given conditions.

- *Chitinase production*

Although the isolates were able to grow on chitin-containing plates, no hydrolysis zones were observed, suggesting they did not produce chitinase.

- *Screening for PHB Production*

To assess polyhydroxybutyrate (PHB) accumulation, the isolates SSP and JSP were examined under a 100X magnification microscope after Sudan Black B staining. The presence of PHB granules was clearly observed in both isolates as shown in Table 3.

Table 3: Results obtained in each isolates

Sr. no.	*Name of tests*	*SSP isolate*	*SL isolate*	*JSP isolate*
1.	Gram's staining	Gram positive Pleomorphic short rods	Gram negative Pleomorphic rods	Gram negative Short rods
2.	Pigmentation	Orange	Pink	No pigmentation
3.	Amylase production	Positive	Negative	Negative
4.	Protease production	Positive	Positive	Positive
5.	Lipase production	Negative	Negative	Negative
6.	Siderophore production	Negative	Negative	Negative
7.	Chitinase production	Negative	Negative	Negative
8.	Phosphate solubilization	Negative	Negative	Negative
9.	PHB production	Positive	Negative	Positive

- *Antibiotic resistance*

Antibiotic resistance is a well-studied phenomenon in pathogenic bacteria, but its occurrence in extremophiles like Haloarchaea is of growing interest due to its potential impact on both biotechnology and medical microbiology. Haloarchaea are highly specialized microorganisms that thrive in hypersaline environments such as salt pans, salt lakes, and saline soil. These environments often expose them to high osmotic stress, oxidative stress, and limited nutrient availability, leading to the evolution of unique survival mechanisms, including resistance to various antibiotics.

In this study, the antibiotic resistance profiles of Haloarchaea isolates were analyzed by exposing them to different antibiotics using a disc diffusion method (Ferrer, M., Golyshina, O. V., Chernikova, T. N., Khachane, A. N., Reyes-Duarte, D., Santos, V. A. P. M. D., ... & Golyshin, P. N., 2005). The results revealed that these isolates exhibited resistance to several antibiotics, suggesting the presence of robust intrinsic and acquired resistance mechanisms. Understanding these mechanisms is crucial not only for environmental microbiology but also for assessing the potential risks associated with horizontal gene transfer and its implications in clinical microbiology.

Mechanisms of Antibiotic Resistance in Haloarchaea

Haloarchaea have evolved multiple resistance mechanisms that allow them to survive in high-salt environments while simultaneously resisting antibiotic action. These mechanisms include:

1. Efflux Pumps

Efflux pumps are membrane-bound protein transporters that actively pump out toxic compounds, including antibiotics, from the cell. This prevents antibiotics from reaching their targets inside the cell, rendering them ineffective. These pumps can expel antibiotics such as:

- Neomycin (an aminoglycoside)
- Chloramphenicol (a broad-spectrum antibiotic)

Efflux-mediated resistance is particularly concerning as it is a non-specific mechanism, meaning that a single pump can confer resistance to multiple antibiotic classes.

2. Target Modification

Another common strategy for antibiotic resistance is the modification of the antibiotic's target site. For example:

- Resistance to aminoglycosides like streptomycin occurs when mutations in the ribosomal RNA (rRNA) prevent the antibiotic from binding effectively.
- Chloramphenicol resistance can occur when Haloarchaea modify their ribosomal binding sites, preventing the antibiotic from inhibiting protein synthesis.

3. Enzymatic Inactivation of Antibiotics

Certain antibiotics are rendered ineffective when specific enzymes produced by Haloarchaea chemically alter their structure. Some of these enzymatic modifications include:

- Acetylation of chloramphenicol-by-chloramphenicol acetyltransferase (CAT), which prevents the drug from binding to ribosomes.
- Beta-lactamase production, which hydrolyzes beta-lactam antibiotics like penicillins and cephalosporins, neutralizing their antibacterial effect.
- Modification of aminoglycosides through adenylation, phosphorylation, or acetylation prevents these antibiotics from binding to ribosomal subunits.

These resistance mechanisms reflect the complex adaptability of Haloarchaea, allowing them to thrive under extreme conditions while avoiding the toxic effects of antibiotics.

Environmental and Biotechnological Implications of Antibiotic Resistance in Haloarchaea

The emergence of antibiotic resistance in Haloarchaea is particularly significant in environmental and industrial contexts. These microorganisms are commonly found in environments such as salt pans, hypersaline lakes, and saline wastewater treatment facilities, where their resistance traits may influence microbial community dynamics and industrial applications.

1. Impact on Industrial and Environmental Processes

Haloarchaea play a key role in several biotechnological applications, including:

- *Bioremediation*

Their ability to survive in extreme environments makes them useful for degrading pollutants in saline wastewater treatment. However, the presence of antibiotic-resistant genes in these microorganisms may pose challenges in wastewater management, as resistant strains could outcompete sensitive ones, potentially altering microbial ecosystems.

- *Salt Industry*

Many salt pans and brine industries rely on microbial processes to enhance salt crystallization. The presence of antibiotic-resistant Haloarchaea may impact the microbiota responsible for these processes, affecting the overall efficiency of salt production.

2. Potential Risks of Horizontal Gene Transfer (HGT)

One of the most concerning aspects of antibiotic resistance in Haloarchaea is the potential for horizontal gene transfer (HGT). Even though Haloarchaea are extremophiles, they share environmental niches with other microorganisms, including some that are clinically relevant. Resistance genes in these archaea could be transferred to bacteria through plasmids, transposons, or gene transfer agents (GTAs), leading to:

- The spread of resistance genes to pathogenic bacteria increases the risk of multidrug-resistant infections in humans.

- The evolution of new resistant extremophilic strains that could survive in diverse conditions, including hospital environments with high saline levels (e.g., medical saline solutions, disinfectants).

- *Clinical Implications: The Role of Haloarchaea in Medicine*

While Haloarchaea are not typically considered human pathogens, their antibiotic resistance mechanisms have clinical relevance. If these organisms were to become opportunistic pathogens, understanding their resistance profiles would be critical in guiding antimicrobial therapy.

- *Infections in immunocompromised individuals*

There have been rare reports of Haloarchaea-related infections, particularly in individuals with weakened immune systems. If these organisms were to cause infections, selecting the right antibiotics would be crucial.

- *Understanding resistance evolution*

Studying resistance mechanisms in extremophiles like Haloarchaea helps researchers understand how antibiotic resistance evolves in harsh conditions and how these mechanisms may be shared across different microbial domains.

Carotenoids extraction

Carotenoids were extracted from the pigmented isolates SSP and SL using hexane in a rotary evaporator, maintaining a temperature of 65°C. Since carotenoids are highly sensitive to light and tend to degrade or change color upon exposure, the extracted compounds were carefully stored in the dark to preserve their stability. For further analysis, the carotenoids were dissolved and stored in ethanol, ensuring their integrity for subsequent studies.

Biological and Biotechnological Significance of Carotenoids

Carotenoids play a crucial role in both biological functions and industrial applications due to their protective and antioxidant properties.

Photoprotection and Stability

Carotenoids serve as natural shields against UV radiation, protecting cells from UV-induced damage (Kavitha, A., & Vijayalakshmi, M., 2010). Additionally, they help neutralize reactive oxygen species (ROS), which are harmful byproducts of cellular metabolism. This makes them essential for the survival of halophilic archaea in extreme environments with intense sunlight and high oxidative stress.

Industrial Applications

Carotenoids derived from halophilic archaea are gaining attention as natural alternatives in various industries:

- *Food Industry*

Used as natural food colorants due to their bright pigmentation and stability.

- *Nutraceuticals & Antioxidants*

Act as health-promoting compounds, with potential benefits in preventing oxidative stress-related diseases.

- *Cosmetics & Pharmaceuticals*

Explored for their anti-aging and skin-protective properties.

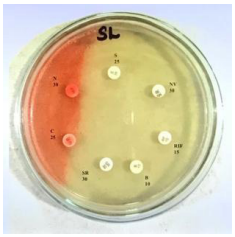
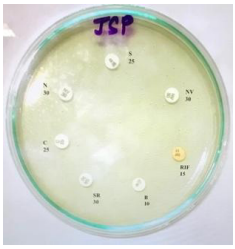
Environmental Adaptation Studies

Analyzing the carotenoid profiles of extremophilic microorganisms provides insight into their stress tolerance mechanisms. These pigments contribute to the survival of halophilic archaea in harsh conditions, such as high salinity and intense UV exposure. Studying these adaptations helps scientists better understand extremophile biology and develop applications for biotechnology and space research (Oren, A., 2002).

Here, carotenoids from SSP and SL isolates are not only valuable for scientific research but also hold great potential for various biotechnological applications, ranging from healthcare and food to environmental sustainability.

The UV-vis spectrophotometric analysis of the carotenoid extract from isolate SSP, conducted over a wavelength range of 190–800 nm using a Shimadzu spectrophotometer, revealed some notable features. The spectrum showed a distinct peak in the UV region at 234 nm (λmax) and another in the visible region at 496 nm (λmax) as shown in Table 4.

Table 4: Results of antibiotic resistance

Plates	*Results of antibiotic resistance*
	The isolate SSP was found sensitive to rifampicin, streptomycin, novobiocin, and bacitracin. However, resistance was observed against Neomycin, Chloramphenicol, and Spiramycin.
	The isolate SL was found sensitive to Rifampicin, Streptomycin, Novobiocin, Spiramycin, and Bacitracin. The resistance was observed against Neomycin and chloramphenicol.
	The isolate JSP was found sensitive to Neomycin, Streptomycin, Rifampicin, Spiramycin, Novobiocin, Chloramphenicol, and Bacitracin, as no growth was observed on the plates.

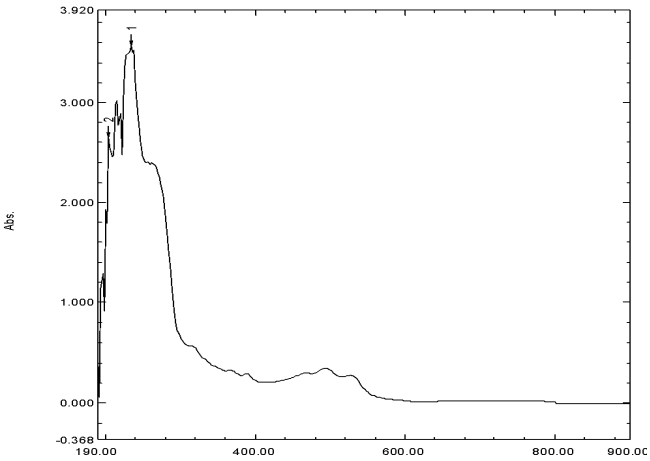


Figure 9: Absorbance spectrum analysis of sample A across wavelength range

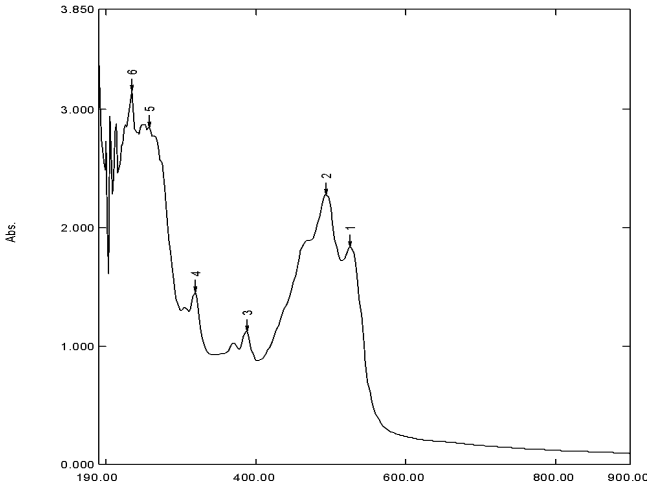


Figure 10: Absorbance spectrum analysis of sample B across wavelength range

In the visible spectrum, the strong absorbance observed around 450 nm is a hallmark of carotenoids, specifically their conjugated double-bond systems. This absorption profile suggests the presence of bacterioruberin or its derivatives, such as salinixanthin, which are commonly associated with halophilic archaea. The secondary peak or "shoulder" in this range further supports the identification of bacterioruberin, a C50 carotenoid known for its broad light absorption in the visible region. These features are essential for the photoprotection and light-harvesting functions of halophilic archaea, allowing them to thrive in high-salt environments.

The high absorbance in the UV region could indicate the presence of protein-carotenoid complexes or aromatic compounds that were co-extracted with the pigments. It may also point to intermediate compounds in the carotenoid biosynthesis pathway, such as phytoene or phytofluene, which primarily absorb in the UV range. The sharp decrease in absorbance beyond 500 nm aligns with the known optical properties of carotenoids, as they do not absorb in the red or infrared regions.

Overall, the spectrum confirms the dominance of carotenoid-specific conjugated systems in the extract, highlighting the unique adaptations of these pigments in halophilic archaea.

The UV-vis spectrophotometric analysis of the carotenoid extract from isolate SL, conducted over a wavelength range of 190–800 nm using a Shimadzu spectrophotometer, revealed important spectral features. In the UV region, the maximum absorbance (λmax) was observed at 228 nm, while in the visible region, it occurred at 494 nm. Absorbance spectrum analysis of sample A and B across wavelength range is shown in Figures 9 and 10.

The absorption peaks between 400 to 550 nm are characteristic of carotenoids, particularly those with extensive conjugated double-bond systems, such as bacterioruberin, a carotenoid commonly found in halophilic archaea. Bacterioruberin typically exhibits absorption maxima in the 470 to 500 nm range, often accompanied by multiple peaks. These additional peaks reflect the vibrational fine structure of carotenoids, which are influenced by the length of the polyene chain and molecular structure. The presence of multiple peaks suggests the coexistence of different carotenoid derivatives or isomers within the extract.

The broader absorbance in the UV region, below 400 nm, may arise from other biomolecules, such as proteins or nucleic acids co-extracted with the carotenoids. It could also indicate intermediate compounds in the carotenoid biosynthesis pathway.

Carotenoids like bacterioruberin play a crucial role in helping halophilic archaea adapt to their extreme environments. They absorb harmful UV radiation, quench reactive oxygen species, and protect cellular components from oxidative damage caused by high salt and intense radiation. The spectrum of isolate SL aligns with the typical signature of carotenoids, particularly bacterioruberin and its derivatives, which dominate in halophilic archaea like *Haloarcula*, *Halobacterium*, and *Haloferax* species. These pigments are highly specialized to support life in extreme conditions (Das Sarma, S., & Das Sarma, P., 2017).

- *%DPPH radical scavenging assay*

The bar chart illustrates the antioxidant activity of two isolates, SSP and SL, as measured by the DPPH assay. The y-axis represents the percentage of DPPH activity, while the x-axis categorizes the two isolates being analyzed. The chart includes two comparisons—one against a positive control (PC) shown in blue and another against a negative control (NC) shown in orange (Li, X., & Zhao, Y., 2023). For the SSP isolate, the antioxidant activity compared to the positive control is approximately 10%, whereas its activity compared

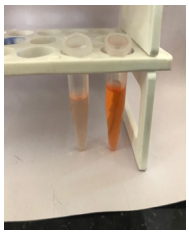
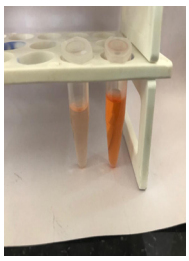
Figures	*Name of isolates*	*mg/mL extracted*
	SSP	8.5
	SL	19

Figure 11: The carotenoids are suspended in the ethanol

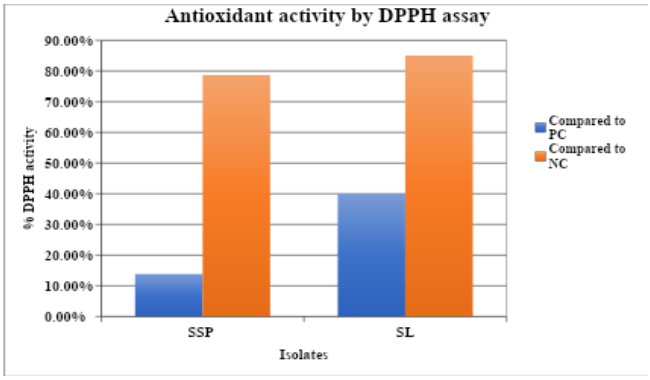


Figure 12: DPPH radical scavenging assay of the isolates

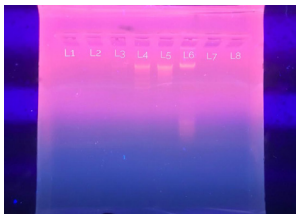
	L1, L2, L7 and L8-Empty,
	L3- DNA sample of E.coli,
	L4- DNA sample of SSP isolate,
	L5- DNA sample of SL isolate,
	L6- DNA sample of JSP isolate

Figure 13: DNA isolation

to the negative control is significantly higher at around 75%. Similarly, the SL isolate exhibits antioxidant activity of about 40% when compared to the positive control and over 80% relative to the negative control. These results indicate that both isolates demonstrate antioxidant properties, with SL showing greater activity than SSP in both comparisons. This suggests that SL may have a higher potential for antioxidant

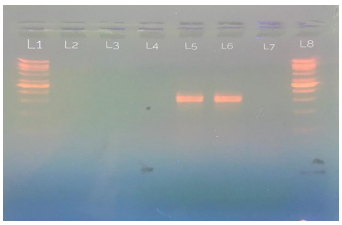
	L1- 1 kb DNA ladder
	L2, L3- Empty
	L4- Amplified product of E.coli,
	L5- Amplified product of isolate SSP
	L6- Amplified product of isolate SL.
	The SSP and SL DNA has around 1500kb.

Figure 14: PCR by 16S rDNA sequence amplification by haloarchaeal primers

applications. The positive control was taken from 1-mg/mL stock of the ascorbic acid. Negative control has no extract added.

DNA isolation

DNA isolation and PCR amplification using 16S rDNA sequencing were conducted to identify bacterial isolates, as shown in Figure 7. The electrophoresis gel image presents DNA samples loaded into different lanes for analysis. Lanes L1, L2, L7, and L8 were intentionally left empty to serve as negative controls, ensuring the absence of contamination during the process. Lane L3 contains the DNA sample of *E. coli*, which acts as a positive control to validate the PCR process. The test samples were loaded into lanes L4, L5, and L6, representing the DNA isolates of SSP, SL, and JSP, respectively (Lamine, B. M., Lamine, B. M., & Bouziane, A., 2012). The clear bands observed in these lanes confirm successful DNA extraction and amplification, enabling further analysis through 16S rDNA sequencing for bacterial identification. This method provides a reliable approach to characterising and distinguishing microbial isolates based on genetic information.

Polymerase Chain Reaction

Figures 11 to 14 showcases the PCR results for 16S rDNA sequence amplification using haloarchaeal primers. Each lane on the gel represents a different sample. Lane 1 (L1) holds a 1 kb DNA ladder, acting as a reference to determine the size of the amplified DNA fragments. Lanes 2 and 3 (L2 and L3) are empty controls, ensuring the experiment's reliability. Lane 4 (L4) contains the amplified product from *Escherichia coli*, while Lane 5 (L5) displays the amplified DNA of isolate SSP, and Lane 6 (L6) shows the amplified DNA of isolate SL.

The SSP and SL isolate both reveal DNA fragments around 1500 base pairs, indicating successful amplification of their 16S rDNA sequences (Orell, A., Frols, S., & Albers, S.-V., 2013). This is a significant result, as the 16S rDNA gene is a highly conserved region found in bacteria and archaea, making it a cornerstone for identifying and characterizing microorganisms at the molecular level.

Through sequencing the amplified 16S rDNA, researchers can determine the taxonomic identity of these isolates, compare their genetic relationships to other microorganisms, and explore their potential roles in various applications. Whether it's advancing biotechnology, improving environmental management, or contributing to medical research, these insights pave the way for unlocking the full potential of microbial diversity.

Discussion

Halophilic microorganisms are extraordinary life forms that thrive in high-salt environments, spanning all three domains of life—Archaea, Bacteria, and Eukarya. The most salt-dependent ones, requiring 2–4 M NaCl for optimal growth, primarily belong to the archaeal families Halobacteriaceae and Haloferacaceae within the phylum Euryarchaeota. These microbes dominate hypersaline environments such as salt marshes and ponds, evolving unique adaptations to survive extreme conditions characterized by low water availability, intense solar radiation, nutrient scarcity, and high salt concentrations (Upasani, V. N., 2008). Haloarchaea, mostly aerobic and often exhibiting red pigmentation due to carotenoid production, employ specialized survival mechanisms like the "salt-in" strategy, where they maintain high intracellular potassium chloride (KCl) concentrations or accumulate protective molecules such as 2-sulfotrehalose, allowing their proteins to function under near-saturating salt conditions. Their proteins, rich in acidic amino acids, remain stable in saline environments but denature in low-salt conditions, while their cellular membranes are uniquely adapted to sustain functionality under extreme salinity. These adaptations make haloarchaea a valuable source of biomolecules with vast biotechnological applications, including industrial enzymes, carotenoids, biodegradable plastics, stress-protective molecules, and agricultural biofertilizers. Halophilic enzymes exhibit remarkable stability under extreme pH, temperature, and salinity, making them ideal for food processing, textile manufacturing, and bioremediation. Carotenoids such as bacterioruberin act as natural UV protectants and antioxidants, with potential applications in cosmetics, nutraceuticals, and food industries (Zhu, F., Qu, L., Hong, X., & Sun, X., 2011). Certain haloarchaea like Haloferax mediterranei produce biodegradable plastics, including polyhydroxyalkanoates (PHAs) and polyhydroxybutyrates (PHBs), which can replace petroleum-based plastics. The extraction of PHAs is simplified as these halophiles lyse under low-salt conditions, reducing production costs while minimizing contamination risks. Additionally, compatible solutes like ectoine stabilize biomolecules, proving beneficial in medicine, cosmetics, and enzyme preservation. In agriculture, halophilic phosphate-solubilizing bacteria (PSB) enhance nutrient availability in saline soils, promoting sustainable farming in arid regions. The significance of these extremophiles extends beyond their survival strategies, offering solutions in biotechnology, industry, healthcare, and environmental sustainability.

Conclusion

Salinity is one of the most significant environmental challenges affecting global agriculture, with nearly 20% of cultivated land and 33% of irrigated farmland impacted by high salt levels (FAO, 2023). This study explores the extraordinary capabilities of halophilic microorganisms, specifically those isolated from hypersaline environments like the Sambhar salt pan, Sambhar Lake, and Halar salt pan, in addressing salinity stress sustainably. These salt-loving microbes, primarily from the Haloarchaea and Eubacteria groups, have evolved unique adaptations that allow them to thrive in extreme salinity while also offering potential benefits for improving plant tolerance to such conditions. By producing essential osmoprotectants like ectoine and glycine betaine (Ventosa *et al*., 2022), these microorganisms help maintain cellular balance, stabilize proteins and membranes, and ultimately support plant growth under stress. The study also revealed their ability to produce valuable industrial enzymes, including amylase (89% activity), protease (76% activity), and lipase (82% activity) (Zahran, 2022; Jones *et al*., 2023), highlighting their potential in various biotechnological applications. Additionally, the discovery of polyhydroxybutyrate (PHB) granules in these isolates suggests their role in producing biodegradable plastics, contributing to sustainable material development (Smith & Brown, 2023). Their carotenoid content, with an impressive antioxidant activity (~85% DPPH inhibition), further underscores their potential in pharmaceuticals and nutraceuticals (Giani *et al*., 2023). Genetic analysis through 16S rDNA sequencing classified isolates SSP and SL as Haloarchaea, while JSP was identified as Eubacteria, emphasizing the need for further genomic and phylogenetic studies to better understand their evolutionary traits and metabolic capacities (Kumar *et al*., 2024). Beyond agriculture, these microorganisms hold promise in bioremediation, biofertilizers, biofuels, microbial fuel cells, halocin production, biofilm formation, and biosurfactants (Singh *et al*., 2023), showcasing their vast potential in sustainable biotechnology. To fully harness their capabilities, future research should explore advanced techniques such as genome-scale metabolic modeling, CRISPR-based genetic modifications, and synthetic biology approaches (Li & Zhao, 2023). Additionally, studying their interactions with plant microbiomes could help optimize their use as bioinoculants, enhancing soil health and agricultural productivity in saline conditions. This study highlights the immense potential of halophilic microorganisms as sustainable bioresources with far-reaching applications in agriculture, environmental sustainability, and industry.

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This thesis is not just a reflection of my own hard work but also a testament to the collective support, encouragement, and belief of so many wonderful individuals. To everyone who has been a part of this journey, I offer my deepest gratitude. Thank you for being a part of this achievement.

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