

Isolation of Halophilic Micro Organism from Marine Soil and Its Enzyme Potential

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Abstract

Halophilic microorganisms represent a unique group of extremophiles that have adapted to survive and thrive in environments characterized by high salinity. These organisms are not merely salt-tolerant; they require specific concentrations of sodium chloride (NaCl) to maintain their structural integrity and physiological functions. The marine ecosystem, with its vast coastal soil and sediment, serves as a primary habitat for such microbes. This research focuses on the systematic isolation of halophilic bacteria from marine soil collected from Dhanushkodi, Tamil Nadu, and the subsequent evaluation of their potential to produce enzymes of industrial significance, specifically amylase and lipase.

Keywords: halophilic microorganisms, marine soil, Bacillus, amylase, lipase, extremozymes, enzyme potential

INTRODUCTION

1.1 Background of the Study

Microbiology has traditionally focused on organisms that grow under moderate conditions. However, the discovery of extremophiles—organisms that thrive in conditions previously thought to be inhospitable—has opened new frontiers in biological research. Among these, halophiles occupy a significant position. Halophiles are "salt-loving" organisms that inhabit environments such as the Dead Sea, Great Salt Lake, and various marine coastal zones. Unlike terrestrial microbes, these organisms have specialized metabolic pathways and protein structures that prevent denaturation in the presence of high salt.

The marine environment is a complex ecosystem where salinity, temperature, and pressure fluctuate. Marine soil, or coastal sediment, is particularly interesting because it acts as a sink for organic matter, providing a nutrient-rich yet high-salinity environment for microbial growth. This specific ecological niche selects microbes that can produce enzymes capable of breaking down complex organic molecules like starch and lipids under extreme conditions.

Industrially, there is a growing demand for enzymes that are robust. Most standard enzymes used in manufacturing lose their activity when exposed to high salt or high temperatures. Extremozymes, particularly those from halophiles, are naturally designed to be stable. This makes the search for halophilic enzyme producers not just a matter of basic science, but a pursuit of significant economic and industrial value. This study seeks to bridge the gap between ecological exploration and industrial application by identifying new microbial sources of stable enzymes.

1.2 Classification of Halophiles

Halophiles are generally categorized based on the concentration of NaCl required for optimal growth. This

classification is vital for understanding their ecological roles and potential applications. Slight Halophiles: These organisms grow best in environments with 1% to 3% NaCl. Most common marine bacteria fall into this category, as seawater typically has a salinity of approximately 3.5%. Moderate Halophiles: These are the most versatile group, growing in 3% to 15% NaCl. They are often found in saline soils and solar salterns and are highly valued for their industrial robustness. Extreme Halophiles: These organisms require very high salinity, often between 15% and 30% NaCl. They are frequent members of the Archaea domain and are found in hypersaline lakes.

1.3 Physiological Adaptations

To survive the high osmotic pressure of their surroundings, halophiles have evolved two primary strategies. The first is the "Salt-in" strategy, where the organism accumulates high concentrations of inorganic ions (like potassium) within the cytoplasm to balance the external salinity. This requires the evolution of specialized proteins that do not aggregate or denature in high salt.

The second strategy involves the production of "Compatible Solutes." These are organic compounds like ectoine, glycine betaine, and various sugars that help maintain osmotic balance without interfering with cellular metabolism. These solutes themselves have high value in the cosmetic and pharmaceutical industries for their protective properties on human skin and proteins. Understanding these adaptations is crucial for the cultivation of these microbes in a laboratory setting.

1.4 The Concept of Extremozymes

Enzymes produced by halophilic microbes are often termed "halozymes." These are a subset of extremozymes. The structural integrity of these enzymes is maintained by a high frequency of acidic amino acids (aspartate and glutamate) on the protein surface. These negatively charged residues form a hydration shell that prevents the protein from collapsing or precipitating in the presence of high salt concentrations. Standard industrial processes often involve high salt concentrations, such as in the treatment of leather, textile processing, and the production of fermented foods. Standard enzymes would fail in these environments. Halozymes, however, remain active and efficient.

1.5 Amylases from Halophiles

Amylases are enzymes that break down starch into smaller saccharides. Halophilic amylases are particularly useful in the food industry for the production of glucose and maltose syrups from starch under saline conditions. They are also used in the textile industry for "desizing," where starch used to strengthen threads must be removed after weaving. If the process involves saline water, halophilic amylases are the only viable option.

1.6 Lipases from Halophiles

Lipases catalyze the hydrolysis of fats and oils. Halophilic lipases are highly sought after for detergent formulations. Since many laundry environments involve high ionic strength and alkaline pH, these enzymes are perfect for removing tough oil stains. Furthermore, they are used in the synthesis of fine chemicals and biodiesel, where water activity is low and salt presence might be high.

2. MATERIALS AND METHODS

2.1 Study Area and Sampling

The study was conducted using marine soil samples collected from the Dhanushkodi coastal area in Tamil Nadu, India. Dhanushkodi is a unique geographic location where the Bay of Bengal meets the Indian Ocean, creating a high-salinity environment with unique microbial profiles. Soil samples were collected from three different locations along the shoreline. At each site, the top layer of debris was removed, and soil

was collected from a depth of 5 to 10 cm using sterile spatulas. The samples were immediately transferred into sterile polypropylene bags and transported to the laboratory under refrigerated conditions (4°C) to maintain microbial viability and prevent the overgrowth of opportunistic mesophiles.

2.2 Enrichment and Isolation

To increase the population of halophiles, enriching culture techniques were employed. One gram of each soil sample was added to 100 ml of Nutrient Broth supplemented with 5% (w/v) NaCl. The flasks were incubated in an orbital shaker at 150 rpm at 37°C for 48 hours. Following enrichment, the samples were subjected to serial dilution. Dilutions ranging from 10⁻¹ to 10⁻⁶ were prepared in sterile saline water (0.85% NaCl).

A volume of 0.1 ml from each dilution was spread onto Halophilic Agar plates (Nutrient Agar containing 10% NaCl). The plates were incubated at 37°C for 3 to 5 days. After incubation, colonies showing distinct morphological features (color, shape, size, margin) were selected. These colonies were further purified by sub-culturing onto fresh halophilic agar plates until pure cultures were obtained. The pure cultures were maintained on agar slants at 4°C and preserved in 20% glycerol stocks at -20°C for long-term use.

2.3 Morphological and Microscopic Characterization

The isolated bacterial strains were first characterized based on their colony morphology on agar plates. Parameters such as colony color, shape (circular, irregular), elevation (flat, convex, umbonate), and margin (entire, lobate) were recorded. Microscopic examination was performed using Gram staining to determine the cell wall structure (Gram-positive or Gram-negative) and cell shape (cocci or bacilli). Motility tests were also conducted using the hanging drop method to observe the movement of the bacteria.

2.4 Biochemical Characterization

A series of biochemical tests were performed to identify the metabolic capabilities of isolates. These included: Indole Test: To determine the ability of the organism to produce indole from the amino acid tryptophan. Methyl Red (MR) and Voges-Proskauer (VP) Tests: To identify the fermentation pathway (mixed acid vs. butanediol fermentation). Citrate Utilization: To see if the bacteria could use citrate as their sole carbon source.

Catalase and Oxidase Tests: To check for the presence of respiratory enzymes. Urease Test: To determine the ability to hydrolyze urea into ammonia and carbon dioxide.

3. ENZYME SCREENING PROCEDURES

3.1 Qualitative Screening for Amylase

The isolates were screened for amylolytic activity using the starch hydrolysis test. Halophilic agar medium was supplemented with 1% (w/v) soluble starch. The isolates were spot inoculated onto the plates and incubated at 37°C for 48 to 72 hours. After growth, the plates were flooded with Gram's Iodine solution. Iodine reacts with starch to produce a dark blue/purple color. The appearance of a clear, colorless zone around the bacterial colony indicates that the organism has produced extracellular amylase, which has degraded starch in its immediate vicinity. The diameter of the clear zone was measured in millimeters to provide a relative assessment of enzyme production.

3.2 Qualitative Screening for Lipase

Lipolytic activity was determined using Tributyrin Agar. This medium contains tributyrin oil, which gives the agar an opaque, cloudy appearance. The halophilic isolates were spot-inoculated and incubated. Lipase production results in the hydrolysis of tributyrin into glycerol and fatty acids, which are soluble and result in a clear halo around the colony. The size of this "zone of clearance" is directly proportional to the

lipolytic potential of the strain. This method is highly effective for identifying microbes capable of breaking down complex lipids, a trait highly valued for detergent and biodiesel applications.

4. MOLECULAR STUDIES AND QUANTIFICATION

4.1 16S rRNA Sequencing

To confirm the identity of the most promising enzyme producers at the species level, molecular analysis was performed. Genomic DNA was extracted from the pure cultures using a standard DNA isolation kit. The 16S rRNA gene, which is a universal marker for bacterial identification, was amplified using Polymerase Chain Reaction (PCR) with universal bacterial primers (27F and 1492R). The PCR conditions involved an initial denaturation at 95°C, followed by 30 cycles of denaturation, annealing, and extension. The resulting DNA fragments (approx. 1500 base pairs) were visualized using agarose gel electrophoresis. The PCR products were then sequenced. The obtained sequences were compared against the NCBI database using the Basic Local Alignment Search Tool

4.2 Quantitative Amylase Assay (DNS Method)

The amount of amylase produced was quantified using the Dinitrosalicylic Acid (DNS) method. The isolates were grown in a production broth, and the cell-free supernatant (crude enzyme) was collected by centrifugation. The enzyme was incubated with a starch substrate at a controlled temperature and pH. The reaction was stopped by adding DNS reagent, which reacts with the reducing sugars (like maltose) released by the enzyme. The mixture was boiled, and the resulting color change was measured at 540 nm using a spectrophotometer. One unit of amylase activity is defined as the amount of enzyme that releases 1 μ mol of reducing sugar per minute under assay conditions.

5. RESULTS AND DISCUSSION

5.1 Isolation and Morphological Findings

The study successfully isolated ten distinct halophilic bacterial strains from the Dhanushkodi marine soil samples. These were labeled S1 through S10. Most isolates exhibited characteristics typical of the *Bacillus* genus: they were Gram-positive, rod-shaped, and formed creamy, circular colonies with smooth margins. The isolates showed a strong preference for high salinity, with optimal growth observed at 10% NaCl, confirming their status as moderate halophiles.

5.2 Enzyme Production Analysis

Out of the ten isolates, five showed significant amylolytic activity and four showed lipolytic activity. Strain S4 and S7 were the most versatile, producing both enzymes. In the qualitative tests, *Bacillus tequilensis* (Strain S4) produced a starch hydrolysis zone of 18 mm and a lipase clearance zone of 15 mm. The quantitative DNS assay confirmed the high activity of the S4 strain. The results suggest that these halophiles have adapted their extracellular enzymes to function at high ionic strengths, where standard mesophilic enzymes would typically lose more than 90% of their activity. The high activity of *Bacillus tequilensis* aligns with previous studies that have identified this species as a prolific producer of secondary metabolites and enzymes in saline environments.

5.1. Isolation of halophilic bacteria's

The marine soil sample was collected under sterile conditions and used as a source for the isolation of halophilic organism (Fig:1).

5.2 Morphological and biochemical characteristics

5.2.1 Colony morphology

The isolated organism showed typical *Bacillus* colony characteristics with slight differences in shape, texture, and surface.

5.2.2 Gram's stain

Isolated Halophilic organism appears as Gram-positive, rod-shaped (bacilli) bacteria that stain purple under Gram staining (Fig:3).

5.2.3 Biochemical characterization

Biochemical tests were performed to characterize and identify the isolated microorganism based on its metabolic activities. The results confirmed typical properties of (Table:1).

5.3. Enzyme screening

Screening of enzyme-producing halophilic bacteria showing (fig10) amylase activity on starch agar with clear zone after iodine staining and (fig11) lipase activity on tributyrin agar with clear zone around colonies.

(Amylase producing Starch hydrolyse bacteria)

5.4. Molecular identification

5.4.1 For *Bacillus* sp.

PCR amplification confirmed the isolates as *Bacillus* species by showing ~1500 bp 16S rRNA gene bands. (fig12) of 16S rRNA gene from the lipase sample

Sanger sequencing of the 16S rRNA gene confirmed that the isolate belongs to *Bacillus* sp. BLAST analysis of the 16S rRNA gene sequence showed high similarity with *Bacillus* sp., confirming the identity of the isolates.

5.2 For *Bacillus tequilensis*:

PCR amplification confirmed the isolates as *Bacillus* species by showing ~1500 bp 16S rRNA gene bands. (fig15)

Sanger sequencing of the 16S rRNA gene confirmed that the isolate belongs to *Bacillus tequilensis*. (fig16)

BLAST analysis of the 16S rRNA gene sequence showed high similarity with *Bacillus tequilensis* confirming the identity of the isolates. (fig17)

The culture broth was centrifuged, and the supernatant was collected as crude enzyme extract for amylase and lipase assays.

6.1 DNSA assay for amylase

DNSA assay confirmed amylase production, with *Bacillus tequilensis* showing higher activity than *Bacillus* sp.

6.2 Spectrophotometric Analysis of DNSA Assay

Absorbance at 546 nm confirmed amylase activity, with *Bacillus tequilensis* showing higher activity than *Bacillus* sp. Amylase activity was confirmed by DNSA assay, with maximum absorbance of 0.247 at 546 nm, indicating effective starch hydrolysis. (fig 20)

Table 1. Biochemical characterization

S. No.	Biochemical Test	Bacillus tequilensis	Bacillus sp.
1	Indole Test	Negative	Negative
2	Methyl Red Test	Positive (+)	Positive (+)
3	Voges-Proskauer Test	Positive (+)	Positive (+)
4	Citrate Utilization	Positive (+)	Positive (+)
5	Urease Test	Negative	Negative
6	Oxidase Test	Positive (+)	Positive
7	Catalase Test	Positive (+)	Positive (+)

Table 2. Enzyme screening results

Isolate ID	Genus and species	Amylase zone (mm)	Lipase zone (mm)
S1	Bacillus sp.	12	10
S4	Bacillus tequilensis	18	15
S7	Bacillus sp.	14	12

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