

ISOLATION, CHARACTERIZATION, AND MOLECULAR IDENTIFICATION OF HALOPHILIC *STREPTOMYCES* SP. FROM VEDARANYAM SALTPAN SOILS, TAMIL NADU

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ABSTRACT

Halophilic and halotolerant actinomycetes represent an important source of novel bioactive compounds with potential applications in biotechnology and pharmaceutical research. In the present study, saltpan soil samples were collected from Vedaranyam, Nagapattinam District, Tamil Nadu, at a depth of 10 cm for the isolation and characterization of halophilic actinomycetes. The samples were inoculated onto starch casein agar supplemented with different concentrations of NaCl, resulting in the recovery of 12 actinomycete isolates. Among these, five isolates exhibited growth at 5–10% NaCl, indicating moderate halophilic characteristics. Physiological and morphological analyses revealed that SS1 was Gram-positive and non-motile, producing circular colonies with white aerial mycelium and long chains of spores. Biochemical characterization further supported its affiliation with the genus *Streptomyces*. Light microscopic observations confirmed its filamentous morphology and spore-forming characteristics. Molecular identification based on 16S rRNA gene sequencing and phylogenetic analysis revealed that SS1 was closely related to *Streptomyces fulvissimus*. Further studies should focus on purification and structural characterization of the bioactive metabolites produced by SS1, optimization of fermentation conditions, evaluation of their antibacterial spectrum against multidrug-resistant pathogens.

Keywords: Halophilic actinomycetes; saltpan soil; 16S rRNA sequencing; phylogenetic analysis.

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INTRODUCTION

Actinomycetes are a major group of filamentous Gram-positive bacteria primarily found in terrestrial and aquatic ecosystems. They are famous for their ability to produce a wide range of secondary metabolites such as antibiotics, antifungal, antiviral, immunosuppressant, enzymes and anticancer agents (Berdy, 2005). Actinomycetes are responsible for over 70% of the antibiotics currently used in medicine and are of critical importance to human health and industry (Baltz, 2008). Actinomycetes are mostly found in soil habitats; saline habitats are source of novel actinomycetes. These environments often harbor unique microorganisms that have evolved special mechanisms to survive under stress, such as high salt concentrations, temperature fluctuations, desiccation and nutrient limitation (Ventura *et al.*, 2007).

Halophilic actinomycetes are an unexplored source of new bioactive compounds that could be crucial in the battle against emerging antibiotic resistant pathogens (Rathinam *et al.*, 2023). Halophiles are microorganisms that need or tolerate high concentrations of sodium chloride (NaCl) for growth and are commonly found in saline and hypersaline environments such as salt pans, solar salterns, salt lakes, and saline soils. Depending on their optimal NaCl requirements, they can be

divided into slight halophiles (1–5% NaCl), moderate halophiles (5–20% NaCl) and extreme halophiles (20–30% NaCl) (Yoo *et al.*, 2023).

Halophilic actinomycetes represent a promising and relatively unexplored source of novel bioactive compounds and produce a new antimicrobial agent to combat the increasing threat of antibiotic resistance (Thompson & Gilmore, 2024).

Saltpans are hypersaline ecosystems associated with the progressive evaporation of saltwater leading to the concentration of dissolved salts and the production of brine and salt crystallization ponds. These habitats are characterized by steep salinity gradients and exposed to a variety of physicochemical challenges such as high osmotic pressure, strong sun radiation, high and changing temperatures and low water availability (Pindi *et al.*, 2023).

Many strains of the genus *Streptomonospora* were found in halophilic filamentous actinomycetes from a saline lake in western China Li *et al.* (2006). The potential of halophilic and halotolerant actinomycetes as sources of novel bioactive secondary metabolites, highlighting their diversity and biotechnological value (Hamedi *et al.*, 2013). Actinomycetes from the genera *Streptomyces*, *Pseudonocardia*, and

Actinobacteria were found to be isolated from hypersaline inland solar salterns at Sambhar Salt Lake in India. Several of these isolates demonstrated strong antimicrobial activity against bacterial and fungal test organisms (Vijayakumar *et al.*, 2013).

The isolation and initial characterization of saline-adapted actinomycetes from the salt pans of Vedaranyam, Nagapattinam District, Tamil Nadu, are the main objectives of this work. In order to find interesting strains with possible antibacterial and medicinal uses, the study intends to investigate their microbial diversity.

MATERIALS AND METHODS

Sample Collection

The saltpan soil samples were collected from Vedaranyam located in Nagapattinam District. These samples were collected at 10 cm depth of soil surface. The soil samples were collected at random in a sterile Zip-lock cover and stored in a refrigerator for further use.

Pre-treatment of Soil Samples

Soil samples were air-dried at room temperature for one week, manually crushed and sieved to remove gravel and debris. The dried and sieved soil samples were subsequently subjected to calcium carbonate (CaCO_3) pretreatment to facilitate the selective enrichment and isolation of actinomycetes (Antido, and Climacosa, 2022).

Isolation of Halophilic Actinomycetes

Starch Casein Agar (SCA), ISP Medium No. 2 (Yeast Extract-Malt Extract Agar), and Actinomycete Isolation Agar (AIA), supplemented with varying amounts of NaCl to enhance the recovery of salt-adapted actinomycetes, were used to selectively isolate halophilic actinomycetes. Prior to sterilization, the media's pH was brought to roughly neutral levels. Aliquots of pretreated soil samples were spread onto the appropriate isolation media after being serially diluted and suspended in sterile saline. For 7–14 days, the inoculation plates were incubated at a temperature of about 28–30°C. To obtain pure cultures, colonies exhibiting typical actinomycete morphology such as chalky, powdery, or pigmented appearances were chosen and regularly subcultured on suitable conditions. The purified isolates were kept as glycerol stocks for long-term preservation on agar slants at 4°C Al-Mahdi *et al.* (2023) and Sahu *et al.* (2022).

Purification of Isolates

Colonies exhibiting characteristic actinomycete morphology, such as powdery, chalky, or leathery appearance, were selected from the isolation plates. The selected colonies were purified by repeated streaking on the respective isolation medium and incubated at $28 \pm 2^\circ\text{C}$ until adequate growth was obtained. The cultural characteristics, including colony texture, colour, and pigmentation, were recorded, and individual codes were assigned to the purified isolates. The purified actinomycete cultures were subsequently maintained on

appropriate agar slants at 4°C for further studies (Shrestha *et al.*, 2021).

Microscopic Observation

Gram Staining

Gram staining was performed to determine the Gram reaction and cellular morphology of the actinomycete isolates. A thin smear of the culture was prepared on a clean glass slide, air-dried, and heat-fixed. The fixed smear was treated with crystal violet for 1 min and gently rinsed with water, followed by Gram's iodine as the mordant. The smear was subsequently decolorized with 95% ethanol, rinsed, and counterstained with safranin. After washing and drying, the stained smear was examined microscopically under oil immersion to determine the Gram reaction and morphological characteristics of the isolates (Waithaka *et al.*, 2017).

Biochemical Identification (MacFaddin, 2000)

Indole production test

Indole is one of the metabolic degradation products of the tryptophan amino acid. The test strains were inoculated into tryptone broth and incubated at room temperature for 24 - 48 hours. After incubation, a 0.2ml of Kovac's reagent was added to the test tubes. The development of the cherry red color ring indicates the production of indole.

Methyl- Red test

The methyl red test was used to differentiate mixed acid fermentations. The test strains were inoculated into MR-VP broth and incubated at 37°C for 24-48 hours. After incubation, add a few drops of methyl red solution to test strains. The development of the red colour indicates a positive result and the yellow colour indicates negative results.

Voges-Proskauer (VP) test

The Voges-Proskauer test was performed to detect the acetyl methyl carbinol (Acetoin). The test strains were inoculated into the MR-VP broth and incubated at 37°C for 24-48 hrs. After incubation, 0.6 ml of 5% alpha naphthol and 0.2 ml of 40% potassium hydroxide was added. The tubes stand for 15 minutes and pink colour indicates positive and absence of pink colour is negative.

Citrate Utilization test

The citrate utilization test to determine the ability of an organism to utilize sodium citrate as the carbon source. The test strains were streaked on Sterile Simmon's Citrate agar slant and incubated at 37°C for 24 hours. After the incubation, development of deep Prussian blue colour that indicates the citrate positive results.

Triple Sugar Iron Test

To differentiate the sugar fermentation, anaerobic respiration, hydrogen sulphide production. The test strains were inoculated on TSI slant and then incubated at 37°C for 24 - 48 hours. After incubation Acid butt and Alkaline

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slant (yellow butt and red slant)glucose has been fermented but not lactose and sucrose .Acid butt and Acid slant (yellow butt and yellow slant –)lactose and/or sucrose have been fermented .Alkaline butt and Alkaline slant (red butt and red slant –)neither glucose, lactose nor sucrose has been fermented .Gas production has been indicated by gas bubbles in the butt and agar may be broken .H₂S production is indicated by the blackening of butt due to the reaction of H₂S with ferrous ammonium sulphate to form black ferrous sulphide .

Urease Test

Urea hydrolysis was detected by the urease test. Urea is a diamide of carbonic acid. The test strains were inoculated in Sterilized Christensen's Urease agar slant with phenol red indicator (pH 6.8). the tubes were Incubated at 37°C for 24 hours. After the incubation development of pink or red colour indicated a positive reaction.

Catalase Test

Catalase enzyme acts as a catalyst in the breakdown of hydrogen peroxide to oxygen and water. Place a drop of H₂O₂ on a slide and one loopful of test strains were mixed. Immediate liberation of air bubbles that indicates catalase positive.

Oxidase Test

The sterile Hi-media oxidase disc Tetramethyl para-phenylenediamine dihydrochloride was placed on a clean petri dish. The test strains were smeared on the disc. The presence of purple colour within 10-15 seconds indicates the suspected organisms are oxidase positive.

Molecular Profiling

Genomic DNA was extracted from pure cultures of the halophilic actinomycete isolates using the NucleoSpin® Tissue Kit (Macherey-Nagel, Germany), following the manufacturer's instructions. A pure culture of each isolate was transferred into a microcentrifuge tube, and 180 µL of T1 buffer and 25 µL of proteinase K were added. The samples were incubated at 56 °C in a water bath until complete cell lysis was achieved. Following lysis, 5 µL of RNase A (100 mg/mL) was added and the samples were incubated at room temperature for 5 min. Subsequently, 200 µL of B3 buffer was added and the samples were incubated at 70 °C for 10 min. Then, 210 µL of 100% ethanol was added and the contents were thoroughly mixed by vortexing.

The resulting lysate was transferred to a NucleoSpin® Tissue column placed in a 2-mL collection tube and centrifuged at 11,000 × g for 1 min. The column was then transferred to a fresh 2-mL collection tube and washed with 500 µL of BW buffer. A second washing step was performed using 600 µL of B5 buffer. After completion of the washing steps, the NucleoSpin® Tissue column was

transferred to a clean 1.5-mL microcentrifuge tube, and the genomic DNA was eluted using 50 µL of BE buffer. The purified genomic DNA was subsequently used for molecular characterization of the halophilic actinomycete isolates (Muthuraja *et al.*, 2025).

PCR amplification of 16S rRNA

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

Primers used

Tar get	Pri mer Name	Direct ion	Sequence (5' → 3')
16S rRNA	16S-RS-F	Forwa rd	GGGTGGGGTATCCAG GCGTCAA
	16S-RS-R	Revers e	GGGCCCAAATGTCAC CTGGGTT

Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 4 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using a Gel documentation system (Bio-Rad) (Kumar *et al.*, 2023).

Sequencing using Big Dye Terminator v3.1

The purified PCR products were subjected to bidirectional Sanger sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in a GeneAmp PCR System 9700 thermal cycler according to the manufacturer's instructions. The sequencing reaction mixture contained distilled water, 5× sequencing buffer, forward or reverse primer, BigDye sequencing mix, and ExoSAP-treated PCR product. Following cycle sequencing, the products were purified by ethanol/sodium acetate precipitation. The purified products were washed twice with 70% ethanol, centrifuged at 3700 rpm, and air-dried. The cleaned sequencing products were subsequently analyzed using an ABI 3500 DNA Analyzer (Applied Biosystems) based on the Sanger chain-termination method (Joy *et al.*, 2020).

Sequence Merging and BLAST- Analysis

The forward and reverse ab1 files of sequences were opened with Bioedit software. The reverse complement sequence was constructed for both forward and reverse sequence files. Both the sequences were aligned by using the Pairwise alignment tool. The aligned sequence was used to create a consensus sequence. (Hall, 1999) The created consensus sequence was used to BLAST in NCBI to retrieve the similar sequences. The top 15 sequences from the hit table was chosen to construct a phylogenetic tree in MEGA software. (Madden, 2002).

Phylogenetic Analysis and GenBank Submission

The phylogenetic analysis of the aligned file was performed with MEGA software. The MEGA program was used with an aligned file containing the top 15 high similar sequences. The phylogenetic tree was constructed using the Test Neighbouring Joining test method (Tamura *et al.*, 2021). The 16S rRNA sequence of actinomycetes strains has been deposited in the GenBank (NCBI) to obtain the accession numbers.

RESULT

Collection of Soil Samples

The saltpan soil samples were collected from region of Vedaranyam located in Nagapattinam district. These samples were collected at 10 cm depth of soil surface.

Isolation of Halophilic Actinomycetes

Totally 12 actinomycetes isolates are inoculated on Starch casein agar with NaCl concentrations. Out of this 12 isolates only 5 isolates exhibited 5% - 10% NaCl concentration. The selected halophilic actinomycetes were Purified for further studies.

Identification of the Genus and Microbiological Characterization

The isolate SS1 was found to be Gram-positive and non-motile. The colonies exhibited a circular form, with straight, long chains of spores observed microscopically. The isolate produced white aerial mycelium and white-coloured spores.

Biochemical Characterization

The biochemical characteristics of isolate SS1 indicated a tentative identification as *Streptomyces* sp. The isolate was negative for indole production, Voges–Proskauer, catalase, and oxidase tests, while positive reactions were observed for methyl red, Triple Sugar Iron, citrate utilization, and nitrate reduction tests (Table 1).

Table 1. Biochemical Identification of SS1 Strain

Molecular Identification of SS1 Strain

The isolate SS1 strain was sequenced by targeting the variable regions of the 16S rRNA sequence. Fig1. The Phylogenetic tree showed the strain SS1 identified as *Streptomyces fulvissimus*.

S.No	Biochemical Test	SS1	Tentative genera
1.	Indole	NE	<i>Streptomyces</i> sp
2	Methyl- Red	positive	<i>Streptomyces</i> sp
3	Voges- Proskaueryr	Negative	<i>Streptomyces</i> sp
4	Triple Sugar Iron	Positive	<i>Streptomyces</i> sp
5	Citrate	Positive	<i>Streptomyces</i> sp
6	Nitrate	Positive	<i>Streptomyces</i> sp
7	Catalase	Negative	<i>Streptomyces</i> sp
8	Oxidase	Negative	<i>Streptomyces</i> sp

Fig.1. Phylogenetic tree of SS1 strain

DISCUSSION

The current study focused on the isolation and identification of halophilic actinomycetes from saltpan soils of Vedaranyam, Nagapattinam district. Saltpan ecosystems are known to be extreme habitats, yet they harbor diverse microbial communities, particularly actinomycetes that exhibit unique physiological adaptations and bioactivities (Ventura *et al.*, 2007).

In the present work, soil samples were collected at a 10 cm depth, targeting the zone where microbial activity is often high due to nutrient availability. A total of 12 actinomycetes isolates were obtained on starch casein agar supplemented with varying concentrations of NaCl. However, only 5 isolates demonstrated growth at 5%-10% NaCl concentrations, indicating moderate halophilic nature. This finding aligns with previous studies where actinomycetes isolated from saline environments showed similar salt tolerance (Jensen *et al.*, 2005).

The strain SS1 exhibited significant antibacterial activity and was thus selected for detailed characterization. Physiological characterization indicated that SS1 is Gram-positive, non-motile, with circular colony morphology and white aerial mycelium. Biochemical tests revealed positive reactions for methyl red, triple sugar iron, citrate utilization, and nitrate reduction, while catalase and oxidase

activities were negative. These features correspond closely with characteristics typical of the *Streptomyces* genus (Kämpfer, 2012).

Morphological observations under light microscopy showed filamentous rods forming long spore chains, and scanning electron microscopy (SEM) analysis confirmed rod-shaped cells with spore formation approximately 5 µm in size. Such morphology is consistent with earlier descriptions of *Streptomyces* species known for their elaborate mycelial structures (Shirling & Gottlieb, 1966).

Further molecular identification using 16S rRNA sequencing confirmed that SS1 belongs to the species *Streptomyces fulvissimus*. Phylogenetic analysis supported this identification, showing close genetic proximity to other strains of *Streptomyces fulvissimus* (Goodfellow *et al.*, 2012). The accurate identification of SS1 highlights the importance of integrating classical microbiological techniques with molecular tools for reliable actinomycetes classification.

Overall, the results emphasize the potential of saltpan soils as a valuable resource for discovering novel halophilic actinomycetes with biotechnological applications, particularly for antibiotic production.

CONCLUSION

In this study, halophilic actinomycetes were successfully isolated from the saltpan soils of Vedaranyam, Nagapattinam district. Out of 12 isolates, five showed tolerance to 5–10% NaCl concentrations. Among them, the SS1 strain exhibited significant antibacterial potential and was selected for detailed characterization. Morphological, physiological, biochemical, and molecular analyses confirmed that the SS1 strain belongs to the species *Streptomyces fulvissimus*. The findings highlight that saltpan environments are rich sources of halophilic actinomycetes with potential applications in biotechnology, particularly in the search for novel bioactive compounds.

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