Isolation and molecular identification of actinomycetes from sacred grove soil

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Abstract

Actinomycetes, a group of prokaryotes sharing traits with both bacteria and fungi, hold significant economic and commercial importance. They are prolific producers of enzymes, antibiotics, enzyme inhibitors and pharmaceuticals of high commercial value. This study aimed to characterize actinomycetes isolated from sacred groves, focusing on their morphological, biochemical and molecular traits and to assess their bioactive potential. Actinomycetes were isolated using plating and serial dilution methods followed by characterization through Gram staining and biochemical tests such as IMViC test (Indole test, Methyl red test, Voges Proskauer test and Citrate utilization test).

Identification to the genus level was achieved using 16S rDNA gene sequencing with strains A1 and A3 identified as Streptomyces mutabilis and Streptomyces cheonanensis respectively. These strains were also screened for amylase production on starch casein agar plates and enzyme assays showed strain A1 to have the highest activity (54.3±1.5 IU/ml/min after 48 hours of incubation). This research underscores the biotechnological importance of these actinomycetes strains, particularly in amylase synthesis crucial for various industrial applications.

Keywords: Actinomycetes, IMViC test, 16S rRNA sequencing, *Streptomyces mutabilis*, *Streptomyces cheonanensis*, Enzyme assay.

Introduction

Actinomycetes are filamentous bacteria that resemble fungi in shape and are Gram-positive[4]. They are abundant in various environments, especially soil and thrive even in harsh conditions[3]. These bacteria contribute significantly to the microbial community of soils, producing a characteristic earthy smell. Their resilience in soil ecosystems depends on the diversity of microbial species[6]. In particular, actinomycetes play a crucial role in biological soil crusts found in forest floors and sacred groves, influencing soil structure and microbial activities[22]. Research on bacterial communities in soil from sacred groves provides insights into ecosystem functioning. Actinomycetes are known for their ability to produce a wide range of important chemicals used in industry, agriculture and medicine[11]. Microorganisms from extreme environments like halophilic actinomycetes found in high-salinity habitats such as saline environments and salt mines, have attracted significant attention due to their specialized adaptations and production of natural compounds.

However, isolating actinomycetes from extreme environments, including salt habitats, poses challenges due to their slow growth rates[19]. Traditional methods for isolation and cultivation of these microbes need careful consideration to study their unique characteristics and potential applications effectively. Actinomycetes are considered to be the most biotechnologically valuable prokaryotic microorganisms. They are known sources of antibiotics and bioactive molecules [17]. Most of these bioactive molecules can possess antibacterial (streptomycin, tetracycline and chloramphenicol), antifungal (nystatin), antiviral (tunicamycin) and antiparasitic (ivermectin) properties shown.

The selection of screening sources, selective medium, culture conditions and the identification of candidate colonies in primary isolation are only a few of the variables that must be taken into account when using conventional isolation procedures[21]. Furthermore, it is important to choose the right medium and growth conditions, because published media are frequently linked to certain microbial genus or species. With the addition of nucleic acid sequencing techniques, molecular approaches that include both classification and identification, have gained new status[8]. In comparison to other conventional methods, the development of the polymerase chain reaction (PCR) and DNA sequencing techniques has a certain advantage with better authenticity. Additionally, the 16S rRNA gene's phylogenetic analysis is a significant area of evolutionary research[20].

Material and Methods

Collection of soil samples: Soil samples were gathered from sacred groves in Nedumangadu, Thiruvananthapuram, Kerala. These samples were taken from the upper 4 cm of soil where microbial activity is highest and bacterial populations are most concentrated. The samples were collected using sterile sealed polythene bags and transported to the laboratory for subsequent analysis.

Isolation and enumeration of total actinomycetes population: One gram of soil sample was taken and serially diluted up to 10^{-6} using distilled water as a diluent and mixed properly. 0.1 ml of each dilution was poured into starch

casein agar plates. The inoculum was spread properly by using an L-rod. Then transfer the plates to the incubator for the optimum growth at 32˚C for about 5 days. After incubation, average number of bacterial colonies in each plate was counted and calculated. Total bacteria present per gram of soil sample was calculated as:

$$\text{Number of colony forming units (CFU) per gram of soil} = \frac{1}{\text{Dilution factor}} \text{X Number of colonies}$$

Morphological and biochemical characterization of actinomycetes: Morphological and biochemical characterization of the isolated actinomycetes followed guidelines from Bergey's Manual of Systematic Bacteriology.

Colony Morphology: Characteristics such as color, shape, margin type, elevation, surface texture and pigmentation of the bacterial colonies were observed by cultivating the isolates on starch casein agar. These features are important for identifying different species of actinomycetes.

Aerial Mass Color: The color of the mature sporulating aerial mycelium was noted as a key trait for grouping and identifying actinomycetes. Colors observed included white, gray, red and green. In cases where the color fell between two categories, both colors were recorded. Intermediate tints were also documented by noting both possible color series.

Reverse Side Pigments: Strains were categorized based on their ability to produce distinctive pigments on the underside of colonies. This categorization distinguishes strains with characteristic pigments (+) from those lacking such pigmentation (-). Colors with low Chroma, such as pale yellow, olive, or yellowish brown, were included in the latter group.

Gram staining: It involves creating a thin smear on a clean glass slide which is air-dried and then heat-fixed. The smear is then treated with crystal violet solution for 1 minute followed by Gram's iodine solution and then decolorized briefly with 95% ethanol. Afterward, the smear is counterstained with saffranine solution. Finally, the slide is blot dried and observed under a microscope using oil immersion for examination [3].

IMViC test: It is used to differentiate the enteric bacteria of the family Enterobacteriacae. The basis of differentiation is the biochemical diversity of different genera. The IMViC test includes four different tests such as indole production, methyl red test, Voges-Proskauer test and citrate utilization test [18].

Molecular identification of actinomycetes isolates: The potent actinomycetes isolates were selected for 16S rRNA based molecular characterization followed by BLASTn analysis. The genomic DNA was isolated using ucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions and the16S rRNA gene was amplified with a set of primers: 16S-RS-F (5` CAGGCCTAAC ACATGCAAGTC 3`) and 16S-RS-R (5` GGGCGG WGTGTACAAGGC 3`). The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) with 2X Phire Master Mix (5 µl), distilled water (4µl), forward primer (0.25µl), reverse primer (0.25µl) and DNA(1µl) under the following conditions:

Sequencing PCR amplification profile

96ºC	-	2min	
96ºC	-	30sec	} 30 cycles
50ºC	-	40sec	
60 ºC	-	4min	
4 ºC	-	∞	

ExoSAP-IT Treatment: ExoSAP-IT (USB) isolates undesirable primers and dNTPs from a PCR product mixture without interfering with subsequent applications. It does this by combining two hydrolytic enzymes, exonuclease I and shrimp alkaline phosphatase (SAP), in a buffer that has been particularly designed. 5 µl of PCR product is mixed with 0.5µl of ExoSAP-IT and incubated at 37 ºC for 15 minutes followed by enzyme inactivation at 85 ºC for 5 minutes.

BigDye Terminator v3.1 sequencing: The BigDye Terminator v3.1 cycle sequencing Kit (Applied Biosystems, USA) was used to perform the sequencing reaction in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) following manufacturer's protocol.

The sequencing PCR mix consisted of distilled water (6.6µl), 5X sequencing buffer (1.9µl), forward primer (0.3µl), reverse primer (0.3µl), sequencing mix (0.2µl), Exosap treated PCR product (1µl).

Sequencing PCR amplification profile

96ºC	-	2min	
96ºC	-	30sec	} 30 cycles
50ºC	-	40sec	
60 ºC	-	4min	
4 ºC	-	∞	

The PCR products were purified and were sequenced in ABI 3500 DNA analyzer (Applied Biosystems) using Sanger DNA sequencing method.

Sequence Analysis: The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequences obtained after amplification were assembled using capcontig program of Bioedit software. The similarity search for the sequence was done by using NCBI-BLAST.

Primary screening of amylase producing bacteria using starch casein agar plate: The isolated strains were

individually tested for their ability to produce amylase by streaking them onto starch agar plates and incubating them at 37°C for 3 days. Following incubation, iodine solution (0.3% iodine and 1% KI) was spread over the plates. After allowing the plates to sit undisturbed for 5-10 minutes, the iodine solution was removed. Actinomycetes strains showing amylase activity were identified and noted based on the formation of clear zones around their growth on the plates.

Measurement of amylase activity: Amylase activity was determined by measuring the release of reducing sugar from soluble starch by DNS method. 100 ml of starch casein broth with 8% (w/v) inoculum was incubated for 48 hours with continuous agitation (150 rpm). 48h old culture was transferred to micro centrifuge tubes and centrifuged at 4000 rpm for 15 min. Pellets were discarded and supernatant was used as crude enzyme for enzyme assay. The reaction mixture contained 0.2ml of crude enzyme and 0.8ml of 100mM phosphate buffer (pH 7) containing 1% (w/v) of soluble starch. The mixture was incubated at 10min at 80ºC and the reaction was stopped by adding 2ml of DNSA (3,5-dinitro-salicylic acid).

The contents were boiled exactly for 5min in waterbath and cooled for 20-25min after which 1ml of 40% Rochelle salt (Sodium potassium tartarate) was added. Finally, the colour developed was read at 540 nm in a spectrophotometer. The amount of reducing sugar released in the mixture was determined. One unit of enzyme activity is defined as the amount of enzyme that releases 1µ mol of reducing sugar as maltose per minute under the assay conditions.

$$\text{Activity of enzyme (IU/ml/min)} = \frac{\text{mg of maltose X 1000}}{\text{volume of enzyme taken X incubation time}}$$

Data analysis: Presented data were the means and standard deviation (SD) of three replicate determinations. Data were analyzed by using a SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver. 17, 2012). Treatment means were compared by one way analysis (ANOVA) and the Duncans New Multiple range test were applied to the result at 0.05 level of significance ($p < 0.05$).

Results and Discussion

In recent years, actinomycetes have been extensively studied across diverse and often overlooked environments worldwide including India. However, there is limited documentation regarding the isolation of actinomycetes from soil in sacred groves. Therefore, efforts have been focused on isolating actinomycetes from this pristine region. Using the serial dilution method, numerous colonies were obtained from various soil samples collected from the sacred grove area. The total bacterial population (CFU/ml) in the tested soil samples was observed at the 10^{-4} dilution with the soil from the sacred grove showing a high population (2.5 x 10^5 CFU/ml). From the different colonies obtained, three actinomycetes colonies were selected, aseptically transferred and streaked onto starch casein agar plates to establish pure cultures of these colonies (Fig. 1).

Morphological characterization of bacteria: Three isolated actinomycetes were characterized based on their aerial mycelium, substrate characteristics and growth patterns, categorized as either abundant or excellent. Each isolate could exhibit white or brown colors, with variability observed in both substrate and aerial mycelium (Table 1). They were grouped based on their ability to produce pigments on the reverse side of their colonies: distinctive and non-distinctive. Strains A1 and A3 fell into the distinctive group, producing brownish-black and greenish-brown pigments respectively on the colony's reverse side while strain A2 did not exhibit reverse side pigmentation (Fig. 2).

Recent research on actinomycetes isolated from soil samples has highlighted the presence of potent antimicrobial compounds, often associated with yellow, dark, or white/grayish spore mass colors. Natural pigments derived from microorganisms are highly valued for their rapid reproduction, ability to thrive in cost-effective media and ease of processing compared to plant-derived pigments, among other advantages.

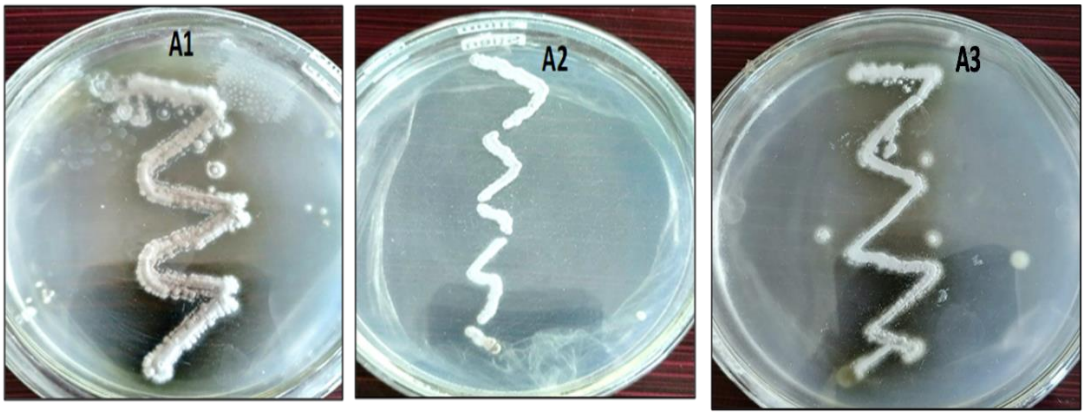

Fig. 1: Pure cultures (A1, A2 and A3) obtained on the starch casein agar plates

Table 1
Colony morphology of isolated soil actinomycetes

Actinomycetes isolates	Colour on starch casein agar	Configuration	Margin	Elevation	Reverse side Pigment
A1	Off White	Circular	Entire	Raised	Brownish black
A2	White	Circular	Entire	Raised	Nil
A3	Gray	Circular	Irregular	Flat	Greenish Brown

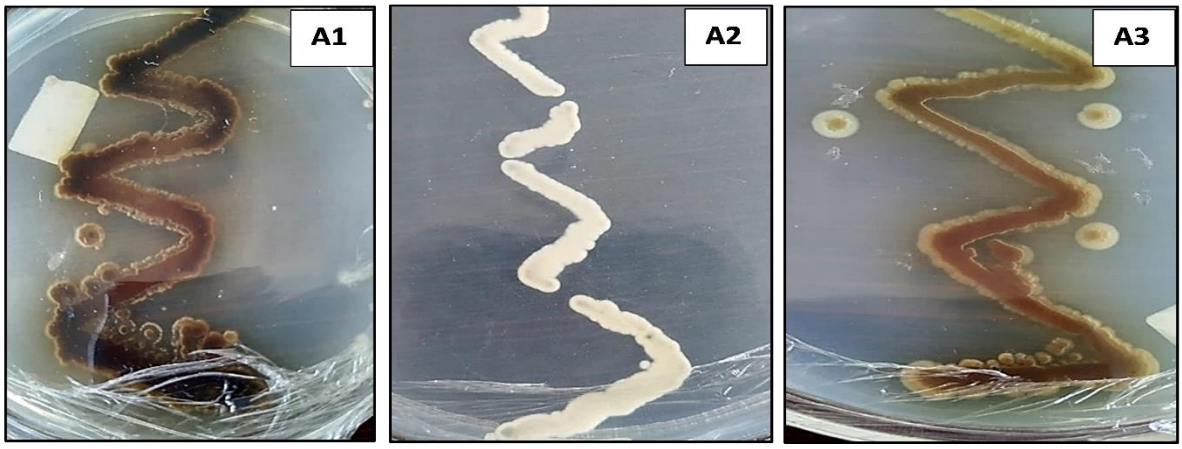

Fig. 2: Reverse side pigments produced by actinomycetes strains (A1, A2 and A3)

Table 2
Biochemical test of isolated soil actinomycetes

Tests	Bacterial Isolates		
	A1	A2	A3
Indole test	Negative	Negative	Negative
Methyl red test	Positive	Positive	Negative
Voges Proskauer test	Positive	Positive	Negative
Citrate utilization test	Positive	Positive	Positive

Currently, there is growing demand for microbial pigments in the global market due to their low production costs and eco-friendly properties, making them preferable over plant pigments and synthetic dyes respectively[12].

Gram staining: The microscopic images of the isolated strains were identified as Gram positive on gram staining (Fig. 3).

Biochemical characterization of actinomycetes: The amylase producing actinomycetes was isolated from soil samples was characterized through several biochemical tests such as IMViC test (Indole test, Methyl red test, Voges Proskauer test and Citrate utilization test) (Fig. 4). This conventional IMViC test method gives results (Table 2) that is similar to an agar plate IMViC method[18]. IMViC test was also carried out in various studies to differentiate the members of family enterobacteriaceae[5].

Molecular characterization

16S rRNA gene amplification: The 16S rRNA gene amplification was done for the identification of isolates A1 and A3 by comparing similar sequences in NCBI database using BLAST search. The gene sequencing was carried using universal forward and reverse primers. Electropherogram of sample A1 and A3 was represented in fig. 5 and fig. 7. The sequences obtained with forward and reverse primers were aligned using Bioedit programme and the resultant contig sequences were subjected to NCBI BLAST analysis. In the case of sample A1 and A3, the sequence obtained showed 98.03% similarity to *Streptomyces mutabilis* and *Streptomyces cheonansis* respectively (Fig. 6 and fig. 8). Biochemical and morphological tests are very laborious and time consuming and are not providing proper results.

Currently molecular methods are used for the isolation and characterization of new bacterial strain from different soil samples. Among them, application of 16S rRNA gene is simpler, yet efficient, in identification of new Streptomyces strains[2]. It is worth noting that although 16S rRNA gene has less changes and transformation through evolution, it is deemed to be a superior candidate for taxonomic studies because of 5' variable areas including α, β, δ, ε and particularly variable γ part which shows relatively high polymorphism at the 5′ end of its structure which could be

exploited for studying the genetic diversity of various Streptomyces species[14].

Identification of new strains of Streptomyces has been frequently described in the literature using amplification of hyper variable regions that can provide strain specific signature. A new strain of Streptomyces with high antibiotic production capacity and higher homology to *Streptomyces echinatus* using the assessment of cultural, morphological and phylogenetic evaluation was provided by 16S rRNA sequence analysis[15]. In the similar work by the 16S rRNA, sequence of the new strain exhibited higher homology with *Streptomyces lavendulae* and *Streptomyces globosus*[9].

Primary screening for amylase production: Each of the isolated actinomycetes (A1, A2 and A3) was individually tested for their ability to produce amylase using starch casein agar plates. A zigzag streaking method was employed for inoculation onto the agar plates followed by an incubation period of three days at 30°C. After incubation, iodine solution was applied over the plates which reacts with starch to produce a visible color.

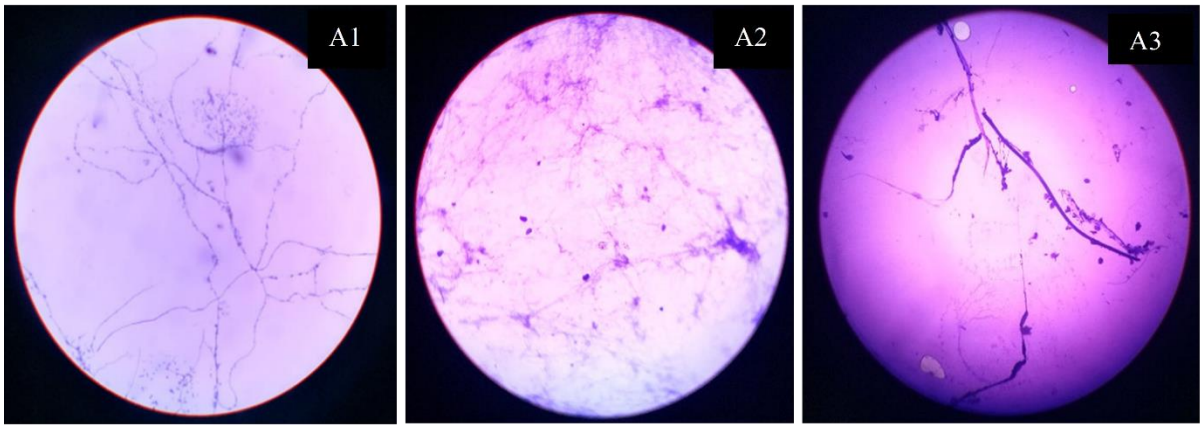

Fig. 3: Gram staining of isolated actinomycetes strains A1, A2 & A3

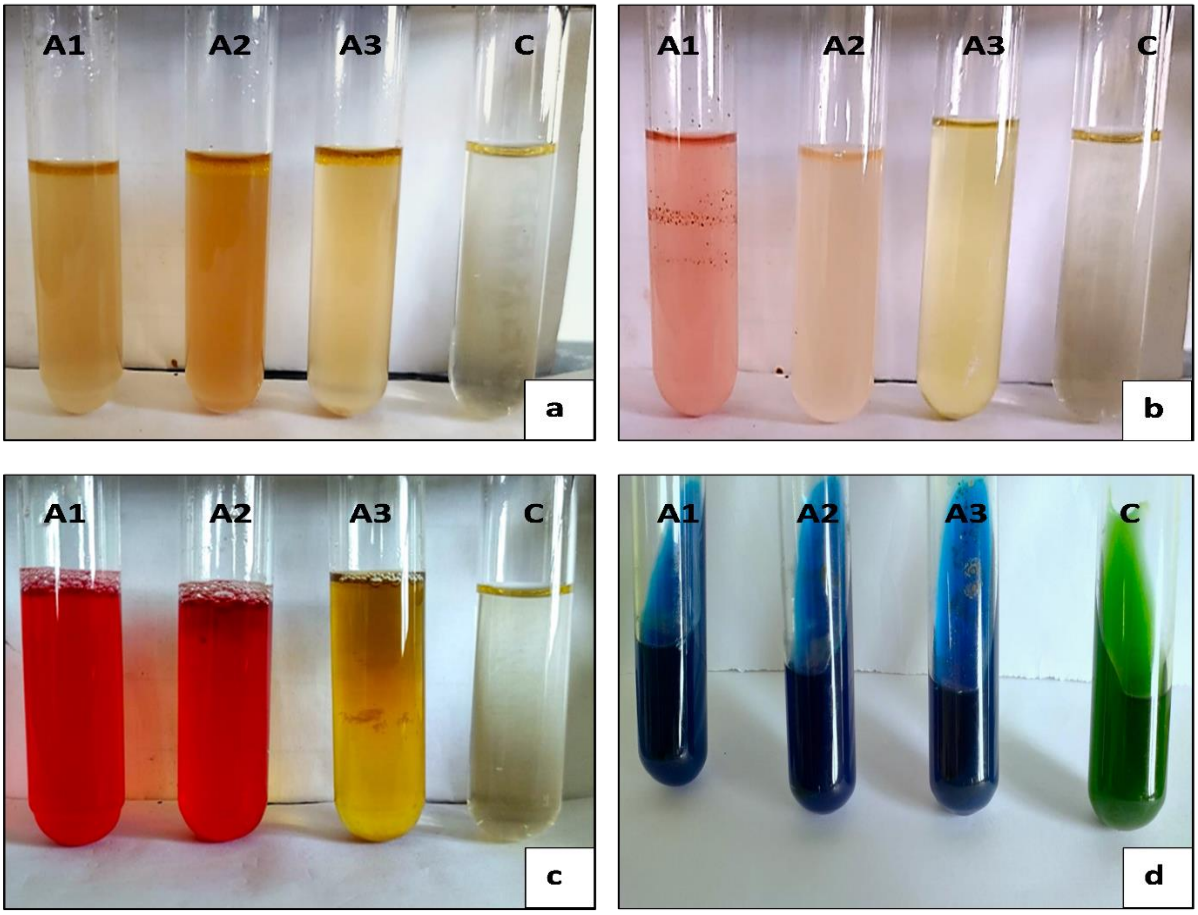

Fig. 4: a) Indole test b) Methyl red test c) Voges Proskauer test d) Citrate utilization test [A1, A2, A3 and C (uninoculated)]

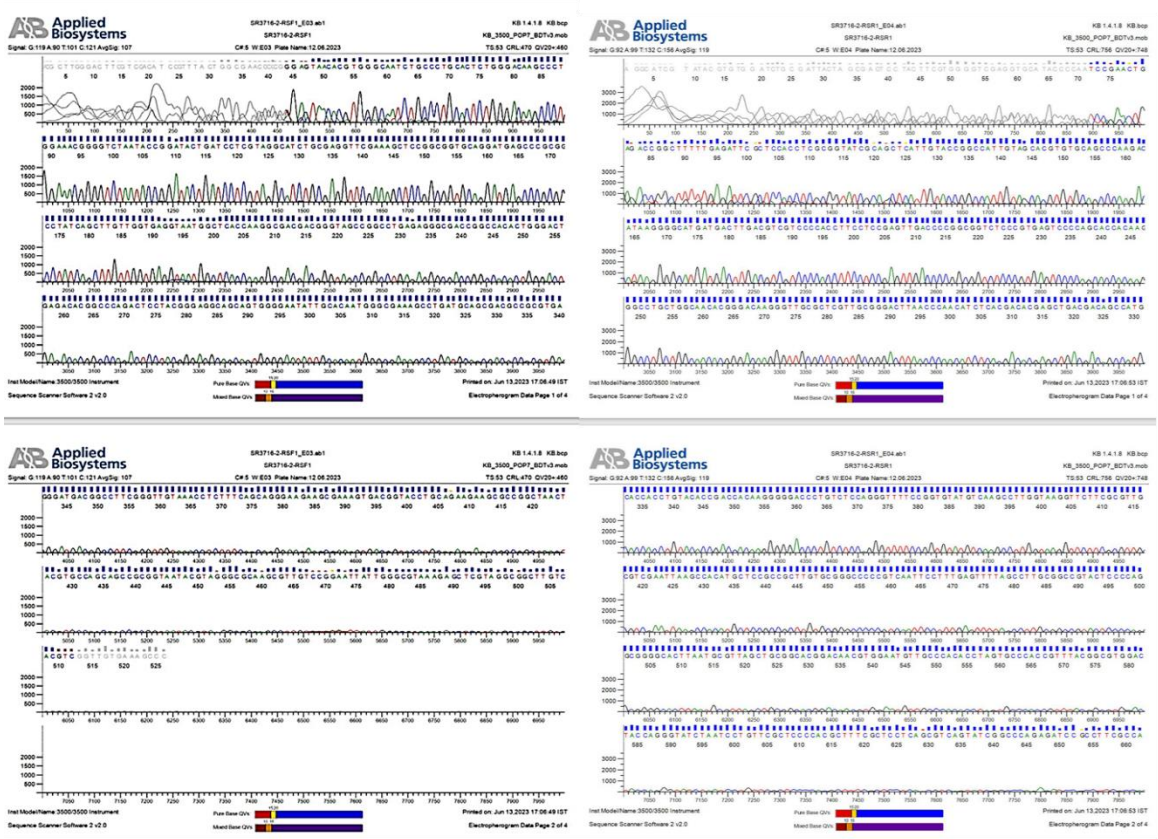

Fig. 5: Electropherogram of sample A1 using forward and reverse primer

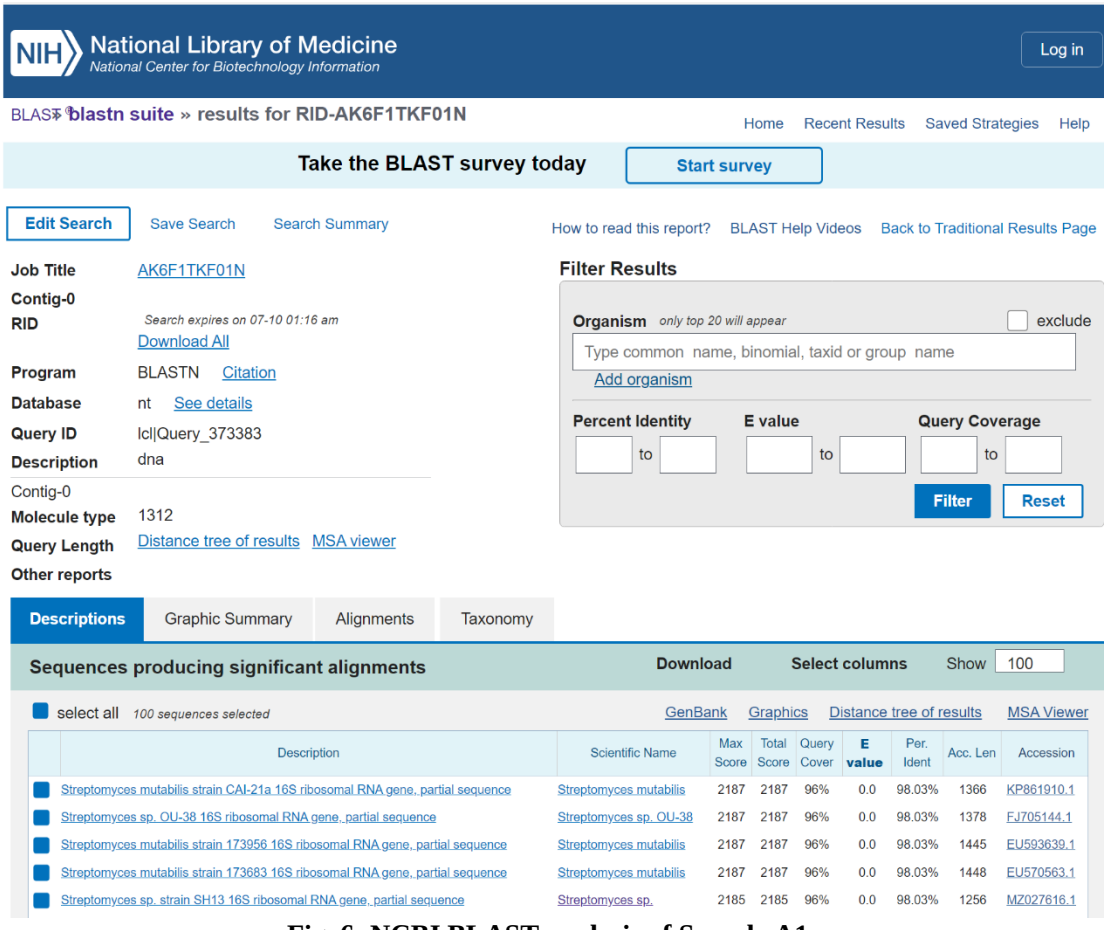

Fig. 6: NCBI BLAST analysis of Sample A1

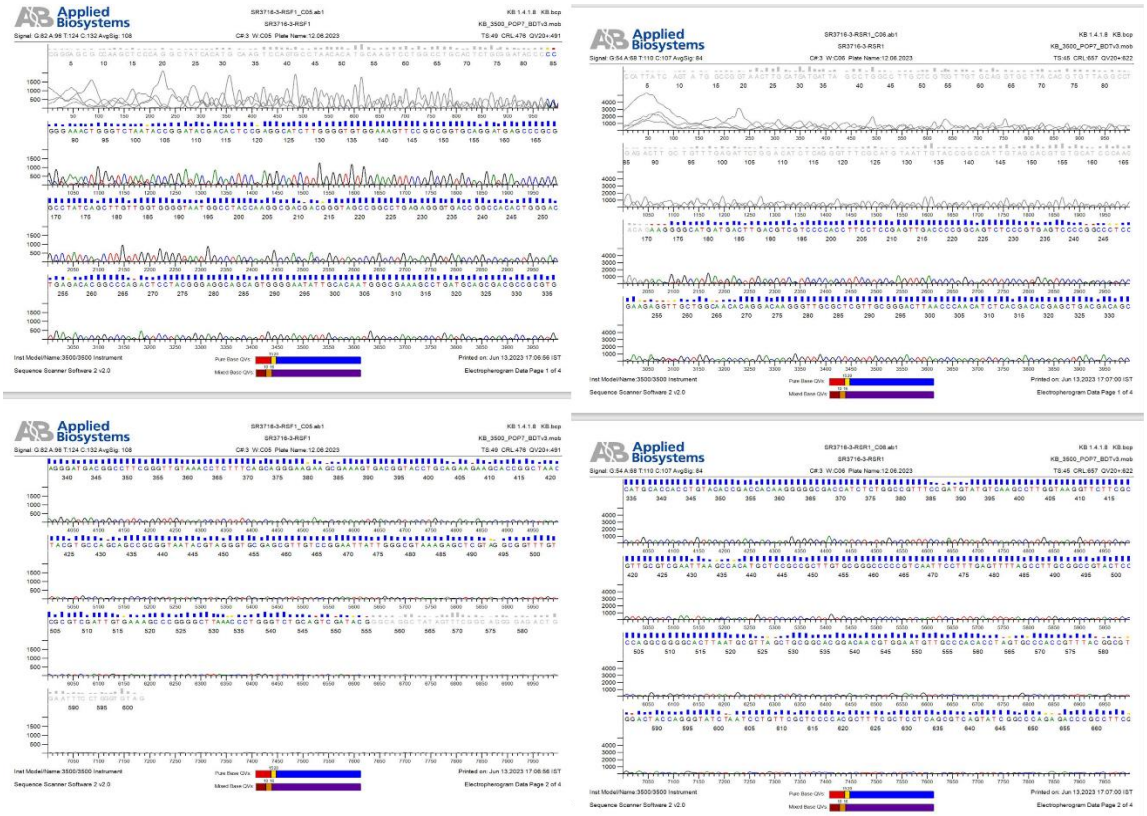

Fig. 7: Electropherogram of sample A3 using reverse primer

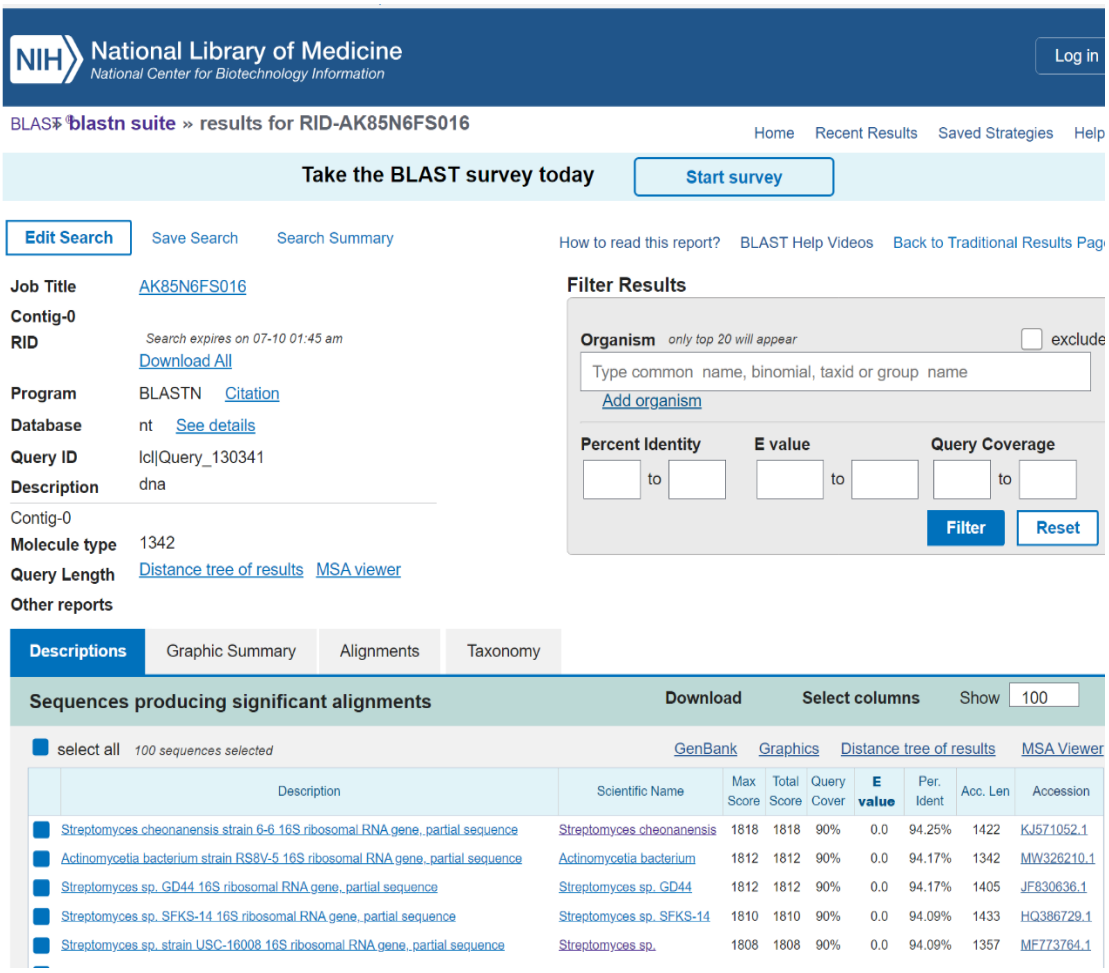

Fig. 8: NCBI BLAST analysis of Sample A3

Fig. 9: The zone formation around the colony due to the hydrolysis of starch by amylolytic enzymes (A1, A2 and A3)

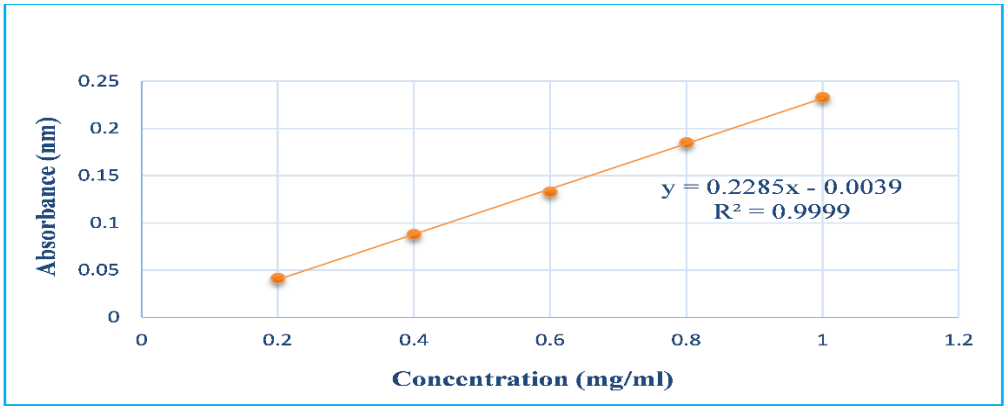

Fig. 10: Standard calibration curve of maltose

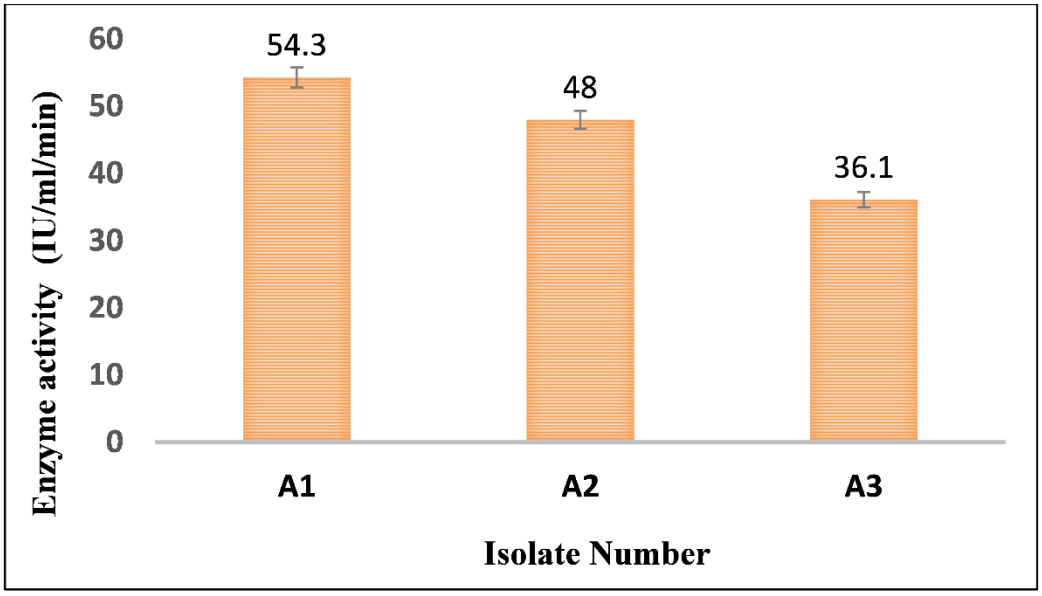

Fig. 11: Enzyme activity of actinomycetes isolates

Absence of color formation around the colonies indicated starch hydrolysis, confirming the production of amylase by the selected actinomycete strains (A1, A2 and A3) on starch casein agar medium (Fig. 9).

This method for detecting amylase-producing microorganisms using starch casein agar and iodine has been previously documented[17]. The clear zone observed around the colonies is attributed to the hydrolysis of starch by amylase enzymes produced during microbial growth, resulting in no staining when iodine is applied.

Amylase Enzyme activity: The enzyme activity of three actinomycete isolates was evaluated using the DNS method, correlating enzyme activity with a maltose standard calibration curve (Fig. 10). The calibration curve indicated that maltose linearity was observed within the range of 0.2 to 1 mg/ml. The study also explored the effect of incubation

time on amylase production, revealing that 48 hours was optimal for maximizing enzyme activity across all three isolates. Beyond this timeframe, enzyme activity showed a decline, likely due to the cells entering a decline phase with reduced amylase production. Among the isolates, A1 exhibited the highest enzyme activity at 54.3±1.5 IU/ml/min, followed by A2 at 48±1.33 IU/ml/min and A3 at 36.1±1.12 IU/ml/min (Fig. 11).

One of the most important industrial enzymes is amylase and it is used in many industrial sectors in the world. Considering this research, amylase production using marine actinomycetes isolated from sediment was investigated[10]. Composition of the medium plays a major role in the growth of actinomycetes and the production of enzymes[1].

Conclusion

Three actinomycete strains were selected from preliminary screening to assess their ability to produce amylase. Physiological and biochemical tests were employed to characterize these strains and to identify their potential for further bioactivity studies. Soil samples from a sacred grove area were serially diluted for isolation, known for its richness in actinomycetes. The aerial masses of the selected strains exhibited off-white, white and whitish-grey colors, with the white series being dominant. Most strains also showed pigment formation.

Through 16S rDNA sequencing, strains A1 and A3 were identified as closely related to *Streptomyces mutabilis* and *Streptomyces cheonanensis* respectively. All three selected strains demonstrated amylase activity. Therefore, strains A1, A2 and A3 exhibit characteristics of amylase-producing enzymes as evidenced by this study.

Acknowledgement

The authors express gratitude to the Department of Biotechnology at Sree Narayana College, Kollam-691 001, Kerala, India, for generously providing the laboratory resources for conducting this study. Additionally, we acknowledge the Regional Facility for DNA Fingerprinting (RFDF) at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, for facilitating DNA Barcoding experiments.

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(Received 06th May 2024, accepted 12th July 2024)