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| RESEARCH ARTICLE

Isolation and Phylogenetic Diversity of *Streptomyces* from Underexplored Soils of Nepal

Ram Prabodh Yadav[1] ✉ and Praveen Kumar Singh[2]
[12]J S Murarka Multiple Campus, Lahan Siraha, Nepal
Corresponding Author: Ram Prabodh Yadav, **E-mail**: ramprabodh30@gmail.com

| ABSTRACT

The genus *Streptomyces* is a prolific source of bioactive secondary metabolites and remains central to antibiotic discovery; however, many ecologically diverse regions remain underexplored for *Streptomyces* diversity. In this study, soil samples collected from four ecologically distinct regions of Nepal were investigated for the isolation and molecular characterization of *Streptomyces* species. Selective isolation was performed using ISP4 medium supplemented with antibacterial and antifungal agents, followed by morphological and microscopic characterization. Genomic DNA was extracted from pure cultures, and the 16S rRNA gene was amplified, sequenced, and subjected to comparative sequence and phylogenetic analyses. Four isolates (PY1–PY4) exhibiting characteristic *Streptomyces* morphology were successfully recovered. High-molecular-weight genomic DNA was obtained from all isolates, enabling reliable amplification of approximately 1.5 kb 16S rRNA gene fragments. Sequence similarity searches and phylogenetic reconstruction confirmed that all isolates belonged to the genus *Streptomyces*, showing close relationships to previously described species while also exhibiting strain-level genetic variation, particularly in isolate PY3. The phylogenetic distribution of isolates from both lowland and high-altitude environments suggests that ecological heterogeneity may contribute to *Streptomyces* diversity in Nepalese soils. Overall, this study provides baseline molecular data supporting Nepalese soils as a valuable and underexplored reservoir of *Streptomyces* diversity and establishes a foundation for future genomic and functional investigations into their biosynthetic and antimicrobial potential.

| KEYWORDS

Streptomyces; 16S rRNA gene; Molecular characterization; Phylogeny; Soil-derived bacteria

| ARTICLE INFORMATION

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1. Introduction

The rapid emergence of antibiotic-resistant bacteria has become a critical global health concern, significantly increasing morbidity and mortality associated with infectious diseases. Gram-positive pathogens, particularly methicillin-resistant and vancomycin-resistant *Staphylococcus aureus*, pose serious therapeutic challenges due to their ability to acquire resistance genes and undergo cell wall modifications that reduce antibiotic efficacy(Abebe & Birhanu, 2023). In parallel, Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* exhibit high rates of multidrug resistance in hospital settings, further limiting available treatment options(Thapa et al., 2025).

Microorganisms remain the most important natural source of antimicrobial compounds, especially through the production of secondary metabolites. Among them, *Streptomyces*, a dominant genus of actinomycetes, is renowned for its extraordinary capacity to synthesize chemically diverse and biologically active secondary metabolites(Sah &

Dhakal, 2023). More than two-thirds of clinically useful antibiotics of natural origin, including aminoglycosides, macrolides, and tetracyclines, are derived from this genus. The prolific biosynthetic ability of *Streptomyces* is attributed to the presence of numerous biosynthetic gene clusters in their large, GC-rich genomes, many of which remain cryptic and unexploited(Krysenko, 2025).

Streptomyces species are filamentous, Gram-positive, spore-forming bacteria that are widely distributed in terrestrial and aquatic ecosystems, particularly in soil. Their complex life cycle, involving mycelial differentiation and programmed cell death, is closely linked to secondary metabolite production(Varghese et al., 2026).Accurate identification and classification of *Streptomyces* are therefore essential for bioprospecting efforts. Molecular approaches based on 16S rRNA gene sequencing have become fundamental tools for genus-level identification and phylogenetic analysis, often complemented by phenotypic and chemotaxonomic data for species-level resolution(Retnowati et al., 2025).

Nepal possesses diverse ecological and geographical conditions that are likely to harbor rich and largely unexplored *Streptomyces* populations. However, systematic molecular studies on soil-derived *Streptomyces* from this region remain limited(Khadayat et al., 2020) . This study aims to isolate and characterize *Streptomyces* species from Nepalese soil using 16S rRNA gene sequence analysis, thereby contributing to the discovery of novel strains and providing a foundation for future investigations into their biosynthetic potential and antimicrobial applications.

Molecular identification of *Streptomyces* using conserved genetic markers provides a reliable framework for understanding their taxonomic position and evolutionary relationships, which is essential for systematic bioprospecting(Law et al., 2018). Among these markers, the 16S rRNA gene remains a widely accepted tool for genus-level identification and preliminary species assignment due to its conserved nature and extensive representation in public databases. Although it does not always provide sufficient resolution for definitive species delineation, 16S rRNA gene analysis is an effective first step for identifying phylogenetically distinct strains that may warrant further genomic and functional investigation(Clarridge, 2004).Therefore, isolating and characterizing *Streptomyces* from ecologically underexplored regions such as Nepal contributes valuable baseline data for future studies focused on microbial diversity and secondary metabolite potential.

2. Materials and Methods

2.1 Sample Collection and Media Preparation

Soil samples were collected from the surface layer (2–3 inches depth) to ensure a high abundance of viable actinomycetes, particularly *Streptomyces*. Sampling was carried out using sterile, dry spatulas, and the samples were immediately transferred into sterile polythene bags and stored at 4 °C until further processing. Four soil samples were collected from ecologically distinct regions of Nepal: Vedetar,Sunsari (1420 m; PY1), Gaighat ,Udaypur (117 m; PY2), Muktinath, Mustang (3710 m; PY3), and Phidim, Pachthar(3,675 m; PY4).

Isolation of *Streptomyces* was performed using ISP4 medium containing soluble starch (10 g/L), dipotassium phosphate (1 g/L), calcium carbonate (1 g/L), ammonium sulphate (2 g/L), sodium chloride (1 g/L), trace minerals, and Bacto agar (18 g/L), with the pH adjusted to 7.2. To suppress the growth of fast-growing bacteria and fungi, nalidixic acid (20 mg/mL) and cycloheximide (50 mg/mL) were added after cooling the medium to approximately 40 °C. Tryptic Soy Broth (TSB) was used for liquid culture prior to genomic DNA extraction (doi:10.1016/j.jksus.2020.02.005).

2.2 Isolation of Streptomyces and Culture Preparation

One gram of each soil sample was suspended in 10 mL of sterile distilled water and vortexed thoroughly. To selectively eliminate vegetative bacterial cells, the suspensions were heat-treated at 80 °C for 30 minutes. Serial dilutions were prepared up to 10^{-6}, and 100 µL aliquots were spread onto ISP4 agar plates supplemented with selective agents. Plates were incubated at 28 °C for 5–8 days. Colonies exhibiting characteristic *Streptomyces*

morphology were subculture and purified using the quadrant streak plate method. Pure cultures were maintained on ISP4 agar and preserved as glycerol stocks at -20 °C(Mahmood et al., 2025).

2.3 Genomic DNA Isolation

Pure isolates were inoculated into 25 mL of TSB broth containing sterile glass beads and incubated at 28 ± 2 °C with shaking at 180 rpm for 4–5 days. Cell pellets were harvested by centrifugation and washed repeatedly with lysis buffer (10% sucrose). Cell lysis was achieved by treating the pellets with lysozyme (8 mg/mL) at 37 °C, followed by the addition of EDTA (0.5 M), proteinase K (10 mg/mL), and SDS (10%). The lysates were incubated at 70 °C and subsequently cooled on ice.

Genomic DNA was extracted using phenol:chloroform:isoamyl alcohol (25:24:1), followed by chloroform extraction. RNA contamination was removed by RNase treatment at 37 °C for one hour. DNA was precipitated using isopropanol and ethanol, washed with 70% ethanol, air-dried, and finally dissolved in TE buffer(Yadav et al., 2024).

2.4 Examination and Quantification of Genomic DNA

The integrity and molecular weight of extracted genomic DNA were assessed by electrophoresis on 0.4% agarose gel in 1× TAE buffer at 50 V for 90 minutes and visualized under UV illumination after ethidium bromide staining. DNA concentration and purity were determined spectrophotometrically by measuring absorbance at 260 nm and 280 nm. The purity of DNA was evaluated using the A260/A280 ratio, and samples were diluted to a working concentration of 125 ng/µL for downstream applications(Lee et al., 2012).

2.5 Amplification and Phylogenetic Analysis of 16S rRNA Gene

The 16S rRNA gene was amplified using universal primers 27F and 1492R. PCR amplification was performed in a 25 µL reaction mixture for 35 cycles under standard conditions, including initial denaturation, denaturation, annealing at 51.4 °C, extension, and a final extension at 72 °C. The expected amplicon size was approximately 1.5 kb.

PCR products were analyzed on 0.8% agarose gel at 100 V for 30 minutes. Amplicons with sufficient concentration (>50 ng/µL) were purified and sequenced commercially. Sequence similarity searches were conducted using BLASTN against publicly available databases. Multiple sequence alignments were performed using ClustalW, and phylogenetic trees were constructed using the Neighbor-Joining method in MEGA X to infer evolutionary relationships among the isolates(Zhou et al., 2025).

3. Results

3.1 Isolation and Morphological Characteristics

Four actinomycete isolates, designated PY1, PY2, PY3, and PY4, were successfully obtained from soil samples collected from ecologically diverse regions of Nepal. When cultured on ISP4 medium at 29 °C, all isolates exhibited morphological features characteristic of the genus *Streptomyces*. Colonies were dry, rough, tough in texture, and slightly elevated.

Gram staining confirmed that all isolates were Gram-positive, filamentous bacteria, showing extensive branching mycelia and thread-like structures. Sporulation was observed within 5–8 days of incubation. Substrate mycelium pigmentation ranged from white to yellow, whereas aerial mycelium colors varied among whitish-pink, whitish-cream, whitish-yellow, and white, further supporting their classification as *Streptomyces* species.

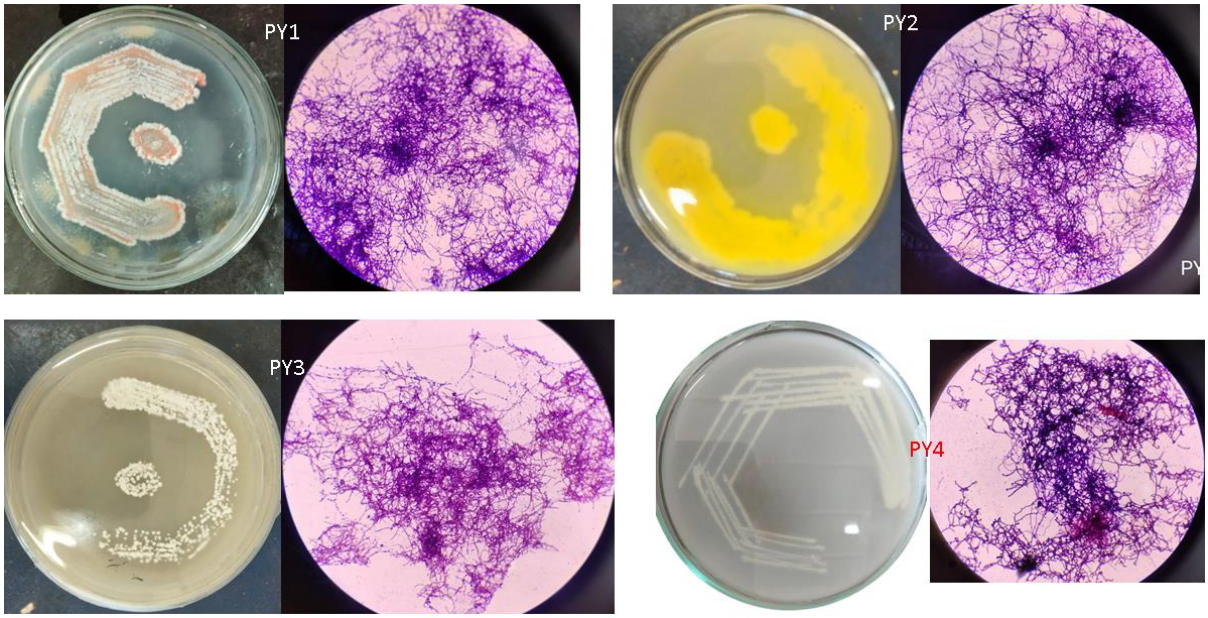

Figure 1: Culture and Gram Staining

3.2 Genomic DNA Isolation and PCR Amplification

High-molecular-weight genomic DNA was successfully isolated from all four isolates. Agarose gel electrophoresis (0.4%) revealed intact DNA bands larger than 10 kb, indicating minimal degradation. Spectrophotometric analysis confirmed measurable DNA concentrations, although A260/A280 ratios were generally below the optimal range of 1.8. Despite this, appropriately diluted DNA templates yielded successful downstream amplification.

PCR amplification of the 16S rRNA gene produced clear amplicons of approximately 1.5 kb for all isolates, as visualized on 0.8% agarose gel electrophoresis. The amplified products showed sufficient concentration and integrity for sequencing (Table 1), confirming the effectiveness of the DNA extraction and amplification protocols.

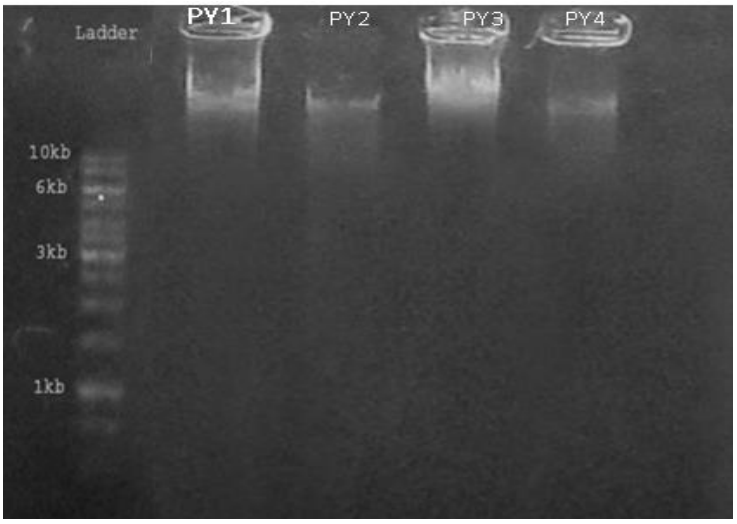

Figure 2: PCR

Table 1. Quantification and purity of PCR-amplified 16S rRNA gene

Strain	OD260	OD280	Purity (A260/A280)	DNA concentration (µg/µL)
PY1	0.138	0.131	1.05	0.72
PY2	0.159	0.134	1.19	8.02
PY3	0.142	0.133	1.07	7.10
PY4	0.137	0.144	0.95	6.60

3.3 Bioinformatic Analysis and Phylogenetic Clustering

BLASTN analysis of the 16S rRNA gene sequences confirmed that all four isolates belong to the genus *Streptomyces*. Sequence similarity searches indicated close phylogenetic relationships with previously described *Streptomyces* species; however, none of the isolates showed complete sequence identity, suggesting possible strain-level variation or potential species-level novelty.

Table 2: Sequences producing significant alignments with the 16S rDNA Sequence of *Streptomyces* species in the database:

Sample	Species (Match in the databases)	Identity %	Accession No.
PY1	*Streptomyces* sp. TN58 m w2gs	99.87%	PX795237
PY2	*Streptomyces yangpuensis* strain CM253	99.93%	PX795246
PY3	*Streptomyces sp.* strain BR123	99.74%	PX795766
PY4	*Streptomyces sp. strain HN25*	98.70%	PX795767

PY1 (1435 bp) exhibited the highest sequence similarity of 98.92% with *Streptomyces globisporus* strain AHS10, followed by 98.10% similarity with *Streptomyces violascens* strain EA27. PY2 (1498 bp) showed close similarity to *Streptomyces* sp. FXJ1.447 (98.36%) and *Streptomyces naganishii* NRRL B-1816 (98.28%). PY3 (1430 bp) shared comparatively lower similarity, showing 97.05% similarity with *Streptomyces pratensis* strain 190525 and 96.98%

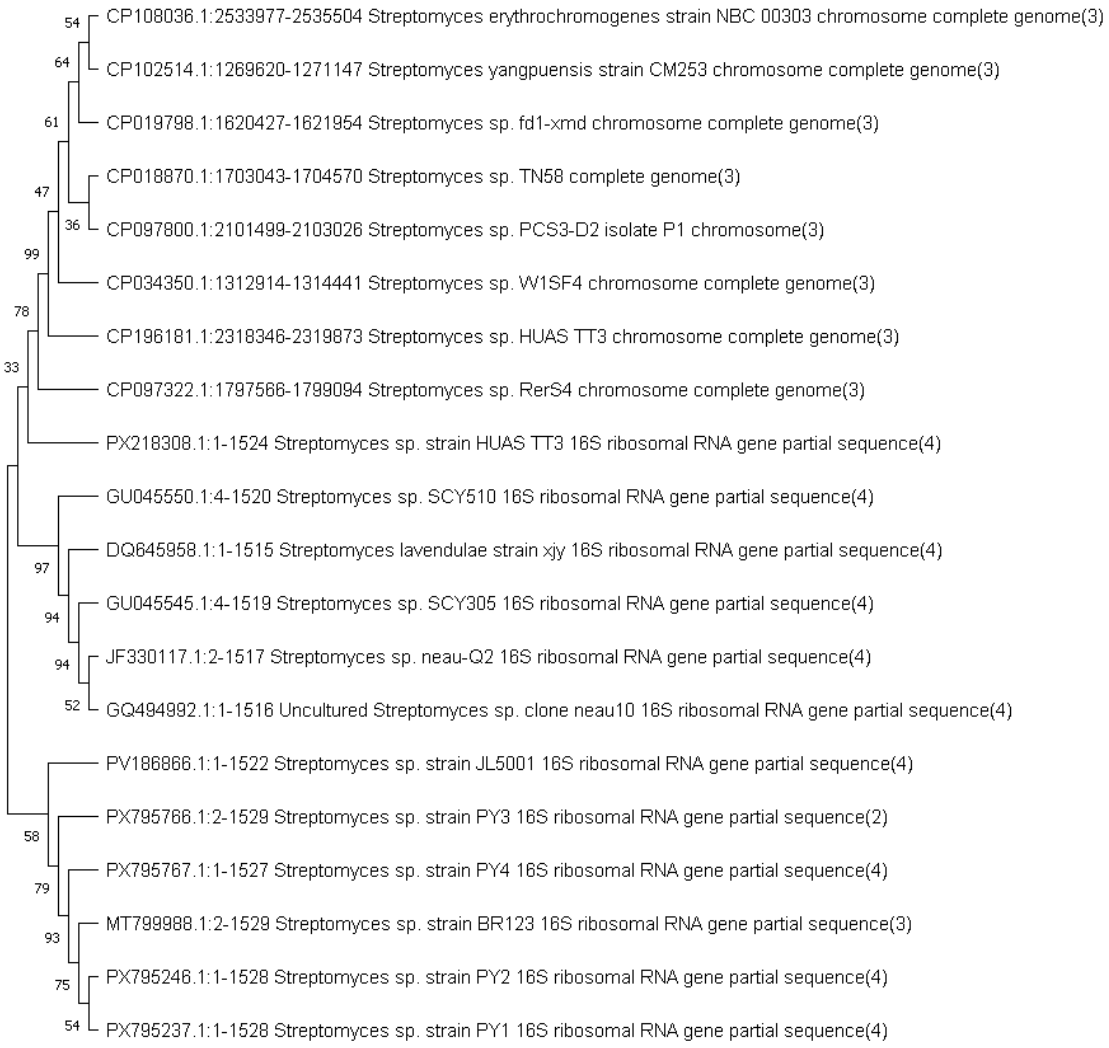

Figure 3: Neighbor-joining phylogenetic tree based on partial 16S rRNA gene sequences showing the evolutionary relationships of Streptomyces isolates PY1, PY2, PY3, and PY4 with closely related Streptomyces taxa retrieved from public databases. The tree was constructed using the Neighbor-Joining method with evolutionary distances calculated by the Maximum Composite Likelihood model. Bootstrap values (expressed as percentages of 1000 replicates) are shown at branch nodes. Escherichia coli was used as the outgroup. The scale bar represents 0.02 substitutions per nucleotide position. Accession numbers of reference sequences are indicated in parentheses.

With *Streptomyces* sp. WA22-1-2. PY4 (1778 bp) demonstrated identical similarity values of 98.62% with *Streptomyces* sp. HN25 and *Streptomyces diastaticus* subsp. *ardesiacus* strain AR-39.

Phylogenetic trees constructed using the Neighbor-Joining method further elucidated the evolutionary relationships among the isolates. PY1 clustered closely with *S. violascens* strains EA27 and EA25, while remaining relatively distinct from other reference taxa. PY2 grouped with *S. naganishii* and *Streptomyces* sp. FXJ1.447. PY3 formed a separate cluster with *S. pratensis* strains, and PY4 clustered tightly with *S. diastaticus* subsp. *ardesiacus* and *Streptomyces* sp. HN25.

Phylogenetic analysis based on 6–10 representative sequences per isolate, with evolutionary distances calculated using the Maximum Composite Likelihood method, supported the BLASTN results and confirmed the placement of all isolates within the genus *Streptomyces*.

4. Discussion

The successful recovery of four Streptomyces isolates (PY1–PY4) from geographically and ecologically diverse soil samples of Nepal demonstrates that these environments harbor a heterogeneous and potentially rich actinomycete community. The colonies exhibited typical Streptomyces morphology, including dry, rough, and leathery textures with well-developed substrate and aerial mycelia (Figure 1), consistent with established descriptions of the genus. The observed variation in aerial and substrate mycelial pigmentation among the isolates further supports phenotypic diversity within the recovered Streptomyces population. The use of ISP4 medium proved effective for selective isolation and phenotypic differentiation, while heat treatment and the addition of nalidixic acid and cycloheximide enhanced selectivity by suppressing non-actinomycete bacteria and fungi(Pandey et al., 2021).

Efficient biomass production in TSB under shaking conditions facilitated downstream molecular analyses. Despite the known difficulty of extracting high-quality genomic DNA from Streptomyces due to their thick and highly cross-linked peptidoglycan cell walls, the combined enzymatic, chemical, and thermal lysis approach employed in this study yielded intact high-molecular-weight DNA (>10 kb), as demonstrated by agarose gel electrophoresis (Figure 2). Although spectrophotometric analysis revealed A260/A280 ratios below the optimal range for some isolates (Table 1), appropriate dilution of DNA templates was sufficient to overcome potential inhibitory effects, enabling consistent amplification of approximately 1.5 kb 16S rRNA gene fragments for all isolates(Nikodinovic et al., 2003).

The 16S rRNA gene remains a cornerstone for bacterial taxonomy because of its conserved structure and extensive representation in public databases. While Streptomyces genomes typically contain multiple rrn operons, previous comparative genomic studies have shown a high degree of intragenomic conservation among these copies, supporting the reliability of single 16S rRNA gene sequences for genus-level identification and preliminary phylogenetic inference(Janda & Abbott, 2007). In the present study, BLASTN analysis confirmed that all four isolates belong to the genus Streptomyces, with sequence similarity values ranging from 98.70% to 99.93% relative to described species (Table 2). None of the isolates showed complete sequence identity, indicating strain-level variation and, in some cases, proximity to conventional species delineation thresholds(Henao et al., 2023).

Phylogenetic reconstruction using the Neighbor-Joining method further resolved the evolutionary relationships of the isolates and supported the BLASTN results (Figure 3). Each isolate clustered within a distinct but closely related Streptomyces clade, with PY1 grouping near Streptomyces violascens, PY2 clustering with S. naganishii and related taxa, PY3 forming a separate lineage associated with S. pratensis, and PY4 clustering closely with S. diastaticus subsp. ardesiacus and Streptomyces sp. HN25. The distinct phylogenetic placement of PY3, combined with its comparatively lower sequence similarity values, suggests a higher degree of genetic divergence relative to the other isolates(Rajeswari et al., 2015).

The recovery of phylogenetically distinct Streptomyces isolates from both lowland and high-altitude environments further suggests that environmental heterogeneity, including differences in altitude, temperature, soil physicochemical properties, and microbial interactions, may influence Streptomyces distribution and diversification. Collectively, these results highlight the genetic diversity of Streptomyces present in Nepalese soils and emphasize the importance of geographically diverse sampling for expanding current knowledge of actinomycete diversity(Antido & Climacosa, 2022).

Although functional assays were beyond the scope of this study, the observed phylogenetic diversity and strain-level differentiation among the isolates provide a basis for considering their potential functional relevance. Members of the genus Streptomyces are well recognized for producing a wide range of secondary metabolites and extracellular enzymes with pharmaceutical, agricultural, and industrial importance. The molecular and phylogenetic characteristics documented here indicate that the Nepalese Streptomyces isolates represent suitable candidates for

future genome-based and functional studies aimed at evaluating their biosynthetic and applied potential(Younis, 2025).

5. Conclusion

This study successfully isolated and molecularly characterized four *Streptomyces* strains (PY1–PY4) from soils collected across ecologically diverse regions of Nepal. The isolates exhibited morphological and physiological features consistent with established characteristics of the genus. ISP4 medium proved effective for selective isolation and differentiation, while TSB supported efficient biomass production for downstream molecular analyses. High-molecular-weight genomic DNA was obtained from all isolates, and the 16S rRNA gene was successfully amplified, sequenced, and analyzed. Phylogenetic analyses confirmed the placement of all isolates within the genus *Streptomyces*, revealing close relationships with previously described species as well as indications of strain-level uniqueness, particularly for PY3. Overall, these findings highlight Nepalese soils as a promising and underexplored reservoir of *Streptomyces* diversity and support the utility of 16S rRNA gene sequencing for taxonomic identification within this genus. The study provides a foundation for future investigations, including whole-genome sequencing, analysis of biosynthetic gene clusters, and functional evaluation of antimicrobial potential in Nepal-derived *Streptomyces* isolates.

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