Original Research Article

Characterization and Antibacterial Activity of Soil *Actinomycetes* from Diverse Land Use Systems in Meru South, Eastern Kenya.

ABSTRACT

Aim: The study isolated, characterized and identified potential *Actinomycetes* candidates with antibacterial activity from diverse land use systems.

Study design: A cross sectional survey design in line transect sampling was used in collection of samples from land use systems for isolation of *Actinomycetes*. A 30 × 7× 3 factorial experiment laid out in completely randomized design was used in screening of antibacterial activity of *Actinomycetes* isolates.

Methodology: The *in-vitro* cultivation of *Actinomycetes* was evaluated using four selective media. The *Actinomycetes* isolates were characterized using morphological, biochemical, and molecular markers. The antibacterial activity screening of crude extracts was conducted against six bacterial pathogens using the agar well diffusion method. The antibacterial activity of *Actinomycetes* isolates were analyzed using Analysis of Variance and means were separated using Least Significance Difference at $\alpha = 0.05$ in SAS version 9.4. The obtained nucleotide sequences were subjected to blast analysis against the NCBI databases to further identify the *Actinomycetes*. Phylogenetic analysis was done using Molecular Evolutionary Genetics Analysis and a phylogenetic tree constructed using the Neighbor-Joining method.

Results: The morphological analysis showed variations in colony morphology, including differences in color, size, and texture. Biochemical tests provided insights into the metabolic capabilities of the isolates, indicating variations in enzymatic activities and substrate utilization. Antagonistic activity of *Actinomycetes* extracts exhibited significant differences (P =.05) against test bacterial pathogens. Notably, isolate C52 from degraded forest zone showed the highest antibacterial activity against *Staphylococcus aureus* (12.21 mm), isolate L6 (16.23 mm) against *Listeria monocytogenes* and isolate C50 (15.5 mm) against *Raoutellaplanticola*. *Streptomyces celluloflavus*, *S. griseobrunneus*, *S. pratensis*, *S*. crystallinus, and S*. eurocidicus* were identified in the study.

Conclusion: The crude extracts obtained from *Actinomycetes* showed significant antibacterial activity against the selected test organisms. This suggests the presence of bioactive compounds with potential antibacterial properties within these extracts.

Keywords: Soil Actinomycetes, Characterization, Antibacterial Activity, Land use systems

1. INTRODUCTION

Antibiotic resistance has become a major public health challenge, leading to severe health issues and a concerning increase in morbidity and mortality rates [1] [2]. Addressing the prevention and treatment of infectious diseases caused by antibiotic-resistant bacteria has become an urgent priority [3]. Microorganisms produce natural products that are a good source of antibiotics, including *Actinomycetes* [4] [5]. In order to tackle existing medical challenges and preempt potential future pandemics, it is imperative to investigate novel bioactive compounds, particularly those exhibiting distinctive modes of action. To meet the demand for new antibiotics, it is essential to investigate a wide range of ecological habitats and land use systems. This is important because these environments could contain distinct populations of *Actinomycetes* that possess untapped capability to produce antibiotics. Instances of such habitats include forest soils, agricultural fields, urban settings, marine sediments, and plant ecosystems, all of which have proven significant in sourcing *Actinomycetes* for screening against resistant bacterial pathogens [6] [7] [8]. Land use systems, which encompass various anthropogenic activities and management practices, significantly influence the microbial communities and their functional potential [9] [10].

Actinomycetes, being integral members of the soil microbiota, can exhibit variations in their abundance, diversity, and functional attributes across different land use systems [11] [12].The characterization of *Actinomycetes* using morphological, biochemical, and molecular markers provides valuable insights into their diversity, physiology, and metabolic capabilities [13] [14]. Morphological analysis, primarily based on microscopy, allows for the observation of cellular and colonial characteristics, spore formation, and branching patterns, providing initial taxonomic insights [15]. Molecular markers, such as 16S rRNA gene sequences, have revolutionized *Actinomycetes* taxonomy and phylogeny studies [16]. By targeting conserved regions within the 16S rRNA gene, molecular markers allow for accurate identification of *Actinomycetes* at the genus and species levels [17].Therefore, comprehensive studies on characterizing *Actinomycetes* isolated from different land use systems was essential to gain a deeper understanding of their biotechnological potential.

Soil *Actinomycetes* can produce a wide range of secondary metabolites and about 70% of the naturally derived antibiotics that are currently in clinical use are produced by them [18]. Almost 80% of the world'santibiotics are known to come from *Actinomycetes*, mostly from the genera *Streptomyces* and *Micromonospora* [19]. Most of their bioactive molecules have been shown to have antibacterial (streptomycin, tetracycline and chloramphenicol), antifungal (nystatin), antiviral (tunicamycin) and antiparasitic (avermectin) properties [20]. The resistance problem demands to discover new antibacterial agents effective against resistant pathogenic bacteria. In this way, the present study characterized and screened *Actinomycetes* from different land use systems for antibacterial action in hope of getting some new *Actinomycetes* strains that produce antibiotics, which have not been discovered yet and are active against drug-resistant pathogens.

2. MATERIAL AND METHODS

2.1 Study Site

The study was conducted in Meru South Sub-County, which is located within Tharaka Nithi County in the Eastern Region of Kenya. The soil sampling sites are shown in figure 1 below. Meru south sub- County is characterized by low and erratic rainfall as well as high evapotranspiration rate [21]. The bimodal rainfall ranges from 1200 - 1400 mm and experiences temperatures range between 24 ^{0}C and 26 ^{0}C. The predominant soil type is humic nitisols, typically deep and weathered soil with moderate to high fertility while the soil texture is predominant sand clay loam [21].

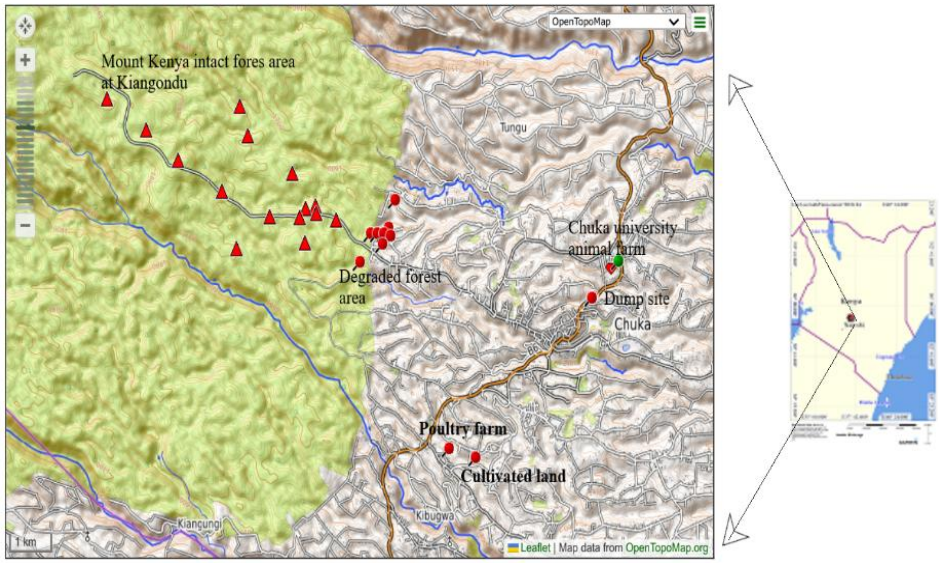


Figure 1: The soil sampling sites in the study area within Meru-South sub county.

2.2 Study design

A cross sectional survey design in line transect sampling was used in collection of samples from land use systems for isolation of *Actinomycetes*. A 30 × 7× 3 factorial experiment laid out in completely randomized design (CRD) was used in screening of the secondary antibacterial activity of *Actinomycetes* isolates.

2.3 Soil samples collection

Soil samples were obtained uncultivated and degraded soils in the Mt. Kenya forest (S0° 18.247' E37° 34.111'), cultivated soils from agricultural land (S0° 19.347' E37° 39.483'), cattle manure from Chuka University, chicken manure from Ikuu farm, and soils from a Chuka municipal solid waste dumpsite at Chuka town(S0° 19.699' E37° 39.176'). The sampling locations were marked using the Etrex 30x Garmin Global Positioning System to ensure accurate spatial referencing (Table 1). The soil samples were collected using a spiral soil auger, which was cleaned with 10% formalin after each sampling to prevent cross-contamination. Five-centimeter soil depth for soil samples from forest zone, dumpsite and cultivated soil was considered during sampling process. The collected soil samples were collected in sterile containers with zip-top lids and then they were transported to the Botany laboratory at Chuka University for further analysis.

Table 1: Soil sampling from different land use systems of Meru South Sub-county

Site(s[1])	Soil sampling area	Ecological importance	Sd[2] (cm)	Elevation	Latitude	Longitude
A	Chuka forest zone	Intact soils	5	1593m	0º19. 30ˈS	37º 36ˈ0ˈˈE
B	Ikuu farm	Cultivated soils	5	1321m	0º11.0ˈˈS	37º 19ˈ00 E
C	Chuka forest zone	Destructed area	5	1558m	0º19.30ˈS	37º36ˈ00ˈˈE
D	Chuka town	Dump site soils	5	1356m	0º 19.699ˈS	37º 39.17ˈ E
E	Chuka University	cattle manure	5	1406m	0º 19.347ˈS	37º39.483ˈE
F	Ikuu farm	Chicken manure	5	1321m	0º11.0ˈS	37º 19.00ˈE

[1]Sampling sites, [2] Soil depth

2.4 Pre-treatment of soil samples

The collected samples from different land use systems were air-dried at room temperature for 7 days [22] [23]. This was done in order to limit the number of moisture-dependent bacteria and fungus. The air-dried soil samples were grounded into a fine powder. Ten (10) g of fine soil sample was mixed with 0.1 g of $CaCO_3$, and the resulting mixture was treated in a hot air oven (Model Memmert UNB400) for one hour at a temperature of 55^0 C.

2.5 Isolation of *Actinomycetes* from soil samples

The soil samples for isolation of *Actinomycetes* were prepared by standard serial dilution method[24]. One gram of soil samples was mixed in distilled sterile water up to 10^{-3} and allowed for shaking with vortex for 5 minutes. After serial dilution, 0.1 ml of each sample was separately plated using spread plate technique in ModiiedLuria Bertani Agar (M1) (Starch 10 g, Peptone 2.0 g, yeast Extract 4.0 g, Agar 18.0 g, distilled water 1000 ml, and pH; 7.0), Starch Casein Agar (Starch 10 g, K_2HPO_4 2 g, KNO_3 2 g, casein 0.3 g, $MgSO_4.7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4.7H_2O$ 0.01 g, agar 15 g,distilled water 1000 ml, and pH; 7.0) and International Streptomyces Project (ISP-1) (Yeast Extract, 3.0g, Tryptone, 5.0gdistilled water 1000 ml, and pH; 7.0) and (ISP-4) media (Soluble Starch, 10.0g, K_2HPO_4, 1.0g, $MgSO_4 \cdot 7H_2O$, 1.0g, NaCl, 1.0g, $(NH_4)_2 SO_4$, 2.0g, CaCO3, 2.0g,distilled water 1000 ml, and pH; 7.0) (HiMedia Laboratories). The plates were incubated at 28°C and colony growth observed on the 7th day after inoculation. After incubation, *Actinomycetes* isolates were distinguished from other microbial colonies using morphological characteristics [25]. Sub-culturing of isolates was done using nutrient agar. The pure cultures were stored at a temperature of 4°C in slant culture on Starch Casein Agar and in glycerol broth [26].

2.6 Morphological Characterization of *Actinomycetes* Isolates

Microscopic characters were observed by isolated strains on starch casein agar, International Streptomyces project-1 (ISP-1), International Streptomyces project-4 (ISP-4) and Modified Luria Bertani agar media. The selected isolates were streaked on the surface of the media by streak plate method and plates were incubated at 28 °C for 7 days. Morphological traits were examined, including colony colour, size, margin, elevation, pigment production, presence or absence of aerial and substrate mycelium. *Actinomycetes* isolates were observed under a microscope to determine the morphology of the hyphae and spores. The spore chain arrangement, size, and shape were determined by Gram stain technique [27].

2.7 Biochemical Characterization of *Actinomycetes* Isolates

2.7.1 Carbon source utilization test

To determine the capacity of the isolates to utilize carbon sources, nutrient agar was used as a basal medium. The following carbon sources; D-Glucose, D-fructose, sucrose, maltose, dextrose and lactose (HiMedia laboratory) were added into separate sterile test tube containing basal medium and mixed with individual *Actinomycete* isolate. After mixing the isolates with the medium at a 1% weight-to-volume ratio, the mixture was incubated at 30 °C for 7 days. The plates were observed for the growth and utilization pattern of the *Actinomycetes* on different carbon sources. Utilization of a carbon source was indicated by a change in the colour of the medium or the formation of a clearing zone around the colony [28].

2.7.2 Casein hydrolysis test

To perform the casein hydrolysis test, a loopful of *Actinomycetes* culture was inoculated onto a skim milk agar (HiMedia Laboratories) plate. The plates were incubated at 30°C. After incubation, the plate were observed for the presence of a clear zone around the bacterial growth [29].

2.7.3 Starch Hydrolysis Test

To determines the ability of microorganisms to degrade polysaccharide starch in the media by producing hydrolytic extra cellular enzymes, *Actinomycetes* isolates were inoculated on a starch agar plate (starch-2g/L, peptone - 5g/L, beef extract-3g/L, agar-30g/L) and incubated at 30°C for 72 hours. After incubation, the surface of the plates were flooded with Iodine solution (0.3% w/v). The plate were observed for the presence or absence of a clear zone around the *Actinomycetes* growth [30].

2.7.4 Urea hydrolysis test

To determine urease activity, the Christensen's urea agar (Ppeptone-1g/L, dextrose -1g/L, sodium chloride- 5g/L, potassium phosphate monobasic- 2g/L, phenol red- 0.012g/L, agar- 15g/L) slant was inoculated with a loopful of Actinomycetes culture which was streaked across the surface of the agar slant. The agar slant was incubated at 30°C for 5 days. After incubation, the slant was observed for colour changes. To confirm the result, a few drops of phenol red solution was added to the slant [31].

2.7.5 Catalase test

To determine catalase activity, a bacterial smear was prepared by gently mixing a small amount of *Actinomycetes* culture with a drop of sterile water on a glass slide. The smear was allowed to air-dry completely and a drop of 3% hydrogen peroxide was added to the bacterial smear using a sterile dropper. The reaction was observed under a microscope immediately.

2.7.6 Citrate utilization test

To determine citrate utilization by *Actinomycetes* isolates, the *Actinomycetes* isolate were inoculated onto a slant of Simmons citrate agar using a sterile loop. The plate were inoculated at 30°C for 24 hours. After incubation, the plate were examined for growth and the colour change of the medium was observed.

2.7.8 Lipase test

To determine lipase activity, the *Actinomycetes* isolate was inoculated onto the Tributryin agar medium (peptone -5g/L, yeast extract- 3g/L, agar-12g/L) plates using sterile loop. The plates were incubated at 30°C for 5 days. After incubation, the plates were observed for a zone of clearing around the bacterial growth, indicating lipase activity [32].

2.7.9 Sulfur Indole Motility test

To determine Sulphur-Indole Motility (SIM) test, *Actinomycetes* isolates were inoculated onto a SIM medium slant using a sterile inoculating loop and incubated at 30°C for 48 hours. After incubation, the Sulphur-Indole Motility medium was examined for the presence of blackening due to the reduction of sulfur for hydrogen sulphide production and presence of motility by the *Actinomycetes* [33]. Presence of Indole was detected by addition of Kovac's reagent to 48-hour cultures of each isolates [34].

2.7.10 Methyl Red test

To determine methyl red test, *Actinomycetes* isolate was inoculated in Methyl Red broth (peptone -7g/L, glucose- 5g/L, potassium phosphate- 5g/L), inoculated with *Actinomycetes* isolates and incubated at 37°C for 48 hours. After incubation, few drops of methyl red indicator were added to the medium and colour change observed.

2.7.11 Voges-Proskauer test

To determine Voges-Proskauer (VP) Test, *Actinomycetes* isolate was inoculated in Voges Proskauer broth (peptone -7g/L, glucose- 5g/L, potassium phosphate- 5g/L). The isolates were incubated at 37°C for 48 hours. After incubation, 5 drops of VP reagent A (5% alpha-naphthol in absolute ethanol) were added to the medium and mixed well followed by addition of 5 drops of VP reagent B (40% potassium hydroxide). Colour change on the medium was observed [35].

2.8 Screening of selected *Actinomycetes* for antagonistic activity against test pathogens

2.8.1 Primary Screening of Antibacterial of *Actinomycetes* Isolates.

Primary screening of *Actinomycetes* was performed on the Mueller–Hinton agar medium employing the perpendicular streak method [23]. In the sterile agar medium, the pure isolate of *Actinomycetes* was streaked along the diameter of the plate. The plate was incubated at 28°C for 5 days. Pure colony of test bacteria *Escherichia coli* (ATCC 25922), *Listeria monocytogenes* (NCTC 11994), *Vibrio furnissii* (NCTC 11218), *Rauotellaplanticola* (NCTC 19528), *Staphylococcus aureus* (ATCC 25923), and *Streptococcus mitis* (NCTC 12261) was transferred into fresh nutrient broth and incubated at 37°C for 24 hours until the visible turbidity. After adjusting the turbidity equal to that of 0.5 McFarland with the cell count of 1.5×10^8, the test organisms were streaked perpendicular to the isolate [36]. The plates were further incubated at 37°C for 24 hours, and the antibacterial activity was estimated from the zone of inhibition of test organism [37]. In order to ensure safety within the laboratory environment, precautionary gear was utilized while working with the test pathogens. Additionally, surfaces and laboratory equipment were routinely disinfected, the frequency of contact was minimized, and laminar flow hoods were employed.

2.8.2 Secondary Screening of *Actinomycetes* Isolates for Antibacterial Activity

Thirty isolates showing positive results in primary screening with inhibition zone above 6.5mm were fermented using the submerged fermentation procedure in boiling tubes containing 30 ml of sterile medium starch casein broth [38]. The inoculated boiling tubes were incubated on rotary incubator shaker (Biotechnich, India) at 28°C and 150 rpm for 7 days. The fermented broth was centrifuged (Remi, RM12C, India) at 10,000 rpm at 4 °C. The supernatant was analysed for antibacterial activity by agar well diffusion method using the same test microorganisms used in primary screening [39]. Five wells of 6 mm diameter were made on the agar plate with the help of sterile cork borers. The test organism was swabbed on the agar surface and 100 µL of supernatant was poured in the wells. The plates containing bacterial strains were incubated at 37ºC for 24 hours. Twenty-five (25) µl of Streptomycin (concentration of 10 µg) was used as positive control left at room temperature for 30 min to allow the compounds to diffuse through the agar. After incubation, the zone of inhibition was measured and expressed as millimetres in diameter [40].

2.9 Molecular characterization of *Actinomycetes* Isolates

Isolates were inoculated in the starch casein agar media (Himedia Laboratories) and incubated at 28°C for 7 days. Isolation of genomic deoxyribose nucleic acid (gDNA) was carried out using standard phenol-chloroform methods [41]. This was followed by Polymerase chain reaction (PCR) amplification using 16S rRNA gene primers. For the identification of bacterial 16S rRNA sequence analysis was performed using universal primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1429R (5'-GGTTACCTTGTTACGACTT-3') [42]. The polymerase chain reaction (PCR) condition involved initial denaturation at 94°C for 5 min, 30 cycles at 95°C for 30 secs, 55°C for 30 secs and 72°C for 120 secs; and a final extension at 72°C for 7 min. The PCR amplified products were analyzed by 0.7% agarose gel electrophoresis. The purified PCR products were sequenced using 27F and 1429R primer with Sanger sequencing methods at Genotech at Macrogen, Germany [43]. The consensus 16s rRNA gene sequence from each potential isolate was saved in FASTA file format and was BLAST in the NCBI database to get the identity of the isolates and later submitted to the same data base for accession numbers. The 16s rRNA gene sequence records were directly entered and submitted electronically at (www.ncbi.nlm.nih.gov/projects/ Sequin/) using Sequin tool/program from national centre for biotechnology information (NCBI) [44]. The accession numbers of 16s rRNA gene sequence for each isolate was received from GenBank database. The aligned sequences were saved as MEGA file to run neighbour joining algorithms using Molecular evolutionary genetics analysis (MEGA) X to generate evolutionary character matrices and distance matrices. Jukes Cantrol model was selected to exclude the gaps and positions with ambiguities. The neighbour joining original tree was generated and the reliability of the resultant neighbour joining tree topologies were assessed and evaluated by bootstrap analysis based on 1000 re-sampling of the neighbour-joining [45] [46]. Both the original tree and bootstrap tree were compared to see the reliability of the tree. Phylogenetic analysis was performed to determine the evolutionary relationship of the *Actinomycete* sample to other bacteria using MEGA X [47].

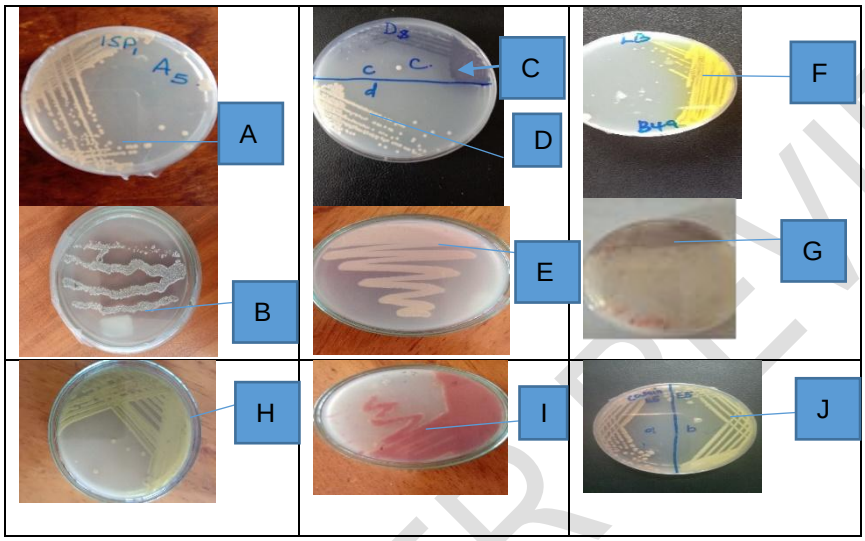
2.10 Statistical analysis

To determine antibacterial activity of *Actinomycetes* isolates against test pathogens, the data collected on diameters of zones of inhibition was subjected to analysis of variance (ANOVA) at significance value(P=.05) using SAS version 9.4 [48]. Significant means were separated using the Least Significant Difference (LSD) at $\alpha = 0.05$.

3. RESULTS

3.1 Morphological Characterization

The *Actinomycetes* colonies exhibited diverse morphological characteristics (Table 2). The majority of the colonies had a circular shape with a round margin and either a raised or flat elevation. The reverse side of the colonies also exhibited colours such as brown, yellow, pink, or grey. The colour of the colonies varied among isolates from grey, purple, pink, brown, cream, yellow, red, green-yellow and cream-white (Plate 1).



Note:A=orange (ISP-1), B= Grey (SCA) C=Purple (SCA), D=cream-white (SCA), E=light pink (M1), F= yellow (M1), G= brown (ISP-1), H= light-green (ISP-4), I= red (ISP-4) and J= green- yellow (SCA). Media used LB=Luria Bertani; SCA= Starch casein agar, ISP-1 and ISP-4 International Streptomyces project media 1 starch and 4 respectively.

Plate 1: Morphological characterization of Actinomycetes isolates from soil samples collected from different land use systems

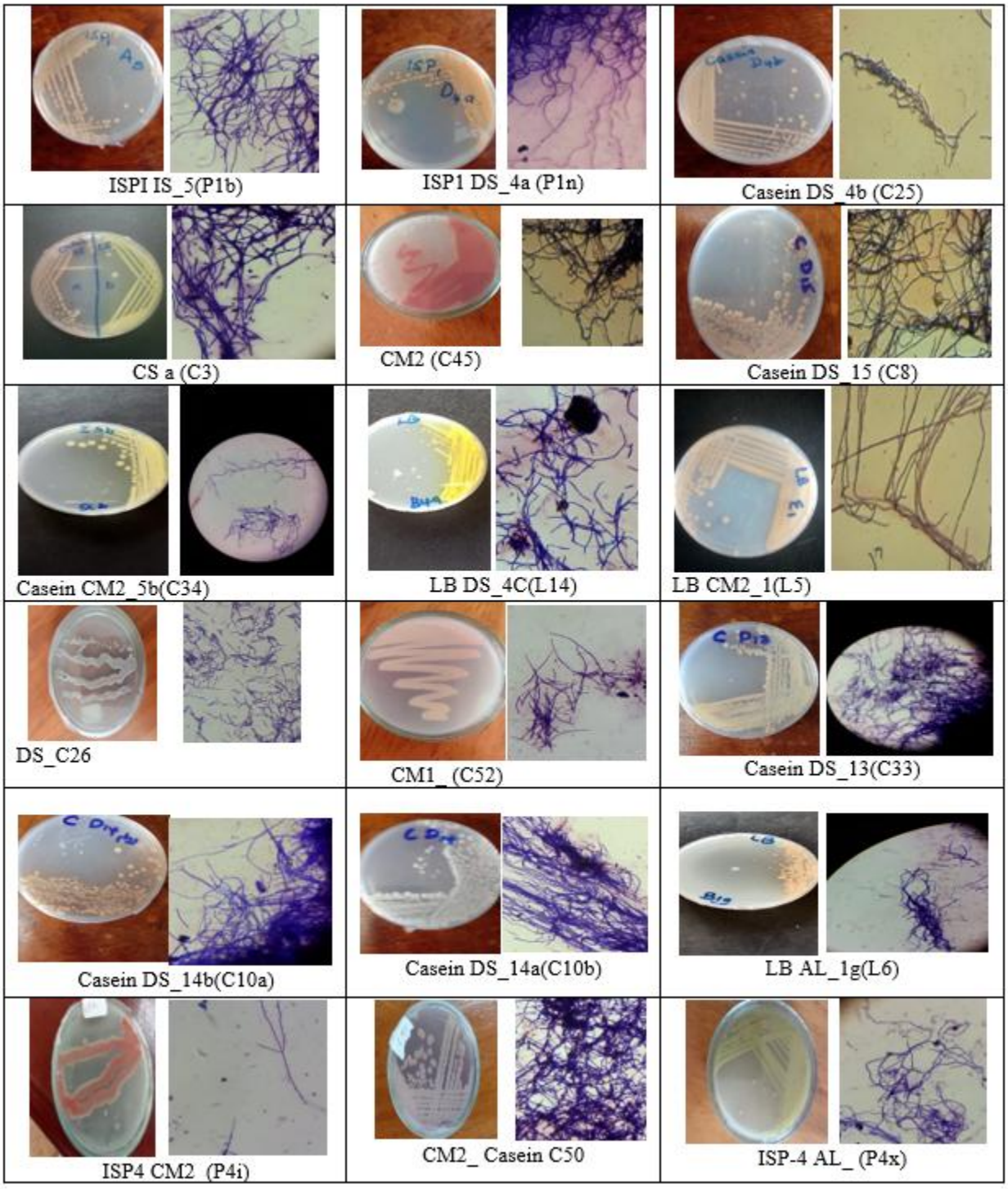
Table 2: Morphological characteristics of selected *Actinomycetes* isolates

Isolates	Colour	Size (mm)	Colony shape	Colony margin	Elevation	Sporulation	Pigments	Constituency	Region obtained
C2	Grey	3	Circular	Smooth	Raised	Grey	Brown	Powdery	CS
C3	Brown	1	Circular	Smooth	Raised	-	none	Leathery	CS
C5	Pinkish white	1	circular	smooth	Raised	pink	None	Leathery	CS
C8	Pink	1	Circular	Smooth	Raised	Pink	Pink	Leathery	DS
C10a	Grey	3	Circular	Smooth	Raised	Grey	Brown	Powdery	DS
C10b	White	1	Irregular	Smooth	Flat	White	yellow	Powdery	CS
C22	Grey	1	circular	Smooth	Raised	Grey	yellow	Powdery	DFZ
C25	Brown	3	circular	Smooth	Raised	Brown	-	Leathery	DFZ
P4i	Grey	2	circular	Smooth	flat	cream	Brown	Powdery	IS
C33	Brown	2	circular	smooth	raised	brown	brown	Leathery	DFZ
C34	White	3	irregular	smooth	raised	white	none	Leathery	DS
C43	Light yellow	3	irregular	rough	raised	white	None	Powdery	DS
C45	Light-green	1	Irregular	serrated	flat	white	green	Powdery	CM2
C52	light green	2	Circular	smooth	raised	white	none	Leathery	CM2
P1b	Light brown	1	Irregular	rough	flat	white	none	Powdery	DS
P1n	Brown	2	Circular	Serrated	Flat	Brown	Brown	Leathery	DS
P4x	Orange	2	Irregular	Rough	Flat	orange	Orange	Leathery	DS
L5	Yellow	1	Circular	Smooth	Flat	yellow	Yellow	Powdery	CM2
L6	Light green	1.5	Circular	Smooth	Raised	white	Green	Powdery	CM1
L14	Cream yellow	1	Regular	Rough	Flat	cream	None	Leathery	DS

NOTE: Symbols used represented media used; C=Starch Casein Agar; P1 and P4=International Streptomyces Project 1 and 4 respectively, L= modified Luria Bertani. Regions where isolates were obtained: CS= cultivated soil; DS=Dumpsite (urban); DFZ=degraded forest zone; CM1=Cattle manure; IS=Intact soil (forest zone); CM2=chicken manure

3.2 Biochemical characterization of *Actinomycetes* and Gram stain test

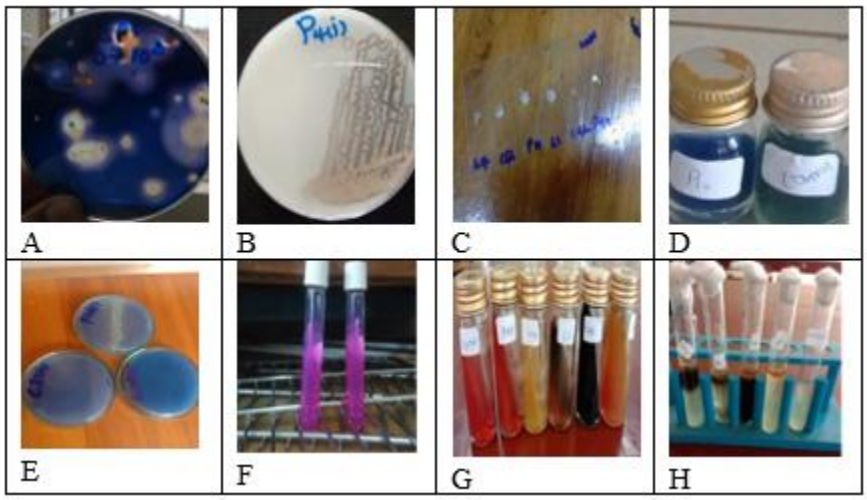
The gram staining analysis of the *Actinomycetes* isolates indicated that the majority of the isolates were gram-positive bacteria, as evidenced by the purple staining observed (plate 2). Further examination of the colonies revealed a diverse array of microscopic characteristics among the *Actinomycetes* isolates. The hyphal structures displayed by the colonies varied significantly, with some colonies exhibiting highly intertwined filamentous hyphae. Additionally, short filaments were observed in some colonies (plate 2). These short filaments may represent a distinct morphological characteristic of certain *Actinomycetes* isolates.



Note: Media used were denoted with; C=starch casein agar; LB= Luria Bertani, ISP= International Streptomyces project-1and 4; Isolates were coded based on media they were isolated from; P4=ISP-4; P1=ISP-1, L=LB, and C= starch casein. Land use systems; IS=Intact soil (forest zone); DFZ= degraded forest zone; CM1= cattle manure; CM2= chicken manure; AL= Agricultural land (cultivated soil)

Plate 2: Colony and microscopic characteristics of selected *Actinomycetes* isolate at 400X magnification on light microscope.

Biochemical characteristics showed that all isolates hydrolysed starch (Plate 3) testing positive for amylase test while those positive for casein and catalase (77%); lipase test (57%); and citrate test (22%). Isolates C26 and C52 fermented D-glucose, dextrose, maltose, fructose, sucrose and lactose used (Table 3).



Note: A= starch hydrolysis test, B= Casein test, C= catalase test, D= Citrate test, E= lipase test, F= Urease test, G=Tripple iron sugar, H= Indole motility

Plate 3: Biochemical tests for active *Actinomycetes* isolates

	C3	C8	C52	C10a	C10b	C42	C45	C34	C43	C50	C25	L6	L14	P1b	P4i	P4x	C26
Hydrolysis test																	
Casein	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-
Amylase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	++	++	-	+	++	+	-	-	+	++	+	++	-	++
Lipase	-	-	+++	++	+	+++	-	-	-	-	+	-	-	+	++	-	-
Urease	Y	P	P	Y	P	P	P	P	Y	P	Y	P	P	Y	P	P	Y
Bt	Y	P	Y	P	P	Y	P	Y	P	P	Y	Y	Y	Y	P	P	Y
Cellulase	-	-	-	-	+	+	-	+	-	+	-	-	-	+	-	-	-
Citrate	-	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-	+
Sulfur	+	-	+	+	-	-	+	+	-	+	+	+	+	-	-	-	+
Indole	+	-	-	+	+	-	-	+	-	-	-	-	-	+	-	-	-
Motility	+	-	+	+	-	+	-	+	+	+	+	+	+	-	+	+	+
MR	-	-	-	-	-	++	++	+	-	++	+	+	-	-	+	+	+
VP	-	-	-	++	-	++	++	-	-	++	-	+	++	-	-	-	++
TSI-A	+	+	+	+	+	-	+	-	-	+	+	+	+	-	-	+	+

B	-	+	+	-	+	-	+	-	-	+	-	-	-	-	-	-	-
Sl	Y	P	P	Y	P	Y	Y	P	Y	P	Y	P	Y	Y	P	P	Y
Bt	Y	Y	Y	Y	P	Y	P	Y	Y	Y	Y	P	Y	Y	Y	Y	P
Carbon source																	
Glucose	+/-	+/-	+	+/-	+,g	-	+	-	-	+	+	+,g	-	+/-	-	+,g	+,g
Sucrose	-	-	+	-	+,g	-	+	-	-	-	+	+,g	+	+	-	+	+
Lactose	+	+	+	+	-	+/-	+	-	+	-	+	+,g	+	+	-	-	+
Fructose	+,g	+	+	+	+	+	+/-	-	+,g	+	+/-	-	-	+	-	-	+
Maltose	-	-	+	+,g	+	-	-	-	+	+	-	-	+,g	-	-	-	+
Dextrose	-	-	+,g	+	+	+	-	-	-	-	+,g	+	+	+,g	-	+	+

Table 3: Biochemical characterization of *Actinomycetes* Isolates on

Enzyme hydrolysis and

Notes: +++ = strongly positive; ++=moderately positive; += positive; +/- = doubtful; - = negative; g=gas production; Y=yellow; P=pink; Sl=slant; Bt= butt. Symbols representing media used; C=Starch Casein Agar; P1 and P4=International Streptomyces Project 1 and 4 respectively, L= modified Luria Bertani, MR= methyl red test VP, TSI=Triple iron sugar

3.3 Antibacterial activity of selected *Actinomycetes*

In the present study, active *Actinomycetes* isolates demonstrated the ability to inhibit the growth of the test bacteria, as evidenced by the presence of halo zones (Plate 4).

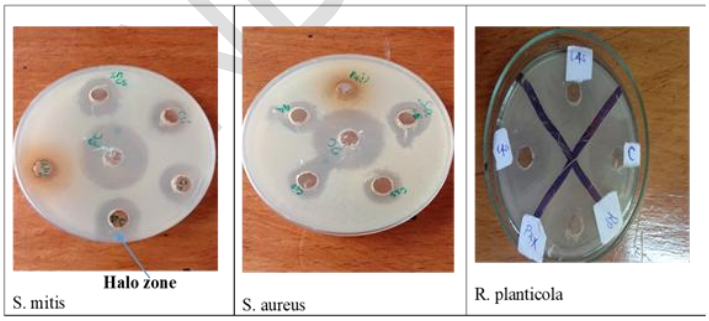


Plate 4: Secondary screening of isolates C5, C25, P4i and C45 showing antibacterial activity against *Streptococcus mitis*, *Staphylococcus auerus* and *Raoutellaplanticola*.

The mean value for antibacterial activity of study isolates against *Staphylococcus aureus* ranged from 6.5 mm to 12.25 mm in diameter. Isolate C52 (12.25 mm) showed the highest antibacterial activity compared to isolate C25 (6.5 mm) which had the least inhibition activity (Table 3). All the isolates under study had antibacterial activity against *S. aureus.*

The mean inhibition zone for *Escherichia coli* ranged from 6.25 mm to 8.75 mm. Isolate L3 showed the highest inhibition activity of 8.75 mm against *Escherichia coli*. There was a significant difference (*P* =.05)among antagonistic isolates against *Listeria monocytogenes*. Isolate L6 showed the highest antagonistic activity of 16.23 mm against *Listeria monocytogenes* compared to the standard; streptomycin (16.3 mm).

Listeria monocytogenes showed resistance to isolates P1b, L3 and L14 (6.0 mm). The difference among the isolates was statistically significant (*P*=.05). Isolates C52, L14, C43, L6, and C8 exhibited the highest activity against *Staphylococcus aureus, Raoutellaplanticola, Vibrio furnissii, Listeria monocytogenes,* and *Streptococcus mitis* respectively). These isolates were isolated from; chicken manure (C52), dumpsite soil (L14, C43, C8), and cultivated soil (L6). Isolate L3 isolated from intact soil (forest zone) showed the highest activity against *E. coli* followed by isolate C3 isolated from cultivated soil.

Table 4: Zones of inhibition (mm) of the selected pathogenic microorganisms in a secondary screening of the *Actinomycetes* isolates

		Zone of inhibition (mm)					
Isolates	LUS	Sa	Sm	Ec	Lm	Vf	Rp
CT(strept)		15.67a	16.67a	16.00a	16.33a	16.00a	16.00a
C52	CM2	12.25b	10.00bcd	6.00d	11.00cde	8.00fghi	12.50cd
L14	DS	11.5bc	10.75bc	6.00d	6.00i	8.00fghi	12.50cd
C8	DS	11.25^{bcd}	11.25bc	7.50cd	8.75ghi	12.00bc	11.00cd
P4x	IS	10.50bcde	9.25bcdefghi	7.50cd	10.25defghi	6.00i	12.50cd
P4i	IS	10.50bcde	9.75bcdefg	6.00d	11.50cd	6.00	13.00bcd
C33	DFZ	10.50bcde	8.00cdefgh	7.50cd	7.50hi	6.7h	6.75h
C5	CS	10.00bcdef	6.25fgh	6.75cd	12.50bc	6.75hi	6.00j
C28	CM2	10.00bcdef	8.75bcdefgh	7.50cd	12.50bc	9.00fgh	9.00cdefgh
C45	CM2	10.00bcdef	6.00f	7.25cd	9.50efgh	9.50efgh	8.50cdefgh
C43	DS	9.50bcdefg	9.75bcdefgh	8.25bcd	11.50cd	11.50bcd	11.50cdef
C50	DFZ	9.50bcdefg	7.25efgh	6.00d	12.50bc	11.50bcd	15.50a

C22	DFZ	9.50bcdefg	11.50b	7.75cd	9.00efgh	9.75cdefg	13.0bcd
C26	DS	9.50bcdefg	7.75efgh	7.75cd	7.50hi	11.25bcd	12.25cde
C61	DS	9.00cdefg	7.75efgh	6.50cd	11.75bcd	8.75efgh	9.75cdefgh
C3	CS	9.00cdefg	8.75bcdefgh	8.25bcd	7.00i	10.50bcde	8.75cdefghi
L6	CM1	8.75cdefg	8.25bcdefgh	6.00d	16.23a	8.00cdefgh	10.50defg
C40	CM1	8.75cdefg	7.00defgh	7.00cd	10.50defg	12.50b	12.25cde
P4ii	CM2	8.50cdefg	7.75defgh	6.25d	7.50hi	10.75bcde	8.75cdefghi
L14	DS	8.25defg	9.00bcdefgh	7.50cd	11.00cde	6.00i	13.25ab
C10b	CS	8.25defg	7.75defgh	6.75cd	8.75ghi	9.75cdefg	8.75cdefghi
C27	CM2	8.25defg	6.00f	6.00d	6.00i	11.25bcd	6.00j
C10a	DS	8.25defg	7.5defgh	8.0bcd	7.0i	10.5bcde	11.0cdefg
C46	DS	8.0efg	9.5bcdefg	8.0bcd	9.5efgh	6.0i	11.0cdefg
C30b	IS	7.75efg	6.25fg	7.0cd	7.5hi	7.75ghi	7.75hij
L3	IS	7.75efg	8.75bcdefgh	8.75bcd	6.0i	10.0bcdef	9.75cdefgh
C2	CS	7.5efg	11.25bc	10.25b	9.0efgh	6.0i	6.0j
P1b	DS	7.25fg	6.25fg	7.7cd	6.0i	10.0bcde	9.25cdefgh
C34	DS	7.0fg	8.5bcdefgh	6.0d	11.5cd	6.0i	6.0j
L1	CM1	6.75g	7.0defgh	7.0cd	13.25b	11.5bcd	7.5hij
C25	DFZ	6.5g	6.5efh	6.0d	10.25defg	6.0i	9.0cdefghi
Mean		9.162	8.537	7.373	9.796	9.105	10.073
LSD		3.125	8.53	2.455	1.715	2.021	2.743
CV (%)		24.16	27.07	23.58	12.34	15.72	19.29

aMeans followed by the same letters are not significantly different at 5% probability level.
NOTE: Test organisms: Ec= Escherichia coli; Lm= Listeria monocytogenes; Vf= Vibrio furnissii; Rp= Raoutellaplanticola; Sa= Staphylococcus aureus and Sm= Streptococcus mitis. Media used represented as C=starch casein, L=Luria Bertani, P1 and P4=ISP-project media. Land use system (LUS) where isolates were obtained: AL=agricultural (cultivated) land; DS=Dumpsite (urban); DFZ=degraded forest zone; CM1=Cattle manure; IS=Intact soil (forest zone); CM2=chicken manure

3.4 Molecular characterization of bioactive *Actinomycetes* isolates

The 16s rRNA sequence analysis revealed that eight isolated strains, C10a, C10b, C3, C8, P41, P4xiv, P1b and C45 belong to the *Streptomyces* species. The 16s rRNA gene sequences of the isolated Actinomycetes in this study were aligned with sequences with similarity indices above 97% retrieved from the NCBI gene bank (https://www.ncbi.nlm.nih.gov), which were then identified up to species or genus level (Table 4). The Neighbor Joining phylogenetic tree (Figure 1) built from the combined 16S rRNA gene sequences showed that isolate P4 (Ac No. ON398794.1) was clustered with *Streptomyces celluloflavus* (Acc No. JQ0066794.1 and Ac No. OK56094.1) from the gene bank at 84% bootstrap support value. Isolate C45 (Accession No. ON398796.1) was identified as *Streptomyces griseobrunneus* (Accession No. KP137823.1 and Accession No. KF703725.1) at similar bootstrap support values. Isolates P4x and C8 (Ac No. ON398793.1 and Accession No. ON398799.1 respectively) were both clustered at 100% bootstrap support with *Streptomyces pratensis* (Ac Nos. OM909122.1 and OK148886.1). Isolate P1b (Ac No. ON398795.1) was identified as *Streptomyces crystallinus* and was clustered with strains NBRC 15401 (NR_041177.1) and CSSP421 (NR_115376.1) at 100% bootstrap support. Isolates C10b, C10a and C3 (Ac No. ON398797.1, ON398798.1 and ON398800.1) clustered with *Streptomyces eurocidicus* strains DLHG12 (HQ844449.1) and IIL-Asp 72 (KX380889.1) at 99% bootstrap.

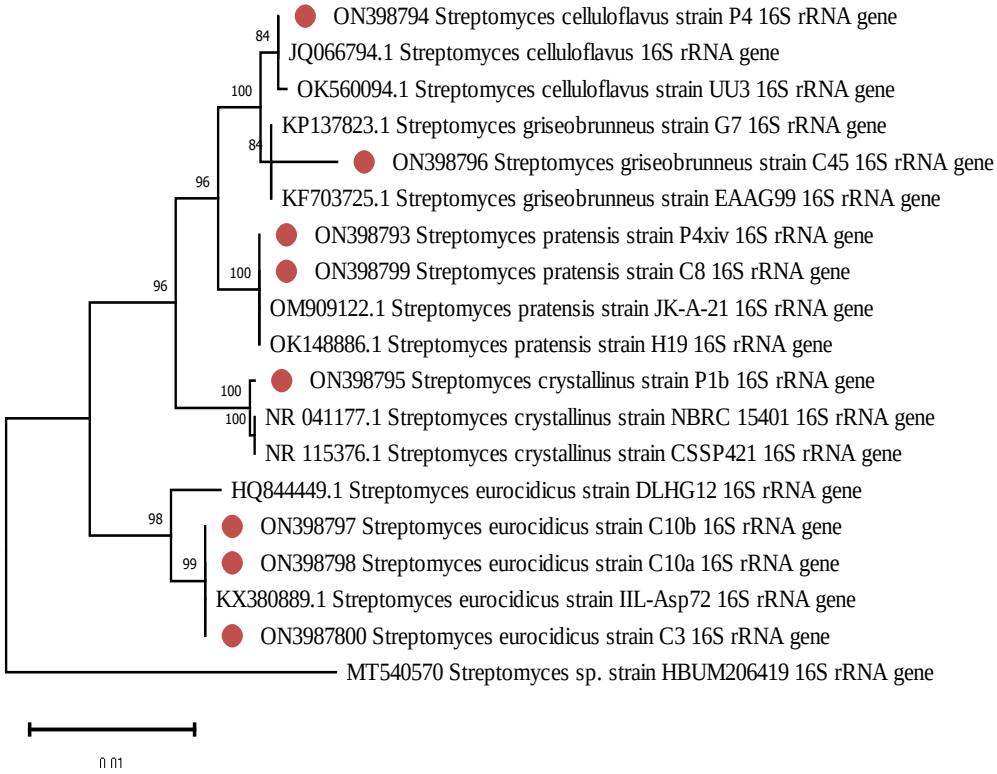


Figure 2. ***A Neighbor-Joining phylogenetic tree drawn from 16S rRNA gene sequences of Streptomyces species isolated from soils from Meru South and other similar sequences retrieved from the NCBI database. The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Tamura et al., 2004). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree***

Table 5: Identities and accession numbers of 5*Streptomyces* isolates obtained from different Land Use Systems in Meru South Sub-County, based on 16S rRNA gene sequences and similar sequences obtained from NCBI

Isolate Identity	NCBI Acc. No. (this study)	Site of isolation	BLAST similarity %	Species Identity	Similar NCBI Acc. No.	Country of origin
P4i	ON398794.1	IS	100	*S. celluloflavus*	JQ0066794.1	-
C45	ON398796.1	CM2	100	*S. griseobrunneus*	KP137823.1	Iran
P4x	ON398793.1	Dumpsite	100	*S. pratensis*	OM909122.1	China
C8	ON398799.1	Dumpsite	100	*S. pratensis*	OK148886.1	China
P1b	ON398795.1	Dumpsite	100	*S. crystallinus*	NR_041177.1	Japan
C10b	ON398797.1	CS	100	*S. crystallinus*	NR_041177.1	Japan
C10a	ON398797.1	Dumpsite	100	*S. eurocidicus*	HQ844449.1	China
C3	ON398797.1	CS	100	*S. eurocidicus*	KX380889.1	Brazil

Note: CM2= chicken manure; CS= Cultivated soil; IS= Intact soil; *S*= *Streptomyces*

4. DISCUSSION

The consistent circular shape of the colonies with round margins and varied elevations across different agar media suggests that these morphological characteristics might be inherent to *Actinomycetes* growth patterns, possibly influenced by their filamentous nature. The diverse range of colony colours observed in this study reflects the heterogeneity of *Actinomycetes* at the species or strain level. Smith *et al*. [49] revealed that there is a greater range of colony colours in *Actinomycetes* isolated from agricultural soils compared to those from forest soils. In the present study the colour of aerial mycelium ranged from white, grey, powdery, leathery, chalky, cream yellow, pink to purple. For the substrate mycelium

the colours ranged from pink, yellow, green, black, red to brown. The findings of this current study are also in agreement with study conducted by Jeffery [50], where it was observed that the colour of aerial mycelium ranging from white, creamy, chalky, powdery, brown, grey, pinkish to violet and substrate mycelium varied from brown, yellow to orange. Additionally, Johnson *et al*. [51] observed notable variations in spore formation patterns and mycelial structures among *Actinomycete* isolates derived from grasslands and agricultural fields.

The gram staining analysis of the *Actinomycetes* isolates indicated that the majority of the isolates were gram-positive bacteria, as evidenced by the purple staining observed. Gram-positive bacteria possess a thick peptidoglycan layer in their cell walls, which retains the crystal violet stain during the decolorization process. This observation is consistent with previous studies that have identified *Actinomycetes* as Gram-positive bacteria [49]. The hyphal structures displayed by the colonies varied significantly, with some colonies exhibiting highly intertwined filamentous hyphae, indicating a dense and complex network of filaments. This observation suggests the presence of *Actinomycetes* strains capable of forming intricate mycelial networks. Moreover, several colonies exhibited branching hyphae, which is a characteristic feature associated with the production of aerial mycelia by *Actinomycetes*. Additionally, short filaments were observed in some colonies in the present study. The presence of short filaments in some isolates may represent a distinct variation in colony morphology or could be attributed to specific genetic or environmental factors influencing their growth [52]. The observed morphological diversity of the *Actinomycetes* colonies, ranging from highly intertwined filamentous hyphae to branching hyphae, is consistent with the known characteristics of this bacterial group [53].

The results of the urease test revealed a diverse range of reactions among the isolates. Some isolates displayed a deep pink colour in both the slant and butt of the urea agar indicating a positive result for urease production. This deep pink colour is indicative of alkaline conditions resulting from ammonia production. Conversely, other isolates exhibited a yellow slant and pink butt, suggesting a negative result for urease activity. In the present study, some isolates tested negative for the Indole test, indicating the absence of Indole production. In casein hydrolysis activity the positive test was determined by the clearing of the agar around the bacterial growth on skim milk agar plate. The presence of clear zones around the colonies of these isolates on the skim milk agar plate indicates their ability to hydrolyse casein. For catalase test, the reaction was recorded positive (the presence of bubbles), indicative of decomposition of hydrogen peroxide by catalase hence production of oxygen gas. In citrate test, the medium turned from green to blue, indicative that the ability of isolate to use sodium citrate as the only source of carbon and inorganic ammonium phosphates as source of nitrogen.

The results of carbon source utilization was made based on the colour change from red to yellow in the phenol red, lactose, dextrose, maltose, and sucrose broths, indicating the conversion of sugars. Gas production was evident in the Durham tubes, suggesting the presence of fermentative activity in the isolates. Among the five different carbon sources tested in our study, the isolates displayed the highest growth rate when cultured with 1% glucose and lactose. Li *et al*. [42]) also observed a wide range of carbon-source utilization patterns among *Actinomycetes* isolated from different land use systems, highlighting their metabolic versatility, in their study on isolation, identification and characterization of *Actinomycetes* from rhizosphere soil of grapevine.

The results from the antibacterial activity tests indicate that the majority of the isolated samples demonstrated stronger effectiveness against gram-positive bacteria in comparison to gram-negative bacteria. These findings are in line with

earlier research conducted by Gebreyohanes*set al*. [24], who similarly observed that *Actinomycetes* strains exhibited antibacterial properties against a variety of bacterial strains, in their study conducted on isolation and characterization of potential antibiotic-producing *Actinomycetes* from water and sediments of Lake Tana-Ethiopia. However, most of the isolates exhibited limited efficacy against *E. coli*, which is consistent with the outcomes reported by Oskay*et al*.[54] on their study on antibacterial activity of some *Actinomycetes* isolated from farming soils of Turkey.

The analysis of the molecular characteristics of bioactive isolates indicated a prevalence of *Streptomyces* representatives (*Streptomyces celluloflavus*, *S. griseobrunneus*, *S. pratensisS. crystallinus* and *S. eurocidicus*). This discovery aligns with earlier research on soil samples collected from a protected region in Kenya [55], which identified five isolates with antimicrobial properties closely related to known *Streptomyces* species. By examining the 16S rRNA gene through phylogenetic analysis in our current study, we confirmed that these isolates were part of the *Actinomycetes* category, with similarity scores ranging from 84% to 100%. Our investigation did not identify a diverse array of *Actinomycetes* in this study. The lack of amplification in other isolates could be attributed to their belonging to rare *Actinomycetes* genera, suggesting that future studies should consider using a broader range of primers, including genus-specific primers.

The analysis from NCBI-BLASTn showed that the sequence of the P4i isolate shares an 84% similarity with *Streptomyces celluloflavus*. The species is acknowledged for its ability to synthesize aurethricin, a compound with the capability to enzymatically degrade cellulose. Additionally, it plays a significant role in the field of pharmaceuticals by generating poly amino acids. The isolates C10a and C3 exhibit a 99% similarity in sequence with *Streptomyces eurocidicus*. This specific strain has the ability to manufacture Azomycin, eurocidin C, D, E, tertiomycin A, B, and 2-nitroimidazole. Isolates P1b and C10b display a complete 100% similarity in sequence with *Streptomyces crystallinus*. This species is well-known for producing hygromycin-B, an aminoglycoside antibiotic that effectively eliminates bacteria, fungi, and higher eukaryotic cells by obstructing protein synthesis [56].The antibacterial impact of Hygromycin A (HA) arises from its capability to hinder protein synthesis, relying on a methylenedioxy bridged-aminocyclitol structure.

5. CONCLUSION AND RECOMMENDATION

The morphological analysis done in the present study provided valuable insights into the colony morphology, spore arrangement, and pigmentation of the isolated *Actinomycetes*, enabling preliminary identification and differentiation of the species. The biochemical tests helped in identifying key metabolic activities and enzymatic profiles of the *Actinomycetes* isolates. This information contributed to the understanding of their potential biotechnological applications. The crude extracts obtained from *Actinomycetes* showed noticeable antibacterial activity against the selected test organisms in secondary screening. This suggests the presence of bioactive compounds with potential antimicrobial properties within these extracts. The identified *Actinomycetes* isolates demonstrate promising antibacterial activity against test pathogens. Therefore, the study recommends further exploration of *Actinomycetes* as a valuable resource for developing novel antimicrobial drugs.

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