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Asian Journal of Research in Biochemistry

Volume 13, Issue 1, Page 26-41, 2023; Article no.AJRB.103306
ISSN: 2582-0516

Characterization of Antibiotic-producing *Actinomycetes* Isolated from River Tana and Lake Elementaita in Kenya

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJRB/2023/v13i1247

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/103306

Original Research Article

Received: 20/05/2023
Accepted: 21/07/2023
Published: 01/08/2023

ABSTRACT

The rise of antibiotic-resistant bacteria has become a global health concern, necessitating the search for novel sources of antibiotics. *Actinomycetes*, a group of microorganisms, have been known for their ability to produce bioactive compounds with antimicrobial properties. This study aimed to isolate, identify, and characterize antibiotic-producing *Actinomycetes* from River Tana and Lake Elementaita. Samples were collected from the study sites, and *Actinomycetes* were isolated using serial dilution and spread plate techniques. The isolates were characterized based on their morphological and biochemical properties. Furthermore, their antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhi* was evaluated using the agar well

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diffusion method. The zones of inhibition were measured (mm), and analysis was done to compare the activity of the isolates using Kruskal Wallis test and medians compared using Wilcoxon with Bonferroni correction at alpha = 0.05 in SAS version 9.4. Analysis of DNA sequences was done using the BLAST program and a phylogenetic tree was constructed using MEGA X version 11. Biochemical tests revealed positive results for catalase, indole, oxidase, and citrate utilization, while coagulase and methyl red tests were negative. In terms of antibacterial activity, 54.5% of the isolates showed activity against *E. coli,* 45.5% against *S. aureus*, and 45.5% against *S. typhi*. Isolate LEL2201 had significant ($p < 0.05$) higher zone of inhibition against *S. aureus* (inhibition zone of 25.0mm), while isolate RT2201 exhibited the highest activity against *E. coli* and *S. typhi* (inhibition zone of 8.5 mm and 8.6 mm, respectively). Molecular characterization through 16S rRNA gene sequencing identified the isolates as belonging to the Actinobacterium order. Phylogenetic analysis revealed their similarity to known *Actinomycetes* species including *Actinomycetales bacterium, Streptomyces intermedius* and *Streptomyces flavomacrosporus* from various countries. The findings of this study demonstrate the presence of antibiotic-producing *Actinomycetes* in River Tana and Lake Elementaita. Thus, further investigations are warranted to identify and characterize the specific antibacterial compounds produced by these isolates.

Keywords: Antibiotic-producing Actinomycetes; characterization; river Tana; Lake Elementaita; bioactivity; molecular sequencing.

1. INTRODUCTION

Natural products have been extracted from plants, animals and bacteria and used in various industries such as pharmacy, medicine, agriculture, and food. Natural products from microbes have acted as a major source of antibiotics used to treat many bacterial infections worldwide. Starting from penicillin discovered in 1929 by Alexander Fleming, studies have indicated that microbes are a great source bioactive compounds [1]. The increased prevalence of bacterial infections has been a major challenge to the human population with devastating high morbidity and mortality rates. This situation has been worsened by increasing antibiotic resistant strains of pathogenic bacteria, reduced effectiveness of antibiotics in the market, and the emergence of new bacterial infections [2].

The yearly report of Global Antimicrobial Resistance and Use Surveillance System (GLASS) indicate rising cases of antibiotic resistance for treatment of sepsis, sexually transmitted diseases, urinary tract infections, and diarrhea [3]. A good example is percentage resistance against ciprofloxacin meant to treatment urinary tract infection is between 4.1% - 79.4% in *Klebsiella pneumoniae* and 8.4% - 92.9% in *Escherichia coli* [4]. Studies show 64% mortality rate amongst people infected with Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Escherichia coli* and MRSA have developed resistance to third generation cephalosporins. *Neisseria gonorrhea* has shown resistance to sulphonamides, fluoroquinolones, tetracycline, macrolides and penicillins. It is only susceptible to extended spectrum cephalosporins ceftriaxone is effective. In 2018 WHO reported about 500,000 incidences of rifampicin resistance and multidrug resistant *Mycobacterium tuberculosis* [3].

The *Actinomycetes* species such as *Streptomyces*, *Actinoallumurus* and *Micromonospora* isolated from various parts of the world has revealed various secondary metabolites of polyketide, cyclo dipeptides, alkaloids and terpenes that have antibiotic activity against pathogenic bacteria [5]. The *Streptomyces* sp. SA32 isolated from India was found to produce biomolecules that were active against multi drug resistance strains of *E. coli*, *K. pneumonia, S. aureus*, E. *cloacae* and *Enterococcus* species [6]. Eleven species of *Actinomycetes* obtained from sediments and water of Lake Tana in Ethiopia had antimicrobial metabolites with activity against *P. aeruginosa*, *S. aureus E. coli,* and *S. typhi* [7]. The species of *Streptomyces* isolated from soil samples in Bangladesh had zones of inhibition against *Bacillus subtilis* [8]. The bacteria isolated from River Wiwi and Lake Bosomtwe showed activity againsst *Proteus vulgaris*, *B. thuringiensis*. *P. aeruginosa, S. aureus,* and *Bacillus Subtilis* [9]. This study aimed at isolation and identification and characterization of antibacterial *Actinomycetes* from river Tana and Lake Elementaita.

2. MATERIALS AND METHODS

2.1 Study Area

The samples for this study were collected from Lake Elementaita and River Tana. River Tana is the longest river in Kenya which is about 1000 km long. Its GPRS coordinates are -2°35'56.42" S 40°20'19.04" E. Lake Elementaita is a soda lake located in the Great Rift Valley about 120 kilometers from Nairobi. Its GPRS coordinates are 0°26'59.99" N 36°14'60.00" E.

2.2 Isolation and Biochemical Characterization of Antibacterial *Actinomycetes* Species from River Tana and Lake Elementaita

2.2.1 Sample collection and inoculation

Using a simple random sampling method, sampling and specific sampling sites identification at R. Tana and L. Elementaita were carried out. During sampling, at each of eight sampling site four water and four sediment samples were collected into UV sterilized 500 ml screw cap bottles and polypots respectively and spaces left to allow mixing and proper aeration [9]. During collection of water samples, the 500 ml bottle were submerged to approximately 10 cm of depth of water then opened to allow water to fill, closed and removed from water. About 5 g of sediments were collected into polypot. The sediment and water material were collected at the shallow parts of the lake and river. The samples were packed and transported at 4°C to the Animal Science Laboratory in Chuka University and stored at same temperature awaiting processing [11].

All the samples were tested within 6-24 hours after collection. The samples were serial diluted to isolate *Actinomycetes*. The stock solution was prepared through dilution of 1 g of sediment in 9 ml of sterile normal saline (8.5 g/l) and 1 ml of water 9 ml of sterile normal saline (8.5 g/l) then shaken well using vortex mixer. From stock solution 1 ml was used to prepare the final dilutions of 10^{-1}, 10^{-2} and 10^{-3} by serial dilution. The 0.1 ml of suspension from 10^{-3} and 10^{-2} were spread on starch casein agar under aseptic conditions [7]. For water samples, 1 ml of the stock solution was used to make ultimate volume of 10^{-1}, 10^{-2} and 10^{-3} by serial dilution. Only the 0.1 ml of 10^{-3} and 10^{-2} suspension were spread on starch casein agar using L-shaped glass rod. To reduce the growth of other bacteria, starch casein was supplemented with doxycline 100 mg/L and 100 ml of nystatin during preparation. The plates were placed at 28˚C and monitored after 24 hrs, 36 hrs and 48 hrs. The resultant colonies were subcultured on nutrient agar to get pure colonies. The pure colonies were maintained in nutrient agar at 4˚C [7].

2.2.2 Identification of isolates by gram staining

Isolated colonies were grown on nutrient agar and Gram-stained using method by Chaudhary et al. [11]. Using a wire loop a colony was picked and placed on a slide and heat-fixed by passing carefully on three times on Bunsen burner. The crystal violet (primary stain) was flooded to the slide for one minute. Then slide rinsed gently with flowing water for about five seconds to remove excess crystal violet. The mordant (Gram's iodine) was applied for a minute and slide washed with distilled water and decolorized using acetone for three seconds. Lastly, safranin (secondary stain) was applied on the slide for a minute and excess safranin washed with distilled water for five seconds. The Gram-positive bacteria retained crystal violet and appear violet/purple under a light microscope (X1000). The Gram-negative bacteria took safranin only, appearing pale red or pink under a light microscope (X1000).

2.2.3 Biochemical characterization *actinomycetes* isolates

The following biochemical tests were performed to identify the isolated *Actinomycetes.*

2.2.3.1 Oxidase test

Dry filter paper method procedure was applied in identification of oxidase property of different *Actinomycetes* species. The strip of Whatman's filter paper were put in 1% solution of tetramethyl-p-phenylenediamine dihydrochloride and drained for 30 seconds, then put on a petri dish and distilled water was added. The colonies of interest were picked with a wire loop and smeared on the moist surface. The positive tests were shown by a deep-purplish blue hue, which appears within 5-10 seconds [12].

2.2.3.2 Indole test

Sterile test tubes each having 4 ml of tryptophan broth were inoculated with 18-hour bacterial

culture, and incubated for 24 hours at 37˚C. Then added Kovac's reagent (0.5 ml) and observed for absence or presence of the ring. The organisms were classified as indole positive and indole negative [13].

2.2.3.3 Methyl red test

Two test tubes containing glucose phosphate broth were inoculated with 24-hour culture and incubated for 4 days at 35˚C. Then five drops of methyl red reagent were added to every test tube. If the broth changes colour to red it implied a positive reaction and no colour change implied a negative test [14].

2.2.3.4 Catalase test

A 24 hour pure colony was collected with sterile wire loop then placed onto the microscope slide. This was followed by addition of a drop of H_2O_2 onto the slide containing the isolate. Appearance of bubbles indicated presence of hydrogen peroxide (positive test). The organisms were classified as catalase-positive [15].

2.3.3.5 Coagulase test

Two drops of physiological saline were applied on a microscope slide followed by addition of 24 hour pure colonies and emulsified in saline to form a cream suspension. Then a drop of rabbit plasma was added. Immediate formation of clumps indicsted a positive test. The organisms were classified as coagulase-positive or catalase-positive [16].

2.2.3.6 Citrate Utilization test

Simmon citrate agar (2.4 g) was placed in 100 ml of distilled water and boiled on electric heater until it completely dissolved. The agar was autoclaved, dispensed in the test tubes to make slants. The isolates were then inoculated and incubated for 24 hours at 37˚C. The positive reaction was indicated by turning of green slant to blue after 24 hours of incubation [7].

2.2.3 Testing for antimicrobial activity of *Actinomycetes* isolates

The isolates were screened for antibacterial activity by agar well diffusion method. To prepare the inoculum, test organisms were grown separately on nutrient agar plates and resultant colonies transferred into 3 ml of normal saline in test tubes [9]. The surface of the Muller-Hinton agar plate was evenly inoculated with *Escherichia coli* with a sterile swab. Then by means of sterile wet swab Muller-Hinton agar plates were inoculated by even streaking of the plate surface [9]. This was repeated for *Salmonella typhi* and *Staphylococcus aureus.* The agar wells (eight milmetres) were made in the inoculated agar using a sterile cork borer and applied with 0.2ml of pure culture of the isolates. The experiment was replicated three times for each of the tests organisms. The plate was incubated at 37˚C for 24 hours. The diameter of the zone of growth-inhibition produced were measured using a ruler and the mean value calculated [9].

2.3 Molecular Characterization of *Actinomycetes* Isolates

2.3.1 Extraction of genomic DNA

The genomic DNA was extracted using a protocol described by Azadi et al. [17] with modifications. The *Actinomycetes* were inoculated in nutrient broth for three days at 28˚C after which the cells pelleted through centrifugation (10, 000 rpm for 10 minutes). The *Actinomycetes* cells were placed in a tube having 500 µl of 0.1mM TE buffer and lysozyme (20mg/ml) and incubated for 30 minutes at 37°C [18]. After which, 20 µl of for each proteinase K and 10% SDS (w/v) were added into the tube and incubated for 30 minutes at 55°C [18]. The lysate was cooled and extracted once with equal volume of phenol: chloroform (v/v, 1:1) at 10000 rpm for 5 minutes then precipitated with ethanol and sodium acetate at 20˚C. The pellet was washed twice with ethanol (90%) and dissolved in 0.1mM TE buffer. Then 20 µl of RNase (20 µg/ml) was added to obtain RNA free DNA and then incubated at 37˚C. The sample was extracted with equal volume of phenol: chloroform. The purified DNA was quantified using agarose gel electrophoresis after staining with ethidium bromide [19].

2.3.2 Amplification of *Actinomycetes* DNA

The 16SrRNA gene of *Actinomycetes* were amplified with universal primers 27F (5'-AGAGTT TGATCMTGGCTCAG-3') to 1492R (5'-CGGTTACCTTGTTACGACTT-3') (Rajivgandhi *et al.,* 2016). The thermo cycler Biometra T Personal (Germany) PCR amplifications were done in 20µl volume of reaction mixture containing 10µl of master mix (10 × DreamTaq Green PCR buffer, 3dNTP and Taq DNA polymerase), 1µl of each primer (20 pmol/µl), 1µl

(approximately 200 ng) of template DNA and 7µl of sterile Millipore water. There were 30 cycles of amplification (94˚C for 5min, 94˚C for 40 sec, 52˚Cfor 1 min and 72˚C for 90 sec and final extension at 72˚C for 10 min) [19].

2.3.3 Sequencing similarities and Phylogenetic Analysis

The PCR products were subjected to Sanger sequencing using ABI3730 genetic analyzer (USA). Then BLAST program (www.ncbi.nlm.nih.gov/blst) was used to determine the degree of DNA similarity [19]. Multiple sequence alignment was done using clustalW and molecular phylogenetic tree drawn MEGA X version 11.0 [20].

2.4 Statistical Analysis

The measurement of the zones of inhibition (mm) for determination of antibacterial activity of *Actinomycetes* was analyzed using Kruskal Wallis test in SAS version 9.4 because the data was not normally distributed even after it was log transformed Significance means were compared using Wilcoxon with Bonferroni correction at alpha = 0.05.

3. RESULTS

3.1 Growth Morphology of *Actinomycetes* Isolates

Eleven *Actinomycetes* species were isolated from 64 samples collected from River Tana and Lake Elementaita. Isolate RT2201, RT2202, RT2203, RT2204, RT2205, RT2206 and RT2207 were isolated from River Tana while isolates LEL2201, LEL2202, LEL2203 and LEL2204 were isolated from Lake Elementaita. The morphological characteristics on starch casein agar and images of each colonies are indicated in the Plate 1and Table 1.

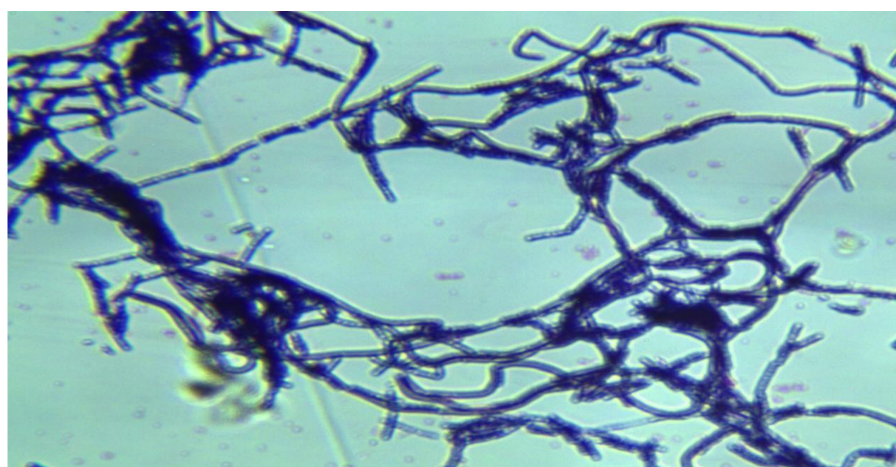
Plate 1. Images of *Actinomycetes* cultures on Starch Casein Agar plates at 28°C for 48 hours. Isolates RT2201, RT2202, RT2205, RT2207, LEL2201, LEL2202, LEL2203 and LEL2204 had smooth margins, raised and white while isolate RT2203, RT2204 and RT2206 had crenated margins, raised and white

Table 1. Growth Characteristics of *Actinomycetes* Isolates on Starch Casein and Nutrient Agar Plates

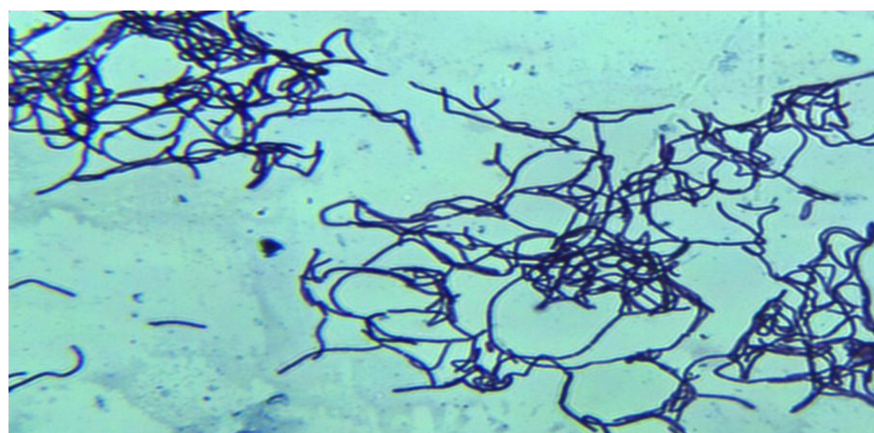
Isolates	Outline	Colour	Smell
RT2201	Entire	Cream	Earth like odour
RT2202	Entire	Cream yellow	Earth like odour
RT2203	Crenated	Cream yellow	Earth like odour
RT2204	Crenated	Cream yellow	Earth like odour
RT2205	Entire	Cream	Earth like odour
RT2206	Crenated	Cream	Earth like odour
RT2207	Entire	Cream	Earth like odour
LEL2201	Entire	Cream	Earth like odour
LEL2202	Entire	Cream	Earth like odour
LEL2203	Entire	Cream	Earth like odour
LEL2204	Entire	Cream	Earth like odour
Control	None	None	None

3.2 Gram Staining Characteristics of *Actinomycetes* Isolates

All the isolates were Gram positive. They appeared as long branched purple filament (Plate 2).



(i). RT 2201



(ii). LEL2201

Plate 2. Microscopic Images of Gram Stains Results for Isolates RT2201 and LEL2201. All Isolates Appeared as Long Branched Purple Filament

3.3 Biochemical Characterization of *Actinomycetes* Isolates

Table 2. Biochemical Tests Results for *Actinomycetes* Isolates

Isolates	Coagulase Test	Catalase Test	Indole Test	Methyl red Test	Oxidase Test	Citrate Utilization
RT2201	-	+	+	-	+	+
RT2202	-	+	+	-	+	+
RT2203	-	+	+	-	+	+
RT2204	-	+	+	-	+	+
RT2205	-	+	+	-	+	+
RT2206	-	+	+	-	+	+
RT2207	-	+	+	-	+	+
LEL2201	-	+	+	-	+	+
LEL2202	-	+	+	-	+	+
LEL2203	-	+	+	-	+	+
LEL2204	-	+	+	-	+	+

The biochemical tests results revealed that 100% of *Actinomycetes* isolates were coagulase and methyl red negative, catalase, indole, oxidase and citrate utilization positive (Table 2).

3.4 Bioactivity of Selected *Actinomycetes* Isolates from River Tana and Lake Elementaita against Selected Test Organisms

There was significantly ($p<0.05$) different in inhibition zones of *Actinomycetes* isolates and standard antibiotics against *E. coli* (Table 3 and plate 3). Isolate RT2202 (8.8 mm) had the largest zone of inhibition compared to all other *Actinomycetes* isolates followed by isolate RT2207 (4.2 mm) (Table 3). Isolate RT2202 performed lower than standard gentamycin (10 mm), cotrimazole (9.9 mm) and chloramphenicol (9 mm) but performed better than standard streptomycin, kanamycin (5 mm), tetracycline, sulphamethoxazole (0.0 mm), and ampicillin (0.0 mm). Isolates RT2201, RT2204 and LEL2201 had the lowest zones of inhibition (2 mm) against *E. coli* however they performed better than ampicillin and equal to tetracycline (2 mm). In total 54.5% of isolates had activity against *E. coli* (Table 3 and plate 3).

There was a significance ($p<0.05$) different in antibacterial activity of *Actinomycetes* isolates against *S. aureus* (Table 3 and Plate 4). Isolate LEL2201 (25.0 mm) had the best inhibition against *S. aureus* followed by RT2201 (8.5 mm) (Table 3 and Plate 4). Isolate LEL2201 performed better than standard gentamycin (7 mm), chloramphenicol (6 mm), co-trimazole (6 mm), tetracycline (4 mm), sulphamethoxazole (4 mm), kanamycin (3 mm), streptomycin (3 mm) and ampicillin (3 mm). Isolate RT2207 (1.5 mm) had the least zone of inhibition against *S. aureus* performing lower than all the standard antibiotics in the study except ampicillin (Table 3 and Plate 4). In total 45.5% of isolates had bioactivity against *S. aureus* (Table 3 and Plate 4).

The antibacterial activity of *Actinomycetes* isolates to *S. typhi* (Table 3 and plate 5) was significantly ($p<0.05$) different. RT2201 (8.6 mm) had the largest zone of inhibition against *S. typhi* followed by isolate RT2202 (7 mm). RT2201 performed better than standard co-trimazole (8mm), sulphamethoxazole (7 mm), chloramphenicol (7 mm), kanamycin (6 mm), gentamycin (5mm), streptomycin (4mm) and ampicillin (5.0 mm). Isolate LEL2201 and RT2207 (4 mm) had the lowest zones of inhibition which are equal to standard streptomycin and better than ampicillin. In total 45.5% of isolates had bioactivity against *S. typhi* (Table 3 and Plate 5).

The isolates were cultured on MHA at 37°C for 24 hours. Isolate RT2201 (2 mm), RT2202 (8.8 mm), RT2204 (2 mm), RT2205 (3 mm), RT2207(4.2 mm) and LEL2201(2 mm) had zones of inhibition. The positive control is a disk with various drug concentrations. TE-tetracycline, K-kanamycin, COT-cotrimoxazole, AMP - ampicillin, S-streptomycin, Sx-sulphamethoxazole, GEN-gentamicin, and C-chloramphenicol. The *E. coli* is sensitive to tetracycline, cotrimazole, streptomycin, kanamycin, gentamycin and chloramphenicol but resistant to ampicillin and sulphomethozazole.

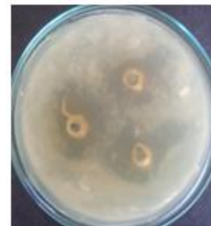
Table 3. The Activity of *Actinomycetes* against E*. coli, S. aureus* and *S. typhi*

Analysis Variable : Median of inhibition (mm)				
Isolates and controls	**N**	***E. coli***	***S. aureus***	***S. typhi***
RT2201	3	2.0^{c}	8.5^{b}	8.6^{a}
Ampicillin	3	0.0^{d}	4.0^{c}	5.0^{b}
RT2202	3	8.80^{a}	3.9^{c}	7.0^{a}
RT2203	3	0.0^{d}	0.0^{e}	0.0^{c}
RT2204	3	2.0^{c}	0.0^{e}	5.1^{b}
RT2205	3	3.0^{c}	7.0^{b}	0.0^{c}
RT2206	3	0.0^{d}	0.0^{e}	0.0^{c}
RT2207	3	4.2^{c}	1.5^{de}	4.0^{b}
Gentamycin	3	10.0^{a}	7.0^{b}	5.0^{b}
LEL2201	3	2.0^{c}	25.0^{a}	4.0^{b}
LEL2202	3	0.0^{d}	0.0^{e}	0.0^{c}
LEL2203	3	0.0^{d}	0.0^{e}	0.0^{c}
LEL2204	3	0.0^{d}	0.0^{e}	0.0^{c}
Tetracycline	3	2.0^{c}	4.0^{c}	5.0^{b}
chloramphenicol	3	9.0^{a}	6.0^{b}	7.0^{a}
cotrimazole	3	9.9^{a}	6.0^{b}	8.0^{a}
kanamycin	3	5.0^{b}	3.0^{c}	6.0^{ab}
Saline	3	0.0^{d}	0.0^{e}	0.0^{c}
streptomycin	3	5.0^{b}	3.0^{c}	4.0^{b}
sulphamethoxazole	3	0.00^{d}	4.0^{c}	7.0^{a}

The figures in the column followed by same letter are not significantly different at α = 0.05

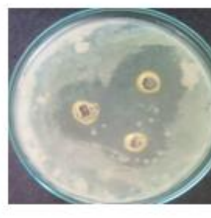
(a). RT2201

(b). RT2202



(c). RT2204

(d). RT2205



(e). RT2207

(f). LEL2201

(g).Positive control

Plate 3. Images of zones of inhibition of selected isolates against *E. coli*

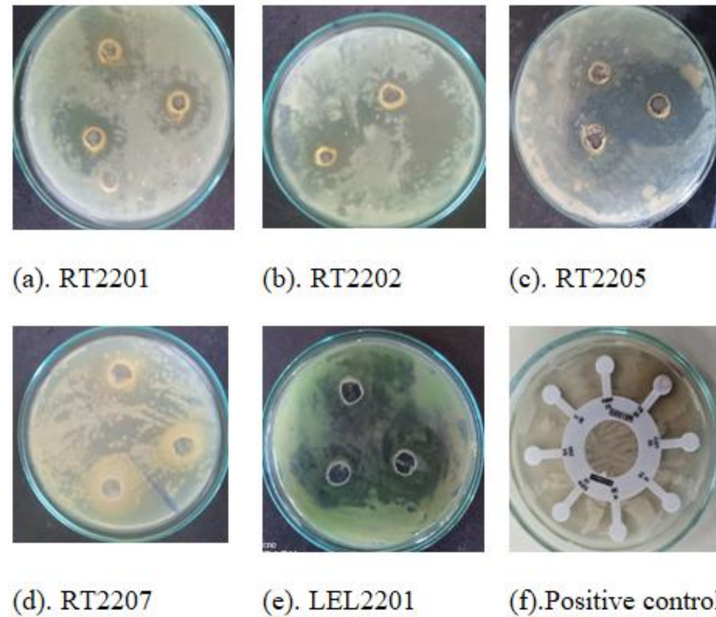


Plate 4. Images of zones of inhibitionof selected *Actinomycetes* isolates (RT2201 (8.5 mm), RT2202 (4.0 mm), RT2205 (7 mm), RT2207 (1.5 mm) and LEL2201 (25 mm) against *S. aureus.* The isolates were grown on MHA for 24 hours at 28°C. The positive control is a disk with various drug concentrations. TE-tetracycline, K-kanamycin, COT-cotrimoxazole, AMP - ampicillin, S-streptomycin, Sx-sulphamethoxazole, GEN-gentamicin, and C-chloramphenicol. The *S. aureus* is sensitive to all of them

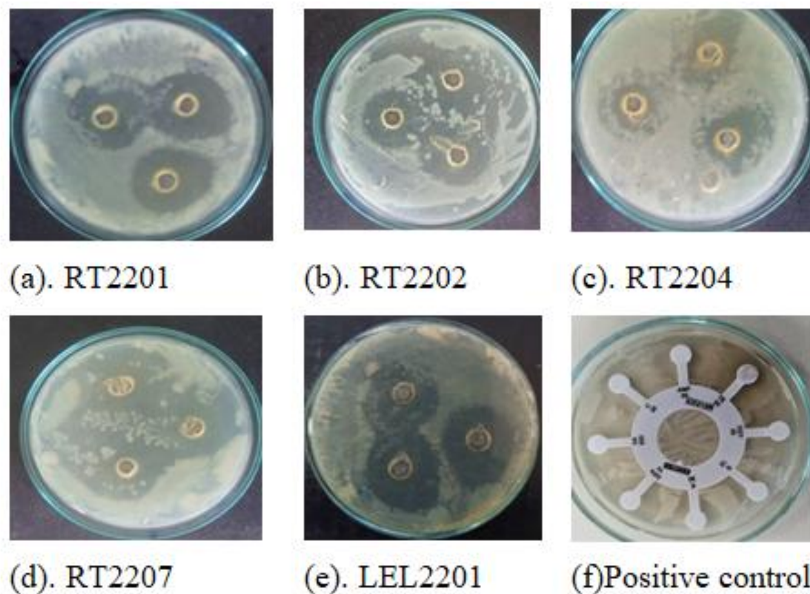


Plate 2. Images of zones of inhibitionof selected *Actinomycetes* isolates RT2201 (8.6 mm), RT2202 (5 mm), RT2204 (5.1 mm), RT2207 (4 mm) and LEL2201 (4 mm) against *S. typhi.* The isolates were grown on MHA for 24 hours at 28°C .The positive control is a disk with various drug concentrations. TE-tetracycline, K-kanamycin, COT-cotrimoxazole, AMP -ampicillin, S-streptomycin, Sx-sulphamethoxazole, GEN-gentamicin, and C-chloramphenicol The *S. typhi* is sensitive to all of them

3.5 Molecular Characterization of Selected *Actinomycetes* Isolates

3.5.1 DNA extraction and polymerase chain reaction

The genomic DNA of the six isolates was extracted and underwent polymerase chain reaction of 16S rDNA and electrophoresis on 1% agarose gel stained with ethidium bromide and observed under UV transilluminator. A positive amplification was seen as a bright band at 1500 bp which is the size of the 16S rDNA gene of *Actinomycetes* species (1250-1500bp) as shown in Fig. 1.

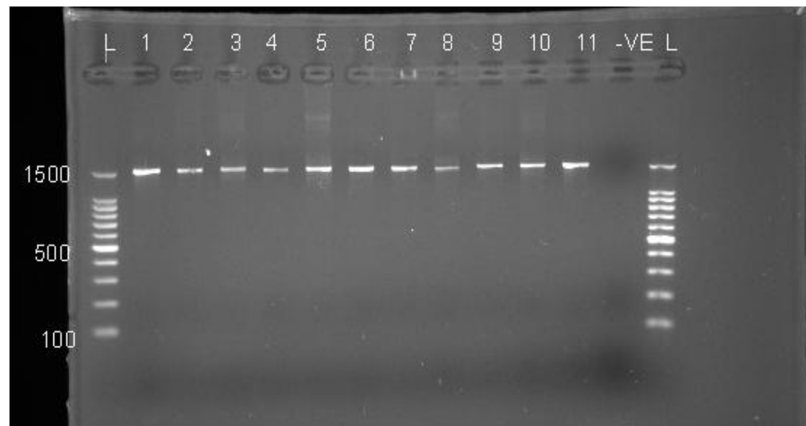


Fig. 1. The agarose gel containing PCR products, primer and template DNA from selected antibiotic producing *Actinomycetes* species. L=molecular ladder, 1=RT2201, 2=Isolate RT2202, 3= isolate RT2203, 4 =Isolate RT2204, 5=RT2205, 6= Isolate RT2206, 7= Isolate RT2207, 8= Isolate LEL2201, 9 = Isolate LEL2202, 10=Isolate LEL2203, 11=Isolate LEL2204, and -VE=Negative control. All the isolates had a bright band at 1.5kb

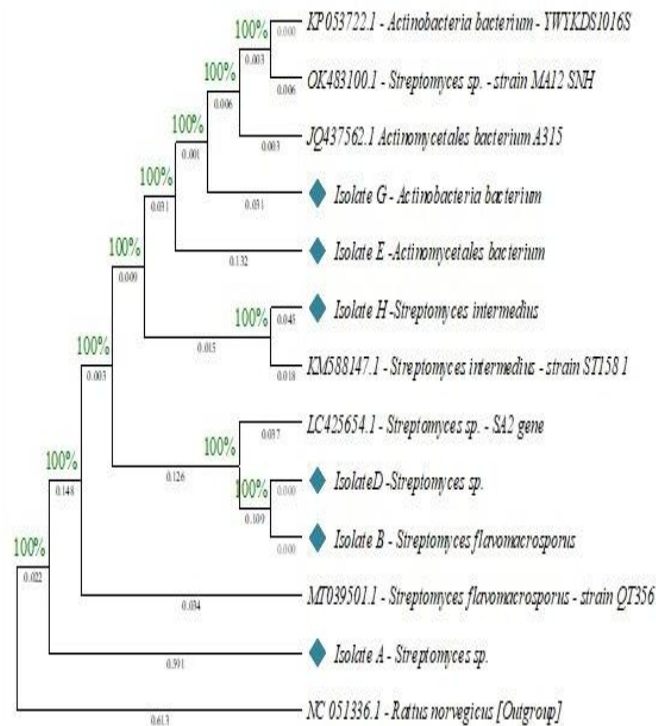


Fig. 1. The phylogenetic tree showing the six isolates divided into four dendrograms. Isolate A =RT2201, B=RT2202, D=RT2204, E=RT2205, G=RT2207 and H=LEL2201

Table 4. The results of molecular sequencing showing percentage similarity of related known organisms to isolates

Isolate	GeneBank Accession No.	% similarity	Scientific name	Country
RT2201	OK483100.1	99.83	*Streptomyces sp.*	India
RT2202	MT039501.1	74.88	*Streptomyces flavomacrosporus*	China
RT2204	LC425654.1	84.59	*Streptomyces sp.*	Nepal
RT2205	JQ437562.1	84.22	*Actinomycetales bacterium*	China
RT2207	KP53721.1	100	*Actinobacteria bacterium*	China
LEL2201	KM588147.1	91.66	*Streptomyces intermedius*	Iran

Table 5. A matrix showing relationship amongst six isolates

Actinomycetes	1	2	3	4	5	6	7	8	9	10	11	12
LC425654.1-*Streptomyces_sp.*_SA2	0											
IsolateD -*Streptomyces sp.*	0.21											
JQ437562.1-*Actinomycetales bacterium*-A315	0.22	0.29										
IsolateE-*Actinomycetales bacterium*	0.33	0.42	0.16									
IsolateG - *Actinobacteria bacterium*	0.24	0.36	0.06	0.28								
KP053722.1– *Actinobacteria-bacterium*-YWYKDS1016S	0.23	0.35	0.01	0.28	0.10							
Isolate H -*Streptomyces intermedius*	0.27	0.38	0.11	0.34	0.19	0.20						
KM588147.1-*Streptomyces-intermedius*-strain-ST158	0.25	0.27	0.05	0.25	0.07	0.04	0.11					
OK483100.1-*Streptomycessp*.strain-MA12-SNH	0.23	0.35	0.01	0.28	0.10	0.00	0.20	0.05				
Isolate A -*Streptomyces sp.*	0.55	0.55	0.51	0.54	0.51	0.49	0.52	0.50	0.50			
MT039501.1-*Streptomyces_flavomacrosporus*_strain_QT356	0.24	0.36	0.07	0.31	0.16	0.05	0.20	0.03	0.05	0.50		
Isolate B -*Streptomyces flavomacrosporus*	0.16	0.00	0.29	0.34	0.30	0.28	0.31	0.29	0.28	0.55	0.29	
MH028054.1-*Nigrospora_sphaerica*_strain_E6_{outgroup}	0.50	0.51	0.42	0.51	0.46	0.44	0.49	0.45	0.45	0.60	0.45	0.48

3.5.2 Molecular sequencing of the 16S rRNA gene

The results of the molecular sequencing of 16S rRNA gene indicated that all the six isolates belongs to order *Actinobacterium* (Table 4).

The phylogenetic tree divided the six isolates into four dendrograms. The RT2207 and RT2205 formed the first dendrograms, LEL2201formed the second dendrograms, RT2204 and RT2202 formed the third dendrograms and RT2201 formed the last dendrograms (Fig. 2). The evolutionary distance matrix of the *Actinomycetes* isolates and various *Actinomycetes* in the gene bank in the above phylogenetic tree.

4. DISCUSSION

4.1 Morphological Characteristics and Antibacterial Properties of *Actinomycetes* Isolates

The *Actinomycetes* isolates exhibited growth on both starch casein and nutrient agar, indicating their ability to utilize the nutrients and minerals present in these media [7,21]. The morphological characteristics observed on both media included smooth-edged to crenated colonies with a raised and hard-to-scrape texture. The isolates had a soil-like odor and appeared in various colors, such as white, cream, or yellow. These morphological characteristics are consistent with previous studies on *Actinomycetes* isolates from Lake Tana in Ethiopia [7] and *Actinomycetes* isolates reported by Gouse et al. [22]. The isolates' had hard to scrap texture is attributed to their substrate hyphae which forms an extensive network in growth media [23]. The characteristic soil-like odour of *Actinomycetes* is attributed to production of volatile essential oils [24].

Gram staining analysis confirmed that the *Actinomycetes* isolates were Gram-positive bacteria, characterized by long branched purple filaments. This is in line with the typical microscopic features of *Actinomycetes*, which exhibit wavy, long-branched filaments that absorb crystal violet stain and resist decolorization by acetone. Similar Gram staining characteristics have been reported in other studies [25,26,24].

All the *Actinomycetes* isolates in this study exhibited positive results for catalase, oxidase, citrate utilization, and indole production, while they tested negative for coagulase and methyl red. The positive catalase activity indicates the presence of the catalase enzyme, which breaks down hydrogen peroxide and protect *Actinomycetes* from effects of hydrogen peroxide [15]. The ability of the isolates to utilize citrate as a carbon source suggests the production of citrate permease, responsible for converting citrate to pyruvate (Jadon et al. 2014). The positive oxidase activity indicates the presence of intracellular cytochrome oxidase or indophenol oxidase, enabling the transport of electrons from NADH to oxygen [12]. Indole production indicates the presence of the tryptophanase enzyme, which catalyzes the deamination of tryptophan to produce indole. These biochemical characteristics are consistent with studies by Dhananjeyan et al. [25] and Gebreyohannes et al. [7]. According to Gebreyohannes et al. [7] approximately 45% of *Actinomycetes* have the ability to metabolize citrate. Though our results on methyl red results and citrate utilization results are in agreement with those of Gouse et al. [22], it worth noting that they reported negative indole production among the four *Actinomycetes* isolates which contradicts our indole results. The biochemical characteristics of the *Actinomycetes* isolates from this study align with previous findings, although some variations were observed. The differences in biochemical characteristics among *Actinomycetes* isolates can be attributed to environmental factors, such as nutrient availability, oxygen levels, and metabolic stress, which can influence the production of metabolic enzymes. The *Actinomycetes* isolates from different environments, specifically from River Tana in Tharaka Nithi and Lake Elementaita in Nakuru, both in Kenya, exhibited similar biochemical characteristics across the six tests. These organisms were aerobic, and differences in factors such as salinity, human activities, nutrient supply, and temperatures might have contributed to the evolution of related metabolic enzymes.

4.2 Bioactivity of Selected *Actinomycetes* Isolates from River Tana and Lake Elementaita against *E. coli*, *S. aureus* and *S. typhi*

The bioactivity screening of *Actinomycetes* isolates from River Tana and Lake Elementaita against *E. coli, S. aureus,* and *S. typhi* resulted in a significant differences. The antibacterial properties of the isolates may be attributed to their secondary metabolites, which are known to possess antibacterial activity. Several isolates

did not show bioactivity against any of the tested bacteria, indicating that their secondary metabolites may not have specific targets in these organisms. On the other hand, some isolates exhibited varying degrees of bioactivity. For instance, isolate RT2201 displayed larger zones of inhibition against S. aureus (8.5 mm) and S. typhi (8.6 mm) compared to *E. coli* (2.0 mm), suggesting a broader spectrum of antibacterial activity. This indicates that the antibacterial metabolites produced by RT2201 may have more molecular targets in S. aureus and S. typhi than in *E. coli* [23].

Isolate RT2205 displayed bioactivity against *E. coli* and *S. aureus*, while no zone of inhibition was observed against *S. typhi*. This suggests that the antibacterial targets for the metabolites produced by RT2205 may be present in *E. coli* and *S. aureus* but lacking in *S. typhi.* Alternatively, *S. typhi* may have mechanisms to evade or resist the activity of the antibacterial metabolites. Similarly, isolate RT2207 exhibited broad-spectrum bioactivity against both Gram-negative (*S. typhi* and *E. coli*) and Gram-positive (*S. aureus*) bacteria, indicating the presence of molecular targets in all tested organisms as put forward by Sharma et al. [23].

The isolate RT2202 and RT2204 had larger inhibition in E. *coli* and then *S. typhi*, *S. aureus*. The antibacterial metabolites of this isolate is also broad spectrum with molecular targets in all test organisms [27]. A difference in bioactivity against (*S. typhi* and *E. coli*) can be that the molecular targets for these antibacterial metabolites are more expressed in Gram negative [28]. Peptidoglycan of the Gram positive *(S. aureus*) can also be a hindrance for the antibacterial metabolite to access the molecular targets [29].

The antibacterial metabolite of isolate RT2205 had bioactivity against *E. coli* and *S. aureus*. Antibacterial metabolites of RT2205 are broad spectrum however their targets are in *S. aureus* and *E. coli* and may be lacking in *S. typhi* or the organism has a way of evading the activity of antibacterial metabolites of RT2205 evident by no zone of inhibition [30]. The isolate RT2207 had broad spectrum bioactivity against Gram negatives (*S. typhi* and *E. coli*) and Gram positive (*S. aureus*). The difference in bioactivity against (*S. typhi* and *E. coli*) can be that the molecular targets for these antibacterial metabolites are more expressed in all test organisms. Peptidoglycan of the Gram positive *(S. aureus*) can also be a hindrance for the antibacterial metabolite to access the molecular targets [29].

The isolate LEL2201 had broad spectrum bioactivity against all test organisms. The antibacterial metabolites in LEL2201 had a larger zone of inhibition against *S. aureus* (25 mm) than Gram negatives (*S. typhi* and *E. coli*) there may be more targets for these metabolite in *S. aureus* than in Gram negatives (*S. typhi* and *E. coli*) [23]. Metabolites of LEL2201 are able to cross both peptidoglycan in Gram positive and complex outer membrane of Gram negative bacterial cell. The secondary metabolites may have ability to inhibit different enzymes in the bacterial cell and inhibiting their growth [31]. The LEL2201 had 25 mm zone of inhibition this is attributed to isoxazole which inhibits the synthesis of purines and DNA leading to bacteriostatic effect [32]. This results are comparable to other studies worldwide which have revealed that *Actinomycetes* have ability to produce molecules with antibacterial activities against bacteria. The Gouse et al. [22] indicated 59% of *Actinomycetes* isolates had antibacterial activity against test organisms. A study by Charousová et al. [33] results indicates that 39.5% of *Actinomycetes* isolates had bioactivity against *E. coli.*

4.3 Molecular Characterization of the *Actinomycetes* Isolates

Genomic DNA of isolates produced good quality DNA that was well amplified by *Actinomycetes* specific 16S rRNA primers [31]. The 16S rRNA gene of the isolated *Actinomycetes* had varying percentage similarity with different *Actinomycetes* isolated at different countries in Asia which indicates that they are organisms of the same species but of different strains [34]. This difference observed can be attributed to accessibility and concentration of nutrients, presence of competitors in the environment, presence of various metabolites in the cell, phage attacks, temperature, pH and density of cell [35]. These factors contributes to evolutionary changes by influencing different gene expressions and enzymatic sets inside the organisms cell which permits the generation of compounds which have potential useful diverse purposes including antibacterial activity [35].

The up regulation of different secondary metabolites gene expression is responsible for synthesis of different potential antibacterial secondary metabolites which had bioactivity

against *S. typhi, E. coli* and *S. aureus* [34]. Phylogenetic results of the six isolates showed that the organisms' falls within four main clusters. However these isolates have produced same classes (long chain hydrocarbons volatile compounds and long chain alcohols) antibacterial metabolites with different concentrations. This indicates that different genera and species of *Actinomycetes* the metabolic pathways of secondary metabolite synthesis are strictly conserved within the order [36].

Sequencing of 16S rRNA gene of RT2201 (99.83%) (Table 4) revealed that it is closely related to *Streptomyces sp.* OK483100.1. The isolate RT2202 (74.88%) is closely related to Streptomyces *flavomacrosporus* MT039501. The *Streptomyces flavomacrosporus* has been reported to have bioactivity against *S. typhi* and Apergillus *species* [37]. The RT2204 (84.59 %) was identified to be related to *Streptomyces sp.* LC425654.1 which had bioactivity against *S. aureus* [38].

The *Actinomycetes* RT2205 (84.22%) and RT2207 (100%) was found to be closely related to *Actinobacteria bacterium* JQ437562 and *Actinomycetales bacterium* KP53721 respectively. The LEL2201(91%) is closely related to *Streptomyces intermedius* KM588147.1 which is closely related to haloalkaliphilic *Streptomyces intermedius* isolated in India that had bioactivity against *E. coli* [39].

5. CONCLUSIONS AND RECOMMENDATIONS

Actinomycetes isolates from both river Tana and lake Elementainta have significance (p<0.05) activity against *E. coli*, *S. typhi* and *S. aureus*. Through molecular screening of 16S rRNA genes all the isolates were determined to be members of *Actinomycetes* species. The isolates should be subjected to further evaluation to satisfy their suitability to produce secondary metabolites with activity against wide variety of bacteria.The genes responsible for various antibiotics in the isolated *Actinomycetes* species should be screened and their potential to produce variety of antibiotics determined.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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