Review Article

CHARACTERIZATION OF ANTIBIOTIC PRODUCING *Actinomycetes* FROM RIVER TANA AND LAKE ELEMENTAITA

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ABSTRACT

The aquatic environment contain wide species of A*ctinomyces* such as *Streptomyces* species that produces anti-tumors, enzymes, antibiotics, antivirals, and antifungals. *Actinomycetes* species have produced antibiotics such as chloramphenicol, streptomycin, gentamycin, among others. 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. This study aimed at isolation and molecular identification of antibacterial *Actinomycetes* species. The experiments for this study was laid out in Complete Randomized Design and replicated three times to determine the difference between the inhibition zones (mm) of isolates against the tests organisms. A total of six antibiotic producing *Actinomycetes* species were isolated from River Tana and Lake Elementaita and identified through morphological and molecular characterizations. There was significant ($p<0.05$) difference on antibacterial activity of *Actinomycetes* isolates against *Escherichia coli, Salmonella typhi* and *Staphylococcus aureus.* The findings of this study can help in developing new or alternative antibiotics that can be used for treatment of pathogenic and resistant bacteria.

Key words: Six *Actinomycetes*, antibiotic resistance, antibacterial activity.

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 (Mohr *et al.,* 2016). 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 (Nordenfjäll *et al.,* 2014).

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 (WHO, 2021). 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* (WHO, 2021). 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* (WHO, 2022).

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 (Rao *et al.,* 2017). 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 (Sanghvi *et al.,* 2014). 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* (Gebreyohannes *et al.,* 2013). The species of *Streptomyces* isolated from soil samples in Bangladesh had zones of inhibition against *Bacillus subtilis* (Ripa *et al.,* 2009). The bacteria isolated from River Wiwi and Lake Bosomtwe showed activity againsst *Proteus vulgaris*, *B. thuringiensis*. *P. aeruginosa, S. aureus,* and

Bacillus Subtilis (Tawiah *et al.,* 2012). 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 (Tawiah *et al.*, 2012). 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 (Pillai *et al.*, 2014).

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 (Gebreyohannes *et al.,* 2013). 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 (Gebreyohannes *et al.,* 2013).

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*. (2013). 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 (Shields and Cathcart, 2010).

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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 (Hari *et al*., 2018).

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 (Das *et al.,* 2012).

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 (Senthilkumar *et al.,* 2021).

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 (Katz *et al.,* 2010).

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 (Gebreyohannes *et al.,* 2013)

2.2.2 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 (Tawiah *et al.*, 2012). 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 (Tawiah *et al.*, 2012). 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 (Tawiah *et al.*, 2012).

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* (2020) 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 (Kumar *et al.,*2010). 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 (Kumar *et al.,* 2010). 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 (Rajivgandhi *et al.,* 2016).

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) (Rajivgandhi *et al.,* 2016).

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 (Rajivgandhi *et al.,* 2016). Multiple sequence alignment was done using clustalW and molecular phylogenetic tree drawn MEGA X version 11.0. (Kumar *et al.,* 2018).

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 1below.

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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

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(i) RT2201

(ii). RT2202

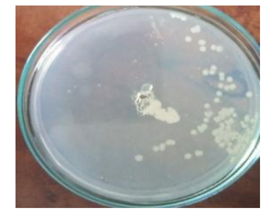
(iii). RT2203

(iv). RT2204

(v). RT2205

(v). RT2206

(vi). RT2207



(vii). LEL2201

(viii).LEL2202 (ix). LEL2203 (x). LEL2204

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

3.2 Gram Staining Characteristics of *Actinomycetes* Isolates

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

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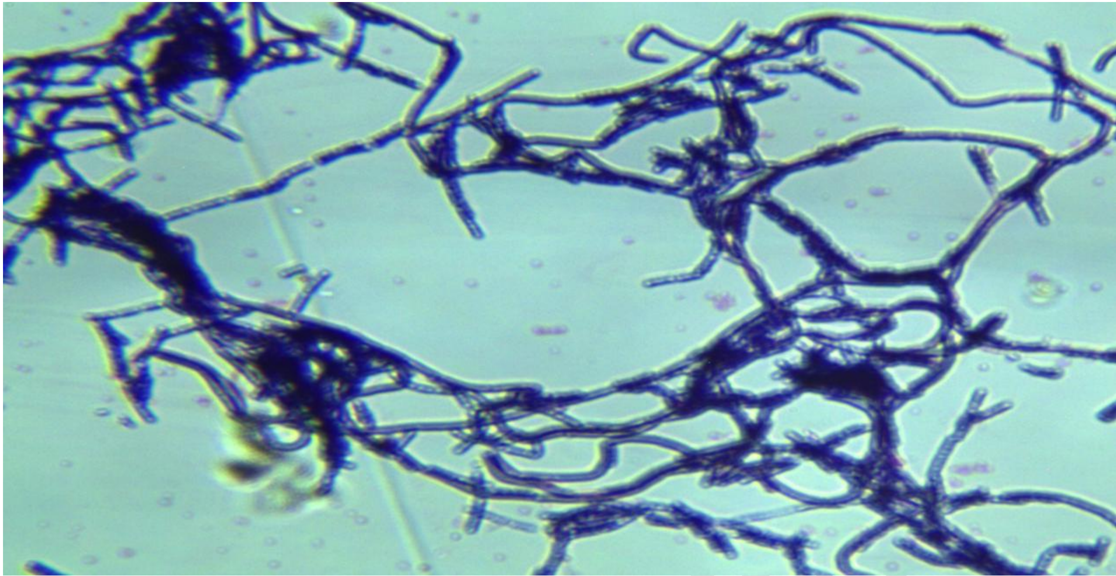
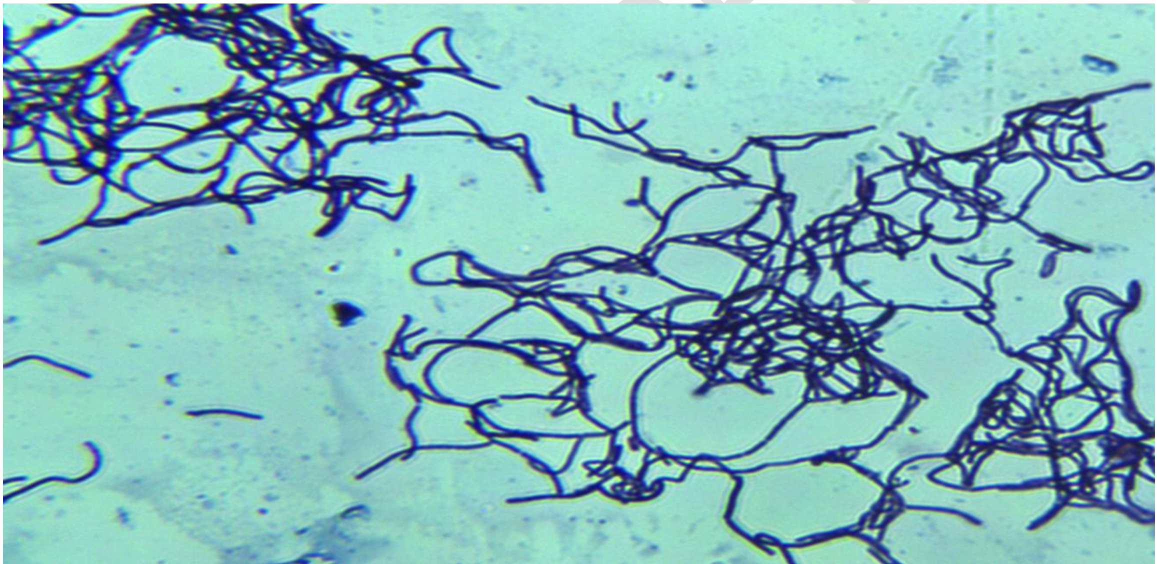


(i). RT 2201



(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

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).

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	-	+	+	-	+	+

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

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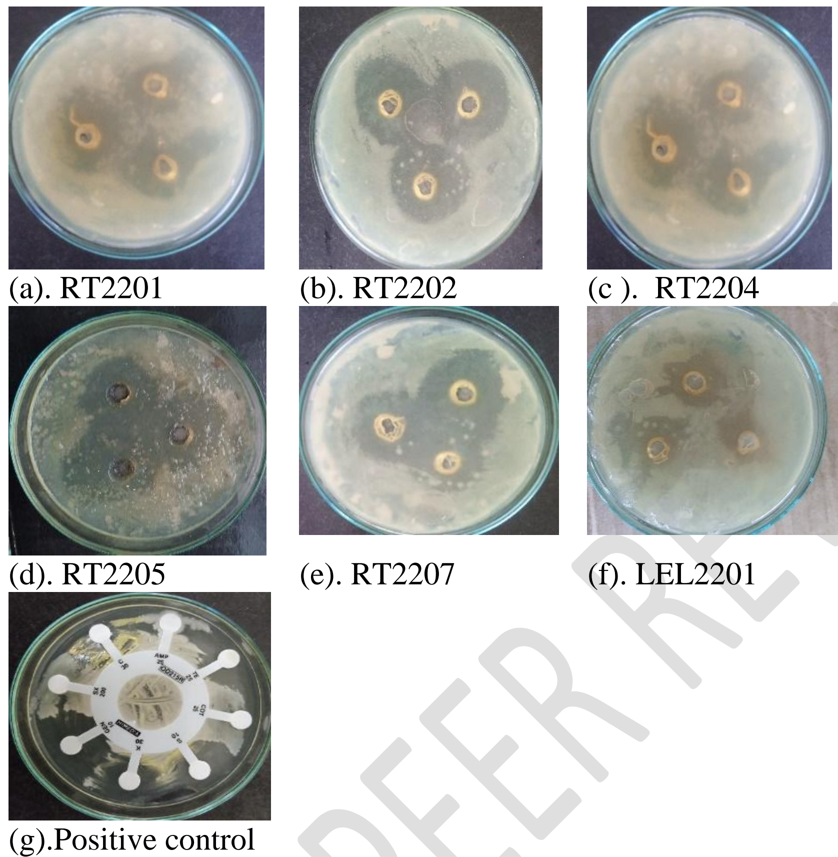
against *S. aureus* followed by RT2201 (8.5 mm) (Table 3 and plate 4). Isolate LEL2201 performed better than standard gentamycin (7 mm), chloramphenicol (6 mm), co-trimazole (6 mm), tetracycline (4 mm), sulphamethoxazole (4 mm), kanamycin (3 mm), streptomycin (3 mm) and ampicillin (3 mm). Isolate RT2207 (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).

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 $\alpha = 0.05$



(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.*

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.

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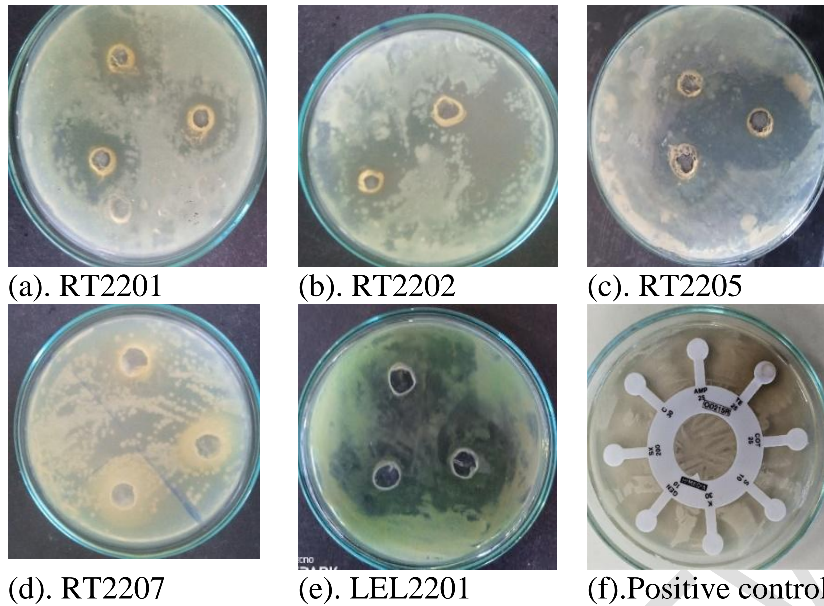

(a). RT2201 (b). RT2202 (c). RT2205

(d). RT2207 (e). LEL2201 (f).Positive control

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.

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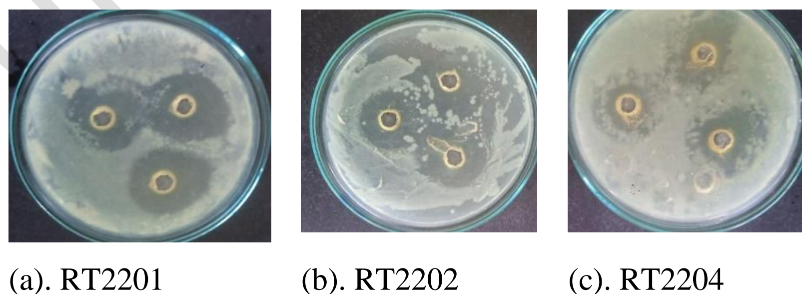
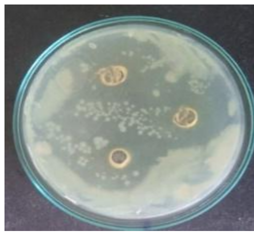
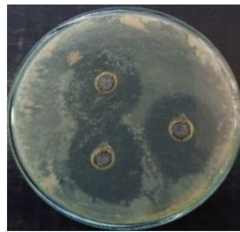
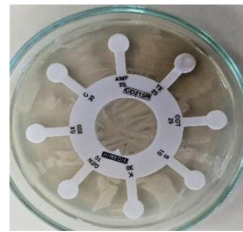


(a). RT2201 (b). RT2202 (c). RT2204







(d). RT2207 (e). LEL2201 (f)Positive control

Plate 5: 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 figure 1 below.

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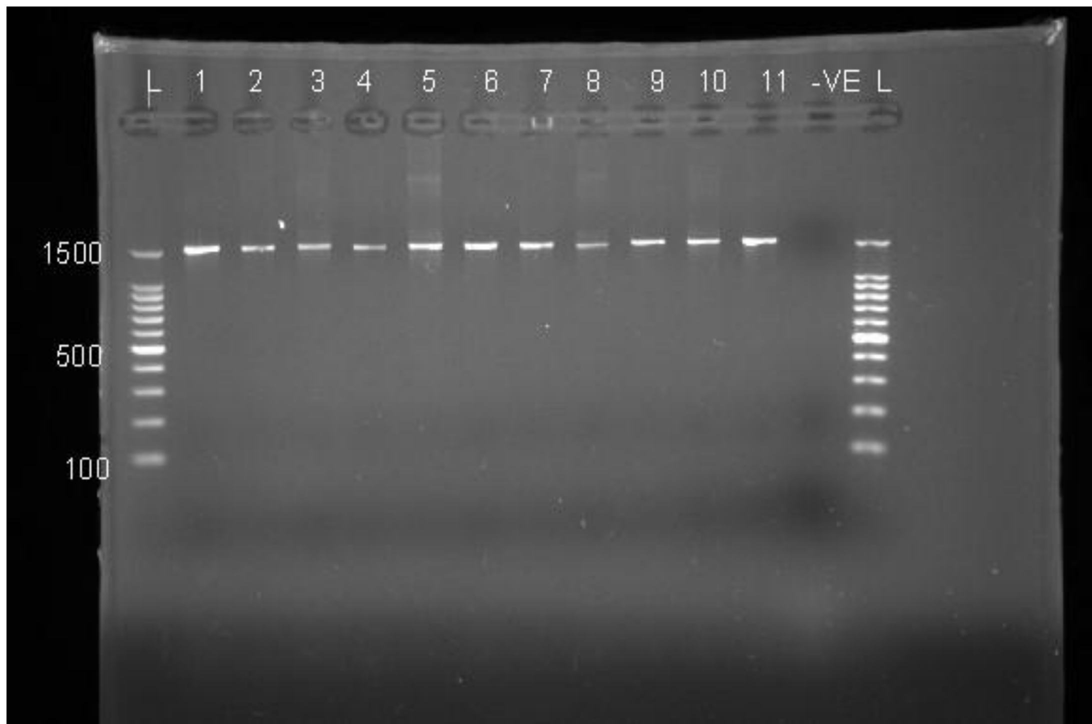

Figure 1: The agarose gel containing PCR products, primer and template DNA from selected antibiotic producing *Actinomycetes* species**.** L=molecular ladder, 1=RT2201, 2=Isolate RT2202, 3= isolate RT2203, 4 =Isolate RT2204, 5=RT2205, 6= Isolate RT2206, 7= Isolate RT2207, 8= Isolate LEL2201, 9 = Isolate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

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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).

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

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 (Figure 2). The evolutionary distance matrix of the *Actinomycetes* isolates and various *Actinomycetes* in the gene bank in the above phylogenetic tree.

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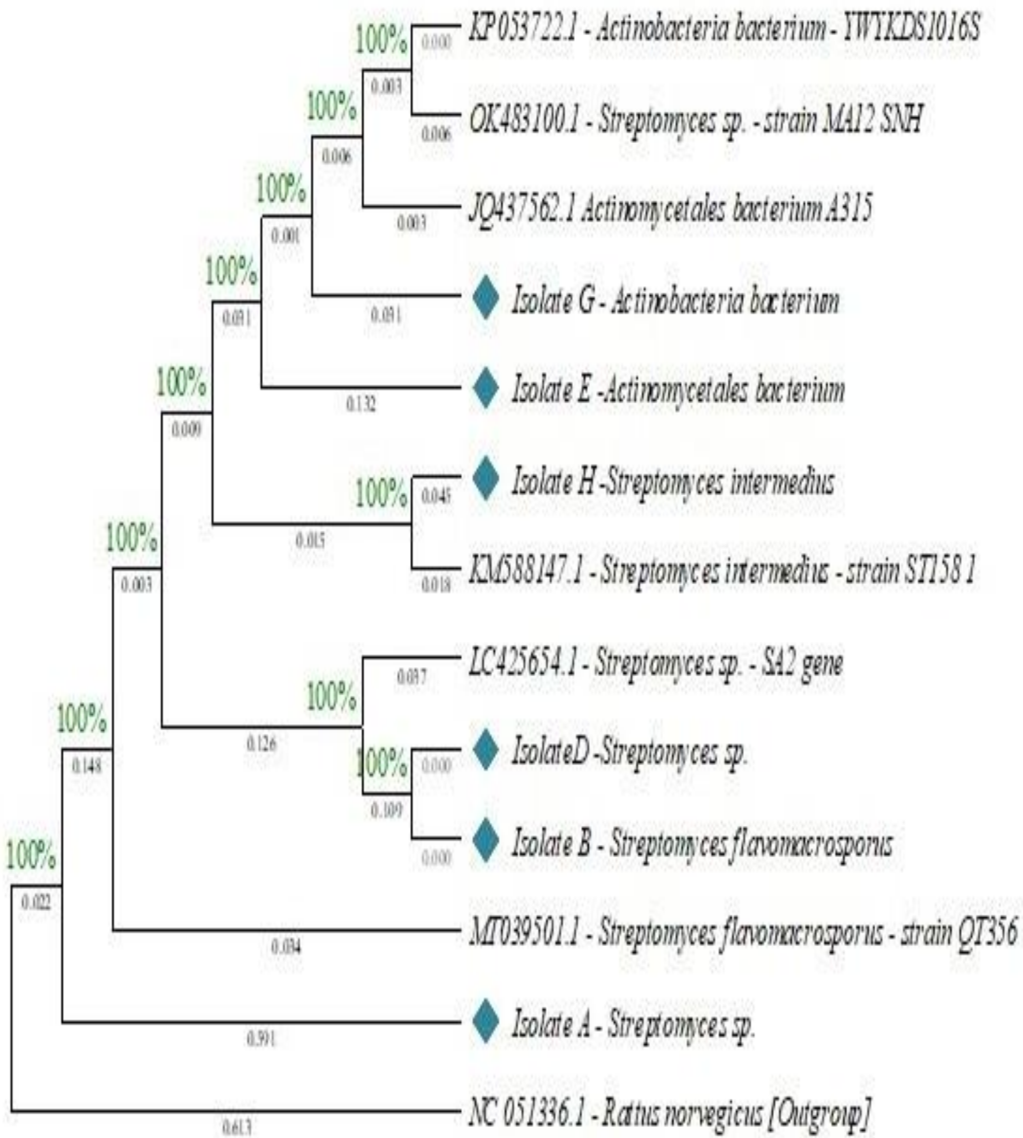

Figure 2: The phylogenetic tree showing the six isolates divided into four dendrograms. Isolate A =RT2201, B=RT2202, D=RT2204, E=RT2205, G=RT2207 and H=LEL2201.

Table 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

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4. DISCUSSION

4.1 Morphological Characteristics and Antibacterial properties of *Actinomycetes* Isolates

The isolated *Actinomycetes* (Plate 1 and Table 1) had growth in both starch casein and nutrient agar which can be due to nutrients and minerals in the two media (Gebreyohannes *et al.,* 2013). The isolates produce numerous enzymes which enable them to grow in both starch casein and nutrient agar by metabolizing the nutrients present in these growth media (Rana and Salam, 2014).

The morphological characteristics on both media were smooth edged to crenated, raised, hard to scrap texture, with soil like odour, and white- cream -yellow in colour (Plate 1, and Table 1). The smooth margins of isolates RT2201, RT2202, RT2205 and RT2207, LEL2201, LEL2202, LEL2203 and LEL2204 can be attributed to the their short and smooth hyphae they produce while the crenated margins of RT2203, RT2204 and RT2206 is attributed to their long substrate hyphae that protrudes on the edges of their colonies (Darshit *et al.,* 2018). The isolates RT2201, RT2205, RT2206 and RT2207, LEL2201, LEL2202, LEL2203 and LEL2204 produce cream pigment which makes their colonies to appear cream while RT2202, RT2203 and RT2204 produce cream yellow pigment which makes them to appear cream yellow (Rana and Salam, 2014). The isolates had hard to scrap texture that is attributed to their substrate hyphae which forms an extensive network in growth media (Sharma *et al.,* 2011). *Actinomycetes* produce volatile essential oils which makes them to have characteristic soil-like odour (Hasani *et al.*, 2014).

These morphological characteristics are similar to Gebreyohannes *et al.* (2013) *Actinomycetes* isolates from Lake Tana in Ethiopia. *Actinomycetes* isolates had good growth in starch casein agar appearing as white or cream colonies that were Gram positive. Observed morphological characteristics of Gouse *et al.* (2017) indicated *Actinomycetes* that were white yellow or cream in colour, wrinkled or smooth edged and Gram positive.

Gram stain analysis of *Actinomycetes* isolates revealed that all of them appeared as long branched purple filament which are Gram positive bacterial strains (plate 2). The microscopic features of *Actinomycetes* are fragmented and no fragmented substrate and aerial mycelium which appear gram positive, wavy long branched purple filaments (Muthu *et al.,* 2013). The hypae of *Actinomycetes* absorbs crystal violet stain during Gram staining procedure and is not decolorized by the acetone making them to appear purple. The Gram staining helps one to view the branching patterns of both aerial and substrate mycelium of *Actinomycetes.* The Gram staining characteristics of *Actinomycetes* isolates is similar to Hasani *et al.* (2014) whose *Actinomycetes* isolates were filamentous Gram positive bacteria, with filaments that fragments to form small spores which were wavy, straight or helical chains under microscope. The results are

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also similar to Dhananjeyan *et al*. (2010). *Actinomycetes* isolates were Gram positive with branched filaments.

All *Actinomycetes* isolates were catalase, oxidase, citrate utilization and indole positive (Table 2) and coagulase and methyl red negative. All the isolates were indole positive (Table 2) *Actinomycetes* which produce tryptophanase enzyme that catalyzes deamination of tryptophan leading to production of indole, ammonium, ATP and pyruvic acid. The indole produced reacts with p-dimethylaminobenzaldehyde and hydrochloric acid in aml alcohol (kovac's solution). The kovac's solution is insoluble water forming an oily ring which appears at the top of broth (Hari *et al*., 2018). All the isolates were methyl red negative *Actinomycetes* which do not metabolize glucose through mixed acid fermentation cycle ensuring a pH of above six of the growth media.

All the *Actinomycetes* isolates were able to grow in Simon citrate agar (Table 2) because they can utilize citrate as carbon source of energy in their metabolism (Jadon *et al.,* 2014). The *Actinomycetes* isolate produces citrate permease that converts citrate to pyruvate that enters the organisms' lactic acid fermentation. During the metabolism of citrate the ammonium salts in the Simon citrate media are broken down to ammonia which makes the media to be more alkaline. The increased alkalinity turns bromothymol blue indicator in the medium from green to blue.

All the isolates were oxidase positive (Table 2) *Actinomycetes* that are cytochrome C containing organisms which have intracellular cytochrome oxidase or indophenol oxidase which oxidises cytochrome C. These enzymes catalyses the transport of electrons from NADH to oxygen (Shields and Cathcart, 2010). The *Actinomycetes* isolates are aerobic in nature because cytochrome C oxidase system is only found in aerobic bacteria with capability to utilize oxygen as the final electron receptor (Shields and Cathcart, 2010).

All the isolates were catalase positive *Actinomycetes* (Table 2) that produce catalase which mediates the breakdown of hydrogen peroxide to oxygen gas and water when a small 24 hour inoculum of the bacteria is introduced. The catalase enzyme is produced by *Actinomycetes* to protect them from effects of hydrogen peroxide (Senthilkumar *et al.,* 2021).

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The biochemical characteristics of this study is similar to Dhananjeyan *et al.* (2010) whose isolates were methyl red, indole and catalase positive but showed negative citrate fermentation. The results of citrate utilization test are similar to Gebreyohannes *et al.* (2013) that indicated 45% of *Actinomycetes* had ability to metabolize citrate. The indole production results differs with Gouse *et al.* (2017) which reported negative indole production among the four *Actinomycetes* isolates while methyl red results and citrate utilization results are in agreement.

The biochemical characteristics of different *Actinomycetes* organisms varies from one environment of isolation to another. The difference in the biochemical characteristics is attributed to exposure to nutrients, oxygen and metabolic stress in the cell which influences the genes responsible for various metabolic enzymes production in these organisms. The organisms from River Tana stretch in Tharaka Nithi and Lake Elementaita in Nakuru both in Kenya have exhibited similar biochemical characteristics for the six tests. The organisms were aerobic and difference in salinity, human activities, nutrition supply and temperatures have evolutionary transformed them to produce related metabolic enzymes.

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

There was significantly ($p<0.05$) different in bioactivity of *Actinomycetes* against *E. coli* (54.5%), *S. typhi* (45.5%) and *S. aureus* (45.5%) (Table 3). The antibacterial properties of isolates is attributed to secondary metabolites which have documented antibacterial properties. The isolates RT2203, RT2206, LEL2202, LEL2203 and LEL2204 did not have bioactivity against *E. coli, S. typhi* and *S. aureus* (Table 3) which may be due to the fact that their secondary metabolites do not have antibacterial targets in these test organisms. The isolate RT2201 had a bigger zones of inhibition in *S. aureus* (8.5 mm) and *S. typhi* (8.6 mm) than *E. coli* (2.0) (Table 3) this indicates that the antibacterial metabolites of RT2201 is broad spectrum . However, it had more molecular targets in *S. typhi* and *S. aureus* than *E. coli* (Sharma *et al.,* 2016).

The isolate RT2202 and RT2204 had larger inhibition in E. *coli* and then *S. typhi*, *S. aureus* (Table 3). The antibacterial metabolites of this isolate is also broad spectrum with molecular targets in all test organisms (Adeyemo *et al.,* 2020). 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 (Hove *et al.,* 2022). Peptidoglycan of the Gram positive *(S. aureus*) can also be a hindrance for the antibacterial metabolite to access the molecular targets (Sperandio *et al.,* 2013).

The antibacterial metabolite of isolate RT2205 had bioactivity against *E. coli* and *S. aureus* (Table 3). 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 (Maria *et al.,* 2015). The isolate RT2207 had broad spectrum bioactivity against Gram negatives (*S. typhi* and *E. coli*) and Gram positive (*S. aureus*) (Table 3). 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 (Sperandio *et al.,* 2013).

The isolate LEL2201 had broad spectrum bioactivity against all test organisms (Table 3). 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*) (Sharma *et al.,* 2016). 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 (Rotich, 2018). 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 (Habib *et al.,* 2020).

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.* (2017) indicated 59% of *Actinomycetes* isolates had antibacterial activity against test organisms. A study by Charousová *et al*. (2019) 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 (Rotich, 2018). 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 (Table 4 and Table 5) (Thomashow *et al.,* 2008). 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 (De Simeis and Serra, 2021). 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 (De Simeis and Serra, 2021).

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* (Tables 3) (Thomashow *et al.,* 2008). Phylogenetic results of the six isolates showed that the organisms' falls within four main clusters (Figure 2, Table 4 and Table 5). 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 (Grasso *et al.,* 2016).

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* (Khalid *et al.,* 2013). The RT2204 (84.59 %) was identified to be related to *Streptomyces sp.* LC425654.1 which had bioactivity against *S. aureus* (Khadayat *et al.,* 2020) (Table 4).

The *Actinomycetes* RT2205 (84.22%) and RT2207 (100%) was found to be closely related to *Actinobacteria bacterium* JQ437562 and *Actinomycetales bacterium* KP53721 respectively

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(Table 4). The LEL2201(91%) is closely related to *Streptomyces intermedius* KM588147.1 which is closely related to haloalkaliphilic *Streptomyces intermedius* isolated in India thathad bioactivity against *E. coli* (Dayma *et al.,* 2019) (Table 4).

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.

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