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Isolation and characterization of soil actinomycetes to produce bioactive compound

Feven Mesfin[1], Minbale Gashu[2], and Asmamaw Tesfaw[3]*

[1]Department of Biology, Debre Berhan University

[2]Department of chemistry, Debre Berhan University

[3]Department of Biotechnology, Debre Berhan University

Abstract

These days, the most difficult issues facing public healthcare globally are the advent of novel diseases and the development of dr resistant human pathogenic microorganisms. To solve this issue, soil-based actinomycetes are used as novel sources of biologically act organisms that produce potent antibacterial agents. Therefore, this study aimed to isolate and characterize antibiotic-produc actinomycetes. The actinomycetes were isolated using starch-casein media, and the isolates were characterized for tests of indole, sta hydrolysis, casein hydrolysis, gelatin hydrolysis, starch hydrolysis, citrate utilization, triple sugar iron, and catalase. The effects of p temperature, carbon sources, nitrogen sources, and salt concentration on isolate growth and bioactive compound production were a investigated. A total of 74 actinomycetes were isolated, of which 21 (28%) isolates showed potential antimicrobial activities against at le two pathogenic test microorganisms. The ethyl-acetate extract of the crude metabolites of six isolates showed antagonistic activity agai at least one tested organism using the disc diffusion antimicrobial assay method. Among the six isolates, DBUMU14, DBUG7, a DBUA4 were found to be classified under the genus Streptomyces. The fractionated crude extracts were found to be active agai *Staphylococcus aureus* ATCC25923, *Salmonella typhi* ATCC13311, and *Candida albicans* ATCC25925. Therefore, isolates such DBUMU14, DBUG7, and DBUA4 are promising candidates for bioactive compound production.

Keywords: Streptomyces, bioactive compounds, actinomycetes

1.Introduction

Infection is one of the most prevalent illenesses triggered by microbiota dysbiosis, which can be caused by bacteria, viruses, parasites, or fungi [1]. Due to this, infectious diseases are becoming a global problem and a regular cause of death for both humans and animals. The creation, evolution, and transmission of new infections are facilitated by factors such as tourism, aging, urbanization, and climate change, all of which contribute to the persistence of this trend [2].

Currently, the capacity to treat bacterial illnesses and administer other medical treatments is becoming more and more threatened by antibiotic resistance. Thus, the development of microorganisms resistant to antibiotics restricts the use of antibiotics in clinical settings [3]. The problem is made worse by a significant decrease in the generation of antibacterial agents through research and development. Therefore, when older classes of antibacterial treatments lose their effectiveness, very few new classes are introduced to the market, and new medications, especially antibiotics, are desperately needed to combat life-threatening diseases and stop the spread of pathogens that are resistant to antibiotics [4].

Over the past few decades, there has been a significant increase in the total number of human pathogenic bacteria that are resistant to one or more antibiotics. The life science community and public health are globally concerned with developing measures to prevent the emergence of antibiotic resistance [5]. Because of this, the World Health Organization (WHO) has determined that antibiotic-resistant bacteria are among the top 10 worldwide public health hazards to humanity [6].

Because of their valuable biological qualities, natural compounds formed from microorganisms are becoming a good source of new drugs. They have also received a lot of interest because of their potential to improve human health [7]. To meet the need for antibiotics, nature continues to be the most abundant, adaptable, and promising source of novel medicines. These secondary metabolites produced by microbes are frequently used in veterinary care, agriculture, industry, and medicine because they may have antibacterial, antifungal, and antiprotozoal effects [8].

Despite this, microorganisms can be found in a variety of natural environments, such as hot springs, deep sea sediment, alkaline, and salted environments [9]. Many

*Corresponding author: asmamawtesfaw@dbu.edu.et

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bacteria that create a wide range of naturally occurring biologically active chemicals, including significant therapeutic medications, depend on the soil as their primary sources.

Among the actinomycetes, various genera, including Streptomyces, *Micromonospora*, *Nocardiopsis*, *Saccharopolyspora*, *Actinomadura*, *Amycolatopsis*, and *Actinoplanes*, are available with excellent biomedical characteristics [10]. The ecological and taxonomic status of actinomycetes that produce antibiotics is well recognized for their metabolic adaptability, which is frequently accompanied by the generation of important secondary and primary metabolites [11].

Most actinomycetes are known to have the capacity to synthesize bioactive secondary metabolites, mainly antibiotics. Worldwide, nearly 70% and 80% of the potent antimicrobial agents are synthesized by actinomycetes and Streptomyces respectively [12].

On the first hand, the number of drug-resistant microorganisms is increasing and this demands the development of new antibiotics. On the second hand, actinomycetes are promising for the development of novel antimicrobial substances. Therefore, this study aimed to isolate actinomycetes from different places in North Shoa, Amhara, Central Ethiopia to evaluate their antimicrobial activities.

2. MATERIAL AND METHODS

2.1. *Description of the Sampling Area*

The samples were collected from Menz-Guassa, Berehet, Mehal-Wenz, and Arbhara-Medihanealem located at Menz Gera Mider, Berehet, Ankober, and Menz Mama Mider respectively (Figure **1**).

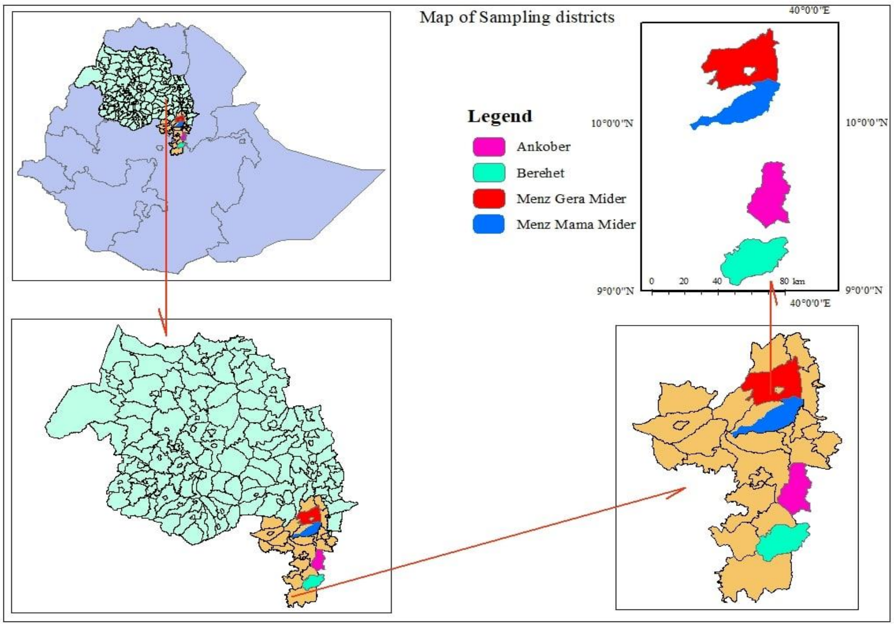

Figure 1 Map The sampling districts of North Shewa, Amhara, Central Ethiopia

2.2. *Sample collection and isolation*

Twenty soil samples were collected randomly from Menz-Guassa (5), Berehet (5), Arbhara-Medihanealem (5), and Mehal-Wenz (5); the upper part of 3-5 inches of soil were removed and at the depth of 10-15 inches of topsoil were collected using a sterile spatula and the samples were placed into a sterile zipped polythene bag. The sample bags were carefully labeled based on their source place and aseptically transported to Debre Berhan University microbiology laboratory and stored in the refrigerator at 4^0c for further analysis.

2.3. *Culture media for cultivation of actinomycetes*

The selective media used in this study were starch casein agar (SCA) g/L: soluble starch 10.0g, casein 0.3g, KNO_3 2.0g, NaCl 2.0g, K_2HPO_4 2.0g, $MgSO_4.7H_2O$ 0.05g, $CaCO_3$ 0.02g, $FeSO_4.7H_2O$ 0.01g and agar 20.0g were dissolved 1,000 ml distilled water; pH was adjusted to 7.0 before sterilization, yeast extract malt agar (YEMA) g/L: Yeast extract 4 g, malt extract 10 g, Dextrose 4 g, agar 20 g; pH 7, glucose asparagine agar (GAA) g/L: glucose 10.0g, asparagine 0.5g, K_2HPO_4 0.5g, agar 15.0g and pH was adjusted to 7 before sterilization. Inorganic salt starch agar (ISSA) g/L: soluble starch 10.0g, K_2HPO4 1.0g, $MgSO_4$. $7H_2O$ 1.0g, NaCl 1.0g, $(NH_4)_2SO_4$ 2.0g, $CaCo_3$ 2.0g, $FeSO_4.7H_2O$ 0.001g, $MnCl_2$. $7H_2O$ 0.001g, $ZnSo_4$. $7H_2O$ 0.001g, agar 20.0g and pH was adjusted to 7.2±0.2 [1]. The media were autoclaved with a temperature of 121^0c for 15 min and cooled down up to 50^0c. Then about 20-25 mL of sterile media was poured on the sterile petridishes and allowed to solidify overnight before use.

2.4. *Isolation and maintenance of actinomycetes isolates*

Actinomycetes cultures were isolated by serial dilution plate technique method [2]. Stock solution was prepared by suspending one gram of the soil sample in a test tube containing 9mL sterile distilled water and mixed well using a vortex. From this, 1mL of aliquot was transferred and mixed with another 9mL of sterile distilled water to make 10^{-2} dilution factor. Similarly, dilution was continued up to 10^{-5} using serial dilution technique [3]. An aliquot of 0.1mL dilution was taken from 10^{-2} 10^{-3}, 10^{-4}, and 10^{-5} and spread evenly over the surface of starch casein agar medium containing cychlohexmaide (50µg/mL) and nystatin (25µg/mL). The inoculated plates were incubated at 28±2^{0}c for 7–10 days [4].

All the isolates were identified as actinomycetes based on colony morphology and color of mycelium. Morphologically identified colonies were selected from a full plate using a sterile wire loop and streaked on starch casein agar medium and incubated at 28±2^{0}c for 7-10 days. The isolates were repeatedly re-streaked to ensure the purity of the culture. Pure actinomycetes cultures were stored at 4^{0}c on starch casein agar slant for further study.

2.5. *Test microorganisms*

Eight human pathogenic bacteria including Gram-positive (*L. monocytogenes* ATCC19115, *S.aureus* ATCC25923, *S. pyogenes* ATCC19615, *B. subtilis* ATCC18112) and Gram-negative (*E. coli* ATCC25922, *P. aeruginosa* ATCC27853, *S. typhimurium* ATCC13311) and yeast (*C. albicans* ATCC25925) were used as a test microorganism to evaluate the antimicrobial activity of actinomycetes. All test microorganisms were obtained from the American Type Culture Collection.

2.6. *Standard inoculum size*

A pure colony of test bacteria was touched on the top of each colony with a sterile loop and inoculated into a test tube containing 6 ml of normal saline until it achieved the turbidity of the 0.5 McFarland standards. The turbidity of the actively growing broth culture was adjusted with sterile broth to obtain that of the 0.5 McFarland. This results in a suspension containing equal to 1.5×10^8 cfu/ml. McFarland Turbidity standard was prepared by adding 0.5ml of BaCl solution into 99.5ml of solution H_2SO_4 [5].

2.7. *Primary Screening*

A total of 21 isolated and purified actinomycetes cultures were screened primarily for their antimicrobial activity by cross streak method against eight test microorganisms [6]. The seven-day-old culture isolate of actinomycetes was streaked at the center of the starch casein agar plate and incubated at 28 ± 2^{0}c for 6 days. This was done to provide enough time for the active organism to produce the bioactive substance which could diffuse into the agar medium. After 6 days, overnight broth cultures of the test microorganism (*S. aureus* ATCC25923, *E. coli* 25922, *P. aeruginosa* ATCC27853, *L. monocytogenes* ATCC19115, *S. pyogenes* ATCC19615, *S. typhimurium* ATCC13311, and *C. albicans* ATCC25925) were streaked at a 90-degree angle in respect to the growth actinomycetes isolates and the plates were re-incubated at 37^{0}c for 24 hrs [7]. Antagonism was observed based on the inhibitory interaction between the actinomycetes and test strains. The isolates that showed a wide spectrum activity against tested microorganisms were selected for further studies.

2.8. *Secondary screening*

2.8.1. *Extraction of crude compounds*

Six isolates were selected for secondary screening in a submerged fermentation system. Two hundred fifty milliliters of starch casein broth was dispensed into a 500 ml Erlenmeyer flask. The production medium was adjusted to pH 7 before sterilization at 121^{0}c for 15 min [8]. The media were inoculated by a loop full of 7-day-old cultures and incubated in the rotary shaker at 28 ± 2^{0}c and 170 rpm for 10 days. After 10 days of fermentation, the culture was harvested and centrifuged at 8000 rpm for 10 minutes to remove cells and debris [9]. All the supernatants were collected by a new sterilized test tube and filtered again using Whatman No.1 paper to remove the remaining debris. Then after, the culture filtrate was extracted with an equal volume of ethyl-acetate (1:1). The mixture was added to the separating funnel and shacked vigorously for 1 hr until two clear immiscible layers (aqueous layer and organic or ethyl-acetate layer) were formed. The ethyl-acetate phase that presumably contained crude extract was separated from the aqueous phase in a separatory funnel. The ethyl-acetate phase that contained crude extract was evaporated and concentrated in vacuum rota-vapor at 40 - 45 ^{0}c [10].

2.8.2. *Disc diffusion assay*

The antimicrobial activity of the crude extract was carried out using the standard disc diffusion method [11]. For this purpose, sterilized Muller Hinton Agar was poured in petridishes and allowed to solidify. The overnight cultures of selected test pathogens (*C. albicans* ATCC25925, *E. coli* ATCC25922, *S. typhimurium* ATCC13311, *S. aureus* ATCC25923, *L. monocytogenes* ATCC19115) were swabbed on the solidify media using glass rod spreader and allowed to set for 10 min.

Sterilized, 6 mm Whatman paper impregnated with 20 µl of crude extract and placed on the surface of inoculated agar plates using sterile forceps. Standard streptomycin antibiotics disc was also placed on an agar medium as a positive control and ethyl-acetate as a negative control. Plates were kept at 4 ^{0}c for 30 min to allow the diffusion of culture filtrates on the medium. All the plates were incubated at 37 ^{0}c for 24 hours [12]. Clear zone inhibition around the disc indicated the presence of antimicrobial activity. The zone of inhibition was measured by mm. The experiment was carried out in triplicates and the mean value of each test isolates was recorded.

2.9. *Characterization and identification of selected isolates*

The selected isolates' identification and characterization were carried out based on the cultural, morphological, physiological, and biochemical characteristics, to identify the isolates in the genus level according to Bergey's Manual of Systematic Bacteriology [13].

2.9.1. Macroscopic characterization

Cultural features, such as the color of aerial and substrate mycelium of selected active strains were examined on the International Streptomyces Project (ISP) media. The standard medium such as yeast extract-malt extract agar, inorganic salts-starch agar, glucose-asparagine agar, and starch casein agar were utilized [14]. A loop full of each isolate from 7-day-old culture was taken and inoculated in duplicate into each of the media by streak plating technique and incubated at 30^0c for seven days.

2.9.2. Microscopic characterization

Morphological characterizations were done by microscopic method. The microscopic examination was performed by coverslip culture and Gram staining method. A sterile cover slip was inserted at an angle of 45^0c at the center of the starch casein agar medium [15]. A loop full of seven days old cultures was inoculated along the surface of the medium that met the surface of the buried coverslip and incubated at 28±2ºc for 7 days; after incubation, the coverslip was carefully removed using sterile forceps and placed upwards on a clean glass slide. The cultures were fixed with a few drops of methylene blue dye for 15 min and then followed by washing with tap water and drying [16]. The morphology of the spore chain and hyphae of substrate and aerial mycelia were observed through the oil immersion (100X).

2.9.3. Biochemical Tests

The biochemical characteristics of the isolates were identified based on their indole production, citrate utilization, starch hydrolysis, casein hydrolysis, oxidase test, catalase test and according to the International Streptomyces Project (ISP), and the results were compared with Bergey's Manual of Determinative Bacteriology [17].

2.9.3.1. ***Indole test***

The purified isolates were inoculated in the test tube containing 8mL of tryptone broth and incubated for 7 days at $28±2^0$c within the inoculated broth that served as a control. After incubation, a few drops of Kovac's reagent were added and the formation of a red or pink colored ring at the top was taken as a positive test and the formation of a yellow color was an indication of a negative test [18].

2.9.3.2. ***Starch hydrolysis test***

The starch hydrolysis test of the isolates was performed with the help of starch casein agar medium plates having a composition of soluble starch 20g, extract 3g, peptone 5g, and agar 15g in 1L distilled water and pH adjusted to 7.5. The 7-day-old cultures were streaked on the media and incubated at 28±2ºc for 7 days and un-inoculated plates served as a control. After proper inoculation and incubation, the iodine solution was flooded on the starch agar medium. After 30 sec, the appearance of a clear zone indicated starch hydrolysis of the isolates due to the production of extracellular enzyme [19].

2.9.3.3. ***Gelatin hydrolysis test***

The culture isolates were taken from 7-day-old culture and heavy inoculum was stabbed into nutrient gelatin tubes with a sterile needle. The inoculated test tubes were incubated for 10 days at $28±2^0$c and un-inoculated tubes were used as a control. After 10 days of incubation, the test tubes were placed into the refrigerator at 4^0c for 15 minutes. If the inoculated gelatin medium tubes liquefied after exposure to 4^0c refrigerator, it was taken as a positive result (the control tubes must be completely solidified at 4^0c within 15 min) and the negative result was considered if the inoculated tubes did not liquefy [20].

2.9.3.4. ***Casein hydrolysis test***

Seven-day-old cultures were inoculated on the skim milk agar plate and incubated for 7 days at $28±2^0$c. Finally, the milk agar plate cultures were examined for the presence and absence of clear zones around the isolates. The appearance of a clear zone confirmed the hydrolysis of casein [21].

2.9.3.5. ***Citrate utilization test***

The isolates were picked up using a sterile inoculating loop and incubated into a slope of Simmon's citrate agar slant. The inoculated slant tubes were incubated at $28±2^0$c for 7 days. The color change from green to blue indicates the utilization of citrate as a positive result [22].

2.9.3.6. ***Triple sugar iron test***

The 7-day-old culture isolates were inoculated using a sterile needle into the triple sugar iron agar slant, from up to the bottom of the TSI tube, and then streaked the needle back and forth along the surface of the slant and incubated at $28±2^0$c for 7 days. After incubation, gas production was determined by observing the cracking of the medium, and the production of H_2S was observed by the blackening of the bottom media [23].

2.9.3.7. ***Catalase test***

The loop full of 7-day-old culture actinomycetes colony was taken by using a sterile loop and transferred to the surface of a clean, dry glass slide. Then two drops of a 3% H_2O_2 solution were added to the glass slide and mixed with a clean toothpick. Rapid formation of bubbles indicates that the isolates are catalase positive, while the absence of bubble formation is an indication of being catalase negative [19].

2.9.4. Physiological characterization

The physiological characteristics of the isolates were studied based on the growth curve, pH tolerance, temperature tolerance, resistance of sodium chloride, the effect of different media, and utilization of carbon and nitrogen.

2.9.4.1. *Growth curve determination*

The growth curve was constructed to determine the accurate days for the isolates to reach the maximum growth phase. One hundred ml of starch casein broth was inoculated with 7-day-old culture isolates. The broth media were incubated at 28 ^{0}c on the incubator shaker for 14 days. Every 24 hours of incubation 1ml was separated and the absorbance was measured at 600 nm [24].

2.9.4.2. Effect of temperature

To determine the optimum temperature for the growth of isolates, the Erlenmeyer flask (250 mL) containing 50ml of sterile starch casein broth medium was inoculated by a loop full of 7-day-old culture isolate and incubated at 28±2^{0}c in the incubator shaker for 7 days at different temperature (20, 25, 30, 35, 40, 45) and their turbidity was recorded by using spectrophotometer at 600nm [25].

2.9.4.3. Effect of pH

Isolated actinomycetes were grown on 50ml of starch casein broth and the medium was adjusted with either NaOH or HCL to give pH values of 4, 5, 6, 7, 8, and 9 to obtain the optimum pH for isolates. The adjusted broth medium was inoculated with a loop full of 7-day-old culture isolates and incubated at 28±2ºc for 7 days on an incubator shaker. The growth was checked by reading their absorbance at 600nm [14].

2.9.4.4. Effect of carbon source

The effect of different carbon sources on the growth of the selected isolates was determined using glucose, starch, lactose, sucrose, mannitol, and fructose. Each carbon source (10g) was added to 50 mL of starch casein broth by replacing the medium carbon source and incubated at 28±2ºc for 7 days. The experiment was done in triplicate and the results were recorded by using a spectrophotometer at 600nm [26].

2.9.4.5. Effect of nitrogen source

Different nitrogen sources including yeast extract, beef extract, casein, urea, and NH_4SO_4 of 0.3g were used to replace the medium nitrogen source. The 7-day-old culture isolates were inoculated on the 50ml of starch casein broth and then incubated at 28±2^{0}c for 7 days. The turbidity of the medium was measured using a spectrophotometer at 600nm [27].

2.9.4.6. Effect of salt-tolerance

The effects of salts were evaluated by growing them on starch casein broth medium supplemented with sodium chloride (0, 2, 5, 7, and 10% w/v). After 7 days of incubation at 28±2^{0}c, the turbidity was measured using a spectrophotometer at 600nm [28].

2.9.4.7. Effect of different media

Different media were used for isolation and identification of actinomycetes spp. Furthermore, to achieve the best types of media (composition) for the growth of actinomycetes, different media with different compositions were used. The 7-day-old culture isolates were inoculated in Erlenmeyer flask (250) containing 50ml 0f sterile starch casein broth (SCB), glucose asparagine broth(GAB), inorganic salt starch broth (ISSB), yeast extract malt broth (YEMB) and incubated on incubator shaker at 28±2ºc for 7 days. After 7 days, the growth in broth media was measured using a spectrophotometer at 600nm to determine the effect of different media on the growth of actinomycetes isolates [29].

2.10. *Thin layer chromatography (TLC)*

The active crude extract was analyzed by analytical thin-layer chromatography (TLC) in the running solvent system with a modified procedure [30]. The TLC plate was cut 5cm×3cm and 1cm was measured from the bottom of the plate using a pencil, a line was drawn across the plate at the 1cm mark. Consequentially, the crude substance was spotted on a single line placed 1cm from the edge of the silica gel plate using a capillary tube and dried. The chromatogram was developed using an ethylacetate: hexane: methanol (3:2:1 v/v) solvent mixture. The solvent mixture was made to cover the bottom of the jar to a depth of 1cm and allowed the solvent to move up to 90% of the plate and taken out of the jar with forcipes and then the solvent front was marked with pencil immediately and allowed to dry. The spot was visualized with vanillin in concentration sulfuric acid spray reagent. The visible spot was circled with a pencil. Retention factors (Rf values) were calculated by dividing the distance traveled by the compound by the distance traveled by the solvent [31].

R_f = distance traveled by the compound/ distance traveled by the solvent

2.11. *Column chromatography*

Partial fractionation and purification of the crude extract were conducted by column chromatography using silica gel. The silica gel was suspended in chloroform for packing the column. The crude extract (100mg) dissolved in chloroform was loaded to the top of the column silica gel. Elution was done with different solvent systems with chloroform (100%), chloroform: methanol (20:5), and chloroform: methanol (10:20). Each collected fractions were dried and monitored with TLC and finally checked for their antimicrobial bioactivities against *S. typhi* ATCC13311, *S. aureus* ATCC25923 and *C. albicans* ATCC25925 test organisms [31].

2.12. NMR

The column-separated fractions were further purified and subjected to NMR analysis for their structure elucidation. NMR analysis was carried out by dissolving the material in deuterated chloroform in the NMR tubes using Bruker ACQ 400 AVANCE spectrometer operating at 400 MHz at Addis Ababa University. Chemical shifts for ^{13}C NMR were referenced relative to chloroform 77.23 ppm. The chemical shifts were expressed in δ values (ppm), and the obtained data were preliminarily analyzed for the functional groups [32].

2.13. *Data analysis*

Data analyses were performed using SAS software version 9 (Inccary NC USA). The experiments were carried out in triplicate. Analysis of variance (ANOVA) and means comparisons were done by Duncan's multiple range test.

3. RESULTS

3.1. *Cultures of actinomycetes isolated from different habitat*

Seventy-four actinomycetes isolates were collected from Menz-guassa (21), Arbhara- Medihanealem (16), Mehal –Wenz (27), and Berehet (10).

3.2. *Primary screening*

From a total of 74 soil actinomycetes isolates, 21 (28%) crude extracts showed antimicrobial activity, and 53 (72%) actinomycetes isolates failed to show any antimicrobial activity against 8 test microorganisms.

Table 1: primary screening of antimicrobial activity of crude extract

Isolate	Test microorganism							
	S. aureus	***E .coli***	***P. aeruginoa***	***L. monocytogenes***	***C .albicans***	***S. typhimurium***	***B. subtilis***	***S.pyogenes***
DBUA1	+	-	-	+	-	-	-	+
DBUA2	+	+	+	-	-	+	+	-
DBUA3	-	-	+	-	-	+	-	+
DBUA4	+	-	+	+	+	-	+	+
DBUA5	+	-	-	-	-	-	+	-
DBUG6	+	+	-	+	-	+	-	-
DBUG7	+	+	+	+	+	-	+	-
DBUG8	-	+	-	-	+	+	+	-
DBUG9	-	-	+	+	-	-	+	-
DBUG10	-	-	-	+	+	-	+	+
DBUG11	+	+	-	+	-	+	-	+
DBUMU12	-	+	+	-	-	+	+	-
DBUMU13	+	+	-	-	+	+	-	-
DBUMU14	+	+	-	+	+	+	-	+
DBUMU15	+	+	-	-	-	-	-	+
DBUMU16	-	-	+	+	-	+	-	-
DBUMU17	+	-	+	-	-	+	-	+
DBUMU18	-	-	-	-	-	+	+	+
DBUB19	-	-	-	+	-	-	+	+
DBUB20	+	-	-	-	-	+	+	+
DBUB21	+	+	-	-	+	-	-	+

Legend: $+_=$ active against test organism, $-_=$ inactive against test organism

Crude extract of 3 isolates (14%), DBUMU14, DBUG7, and DBUA4 showed a wide spectrum of antibiosis against 6 test microorganisms (Table 1). The two isolates DBUA2 and DBUG11 crude extract had antimicrobial activity against 5 test microorganisms. The crude extract of DBUG6, DBUG8, DBUG10, DBUG12, DBUG13, DBUB17, DBU20, and DBUB21 inhibited 4 test organisms. The crude extracts of 7 (33%) isolates were found to inhibit three test organisms. Out of 21 crude extract isolates, only 1 isolate (DBUA5) inhibited 2 Gram-positive test organisms.

Most of the crude extract of 13(62%) isolates inhibited *S. aureus* ATCC25923. 12(57%) isolates showed inhibition against *S. typhimurium* ATCC13311 and *S. pyogens* ATCC19615, followed by crude extract of 11 isolates (52%) and crude extract 10(48%) had antibiosis activity on *B. subtilis* and *E. coli* ATCC25922 respectively. Crude extract of 8 isolates (38%) that showed inhibition on *P. aeruginosa* ATCC27853 and *L. monocytogenes* ATCC19115. Likewise, crude extract of DBUA4, DBUG7, DBUG8, DBUG10, DBUMU13, DBUMU14 and DBUB21 had a potential to inhibit *C. albicans* ATCC25925.

3.3. *Secondary screening*

Based on the primary screening result, out of 21 isolates of actinomycetes, 6 ethyl-acetate of crude extract showed antimicrobial activity using Smf. Table 2 shows the growth inhibition diameter of the organic phase of the crude extract produced by DBUMU17, DBUG7, DBUA11, DBUA2, DBUA4, and DBUMU14 under Smf for 10 days using starch casein broth media against test organisms.

Crude extract of DBUMU14 inhibited *E. coli* ATCC25922, *L. monocytogenes* ATCC19115, *S. aureus* ATCC25923, and *C. albicans* ATCC25925. Four of the six isolates except DBUA2 and DBU11 showed antifungal activity against *C. albicans* ATCC25925. In *E. coli* ATCC25922 the bioactivity spectrum of each organic crude extract was $14.67\pm.25^{a}$ (DBUMU14), 12.67 ± 1.70^{b} (DBUA4) and 9.67 ± 1.25^{c} DBUA4, whereas DBUG7, DBUA11, and DBUMU17 showed no activity against *E. coli* ATCC25922. Organic crude extract of the DBUG11 could inhibit only *S. typhimurium* ATCC13311.

Out of 6 isolates' crude extract, only two (DBUG7, DBUG11) of them showed inhibition spectrum against Gram-negative *S. typhimurium* ATCC13311. Isolates DBUMU14, DBUA2, and DBUG7 crude extract were found to inhibit *L. monocytogenes* ATCC19115. Three isolates of crude extract DBUMU14, DBUMU17, and DBUA4 showed inhibition on Gram-positive *S. aureus* ATCC25923.

In the antimicrobial bioassay screening process (Table 2), high inhibition zone diameter was observed by crude extract of isolate DBUMU14 (18.33 ± 1.25^{a}) against *S. aureus* ATCC25923 followed by crude extract of DBUG7 (15.00 ± 1.63^{a}) against *S. typhimurium* ATCC13311 and the third high inhibition zone was recorded again by crude extract of isolate DBUMU14 ($14.67\pm.25^{a}$) against *E. coli* ATCC25922 and the least inhibition zone diameter was showed by crude extract of DBUA2(9.67 ± 1.25^{c}) against *E. coli* ATCC25922.

Generally, in this antimicrobial bioassay screening process, the crude extracts were tested against various pathogenic Gram-positive, Gram-negative, and yeast test organisms and showed potential activity against most of the test microbes. The zone of inhibition widely varied among the test organisms. Crude extract of DBUMU14, DBUG7, and DBUA4 isolates were selected based on their inhibition zone and spectrum activity for further analysis.

Table 2: Inhibition zone diameter of potential isolates against different test pathogens expressed in mean±SD (positive control was streptomycin and negative control was ethyl-acetate)

Isolates	**Inhibition zone diameter**				
	C. albicans	*E.coli*	*S. typhimurium*	*S.aureus*	*L. monocytogenes*
DBUMU14	12.00±1.63[ab]	14.67±0.25[a]	0.00±0.00[c]	18.33±1.25[a]	12.33±2.05[a]
DBUA2	0.00±0.00[c]	9.67±1.25[c]	0.00±0.00[c]	0.00±0.00[c]	11.33±1.25[a]
DBUG7	11.67±1.70[ab]	0.00±0.00[d]	15.00±1.63[a]	0.00±0.00[c]	13.00±1.63[a]
DBUA4	13.67±1.70[a]	12.67±1.70[b]	0.00±0.00[c]	10.67±2.05[b]	0.00±0.00[b]
DBUG11	0.00±0.00[c]	0.00±0.00[d]	10.33±1.25[b]	0.00±0.00[c]	0.00±0.00[b]
DBUMU17	9.67±1.70[b]	0.00±0.00[d]	0.00±0.00[d]	11.00±1.63[b]	0.00±0.00[b]
Positive control	24.00	20.00	25.00	26.00	22.00
Negative control	-	-	-	-	-

SD, standard deviation; mean, average of three measurements; different letters within the column (a, b, c, d) designate significantly different means as determined by Duncan multiple mean comparison test (P<0.05)

3.4. Morphological characterization

As presented in Table 3, the spore chain morphology of DBUMU14, DBUG7, and DBUA4 actinomycetes isolates were straight to flexuous (rectiflexible) spore chain morphology and showed a branched mycelium structure.

Table 3: Microscopic observation (RC is Rectiflexible)

Characteristic	Isolates		
	DBUMU14	DBUG7	DBUA4
Spore chain	Rc	Rc	Rc

3.5. Cultural characterization

In terms of color, the aerial and substrate mycelia varied depending on the type of different media used. The actinomycetes colonies showed various appearances such as white pink, silver grey, grey, and white on the aerial mycelia and light pink, brown, reddish brown, and pink on the reverse side of the colonies.

Table 4: Cultural characteristics of isolates in different media

Medium	Isolates		
	DBUMU14	DBUG7	DBUA4
SCA	White pink Light pink	Sliver grey Brown	grey Dark brown
ISSA Aerial	White pink Light pink	grey Reddish brown	White grey Brown
YEMA	White pink Pink	Sliver grey Brown	White grey Dark brown
GAA	White Light pink	white Reddish brown	White grey Dark brown

Legend: - SCA= starch casein agar, **YEMA**=Yeast Extract Malt Agar, **GAA**=Glucose Asparagine Agar, **ISSA**= Inorganic Salt Starch Agar

3.6. Biochemical test of actinomycetes

The biochemical properties including casein hydrolysis, catalase, indole, gelatin, starch, and TSI test of the isolates were studied. All the isolates (DBUA4, DBUMU14, and DBUG7) showed positive for starch, gelatin, casein, and catalase test. In the gelatin hydrolysis test, isolate DBUA4, DBUMU14, and DBUG7 produced gelatinase enzymes that liquefy the gelatin present in the medium. DBUA4, DBUMU14, and DBUG7 were positive for the catalase test, the enzyme catalase converts hydrogen peroxide to water and O_2 gas. It was observed that none of the isolates showed positive results on indole, urea, and TSI tests.

Table 5: Biochemical test of the isolates

Biochemical Test	Isolates		
	DBUA4	DBUMU14	DBUG7
Starch hydrolysis	+	+	+
Gelatin hydrolysis	+	+	+
Indole	-	-	-
Urea hydrolysis	-	-	-
Casein hydrolysis	+	+	+
Catalase	+	+	+
TSI	-	-	-
Citrate utilization	-	-	-

3.7. Physiological characterization

Effect of the incubation period

An incubation period of 14 days of growth was studied to find out the maximum growth time of the selected isolates. DBUMU14 and DBUG7 optimum growth time were recorded on the 6^{th} day and isolate DBUA4 maximum growth was shown at the 7 days of incubation period and gradually declined up to 14-day incubation (Figure 2).

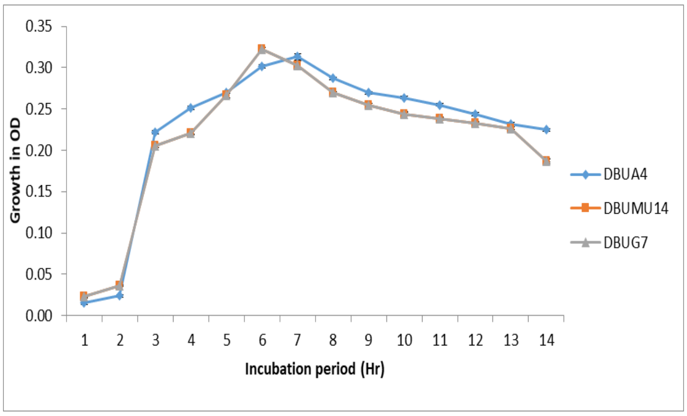

Figure 2: Effect of incubation period on the growth of DBUA4, DBUG7 and DBUMU14 isolates.

Effect of pH

The three isolates DBUMU14, DBUA4, and DBUG7 growth differentiation on various levels of pH were shown in Figure 3. pH 7 was more appropriate for the growth of DBUMU14, DBUA4, and DBUG7.

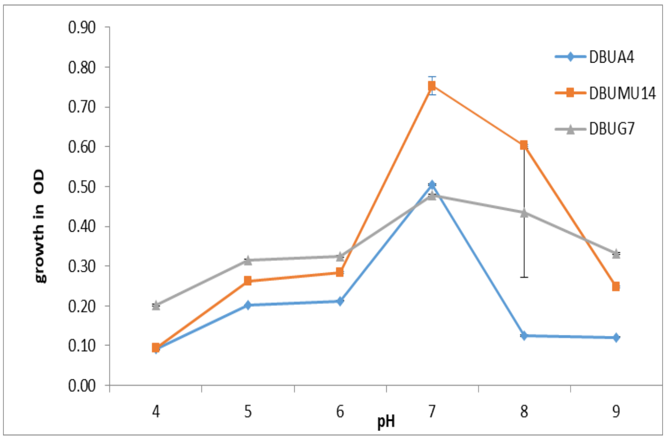

Figure 3: Effect of different pH on the growth of DBUA4, DBUG7, and DBUMU14 isolates measured 600nm using a spectrometer

Effect of temperature

The isolates were grown at a temperature range of 20^0c to 40^0c. The optimum temperature was found at 30^0c (Figure 4). All the isolates did not grow at a temperature of 45^0c.

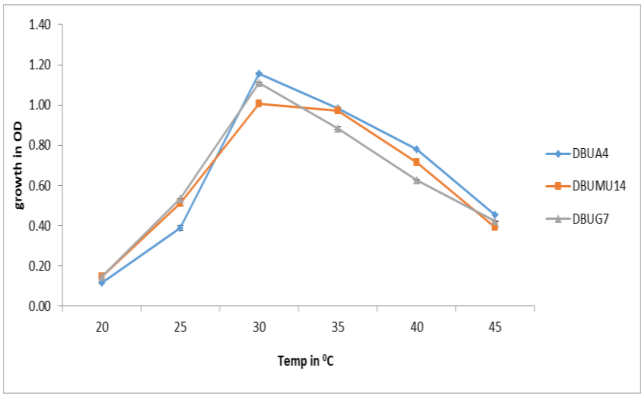

Figure 4: Isolates of DBUMU14, DBUA4, and DBUG7 grown at different temperatures.

Carbon source

Various carbon source utilization capacities were observed in different carbon source mediums. The growths of actinomycetes were greatly influenced by the type of carbon source (Figure 5). DBUMU14 and DBUG7 isolates had the highest growth recorded on the starch medium then followed by glucose. Isolate DBUA4 was observed to grow best in a glucose medium. Generally, starch and glucose were favorable sources for the growth of the three isolates. DBUMU14 and DBUG7 growth was poor on the lactose-supplemented media. DBUA4 was poorly grown on the mannitol medium.

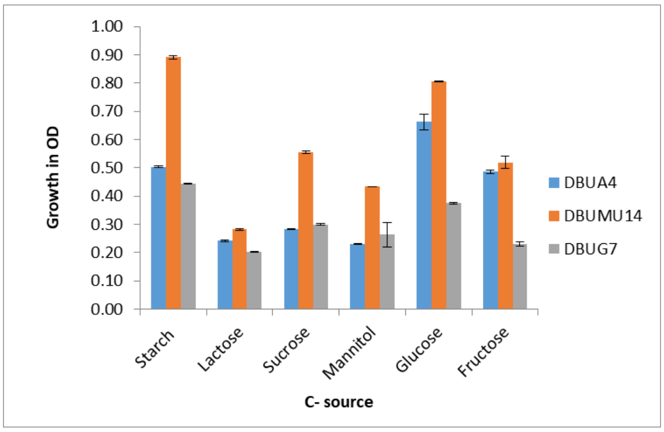

Figure 5: Effect of different carbon sources on the growth of DBUMU14, DBUA4, and DBUG7 isolates

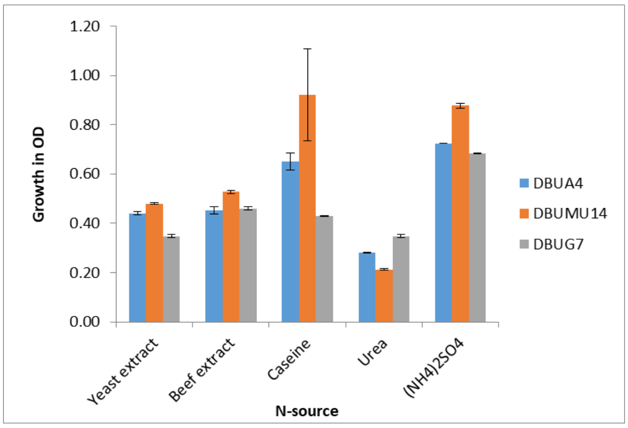

Figure 6: Effect of different nitrogen sources on the growth of DBUMU14, DBUA4, and DBUG7 isolates

Effect of sodium chloride

The study established that a 2% salt concentration was the optimal for the growth of isolates (Figure 6). DBUG7 and DBUA4 isolates were grown better at a 7% concentration of salt than DBMU14. The study revealed that a 2% salt concentration was better for the growth of the selected actinomycetes isolates. All the isolates did not grow at 0% concentration.

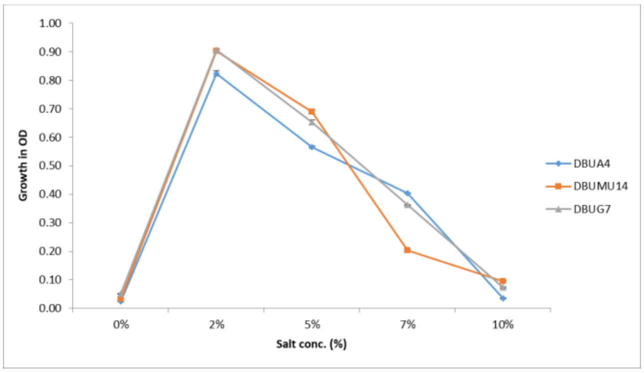

Figure 7: Effect of different NaCl concentrations on the growth of DBUMU14, DBUA4, and DBUG7.

Effect of different media

The result showed that the maximum growth potential of actinomycetes isolates (DBUG7 and DBUA4) was found with the starch casein broth except DBUMU14 (Figure 8).

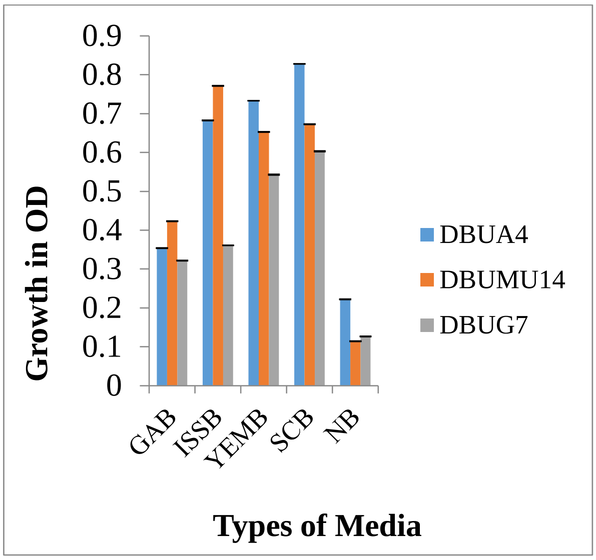

Figure 8: Effect of different media on the growth of DBUMU14, DBUA4, and DBUG7 isolates

3.8. *Thin layer chromatography*

The crude extract was subjected to TLC to separate the bioactive compound. The crude extract was spotted on the silica sheet and different bands were shown on the TLC plate. The three isolates of crude extract R_f value are indicated in Table 6.

Table 6: Retention factor value of the crude extract

Isolates	Rf value
DBUMU14	0.73
DBUA4	0.63
DBUG7	0.67

3.9. *Column chromatography*

Based on the TLC line result, different bands were shown on each crude extract. Different bands of crude extracts were fractionated by using column chromatography based on their polarity with the solvent. Each fraction of the crude extract's antimicrobial activity was detected against *S. aureus* ATCC25923, *S. typhi* ATCC13311, and *C.albicans* ATCC25925 (Table 7). The fraction obtained from the DBUG7 crude extract was seven. Out of the seven different fractions, only three (F3, F5, F7) of them showed antimicrobial activity against the pathogenic bacteria. The DBUG7's crude extract of fraction F3 recorded a high inhibition zone diameter of 10.67±1.25^a against *S. typhi* ATCC13311 pathogenic bacteria and fraction F5 only showed antibacterial activity against *S. aureus* ATCC25923. The DBUG7 fraction F7 crude extract did not inhibit the growth of *C. albicans* ATCC25925.

Table 7: Inhibition zone diameter of DBUG7 fraction against 3 test pathogens expressed in mean±SD

	Inhibition zone diameter (Mean±SD)		
fraction	***S. aureus***	***S. typhi***	***C.albicans***
F3	10.33±1.25^a	10.67±1.25^a	9.00±0.82^a
F5	10.00±1.41^a	0.00±0.00^a	0.00±0.00^b
F7	9.67±1.70^a	10.33±1.25^a	0.00±0.00^b

SD, standard deviation; mean, average of three measurements; different letters within the column (a, b,) designate significantly different means as determined by Duncan multiple mean comparison test (P<0.05)

The fraction of the DBUA4 crude extract result is shown in Table 8. The crude extract obtained from DBUA4 was seven fractions and the fractions of F2, F4, F5, and F7 had antimicrobial activity against the pathogenic bacteria.

Table 8: Inhibition zone diameter of DBUA4 fraction against 3 test pathogens expressed in mean±SD

Fraction	**Inhibition zone diameter (Mean±SD)**		
	S. aureus	*S. typhi*	*C. albicans*
F2	12.00±1.41^c	0.00±0.00^b	0.00±0.00^b
F4	10.00±1.41^a	10.67±1.25^a	8.33±0.47^d
F5	9.67±1.70^a	10.33±1.25^a	8.67±0.94^d
F7	9.33±1.25^a	8.33±1.25^a	0.00±0.00^b

SD, standard deviation; mean, average of three measurements; different letters within the column (a, b, c, d) designate significantly different means as determined by Duncan multiple mean comparison test (P<0.05)

The antimicrobial activity of fractions of DBUMU14, such as F3, F4, and F6 crude extract is displayed in Table 9. The fraction of F3 had the highest inhibition against *S. typhi* ATCC13311 with a clear zone diameter of 12.33±0.47^a and the lowest was observed against *C.albicans* ATCC25925 with inhibition of 8.00±1.41^b. The F4 fraction did not inhibit the growth of *C.albicans* ATCC25925. Fraction F6 crude extract resulted in less inhibition zone diameter against the test pathogen when compared to the rest of the two fractions and showed no inhibition zone against *S. typhi* ATCC13311.

Table 9: Inhibition zone diameter of DBUMU14 fraction against 3 test pathogens expressed in mean±SD

Fraction	**Inhibition zone diameter (Mean±SD)**		
	S. aureus	*S. typhi*	*C. albicans*
F3	10.00±2.16^a	12.33±0.47^a	9.00±1.63^a
F4	9.33±1.25^a	9.67±1.25^a	0.00±0.00^c
F6	9.33±1.70^a	0.00±0.00^b	8.00±1.41^b

SD, standard deviation; mean, average of three measurements; different letters within the column (a, b, c, d) designate significantly different means as determined by Duncan multiple mean comparison test (P<0.05)

3.10. *NMR*

The F1 proton NMR spectra displayed signals of aromatic protons appearing at δ 7.7 (dd, 1H) and δ 7.5 (dd, 1H). The spectrum also showed signals of protons on the oxygenated regions δ 4.0 (d, 2H). The carbon-13 together with DEPT-135 NMR spectra of the extracts indicated the presence of more than seven carbon resonances at δ 167, 132, 130,128, 71, 29, 19, etc. Those carbon resonances appearing at δ 167 and 132 ppm were found to be quaternary (C). The other signals appearing at δ 130, and 128 were as a result of methane (CH) carbons. One methyl (CH3) signal appeared at δ 19. The remaining signal that appeared at δ 71 was due to oxygenated methylene carbon (OCH2). Furthermore, unresolved and different signals were observed in the hydrocarbon region of the compound and found mixtures of unknown compounds.

4. Discussion

The discovery of new bioactive compounds is a never-ending process to meet the endless demand for novel biochemical with antimicrobial properties to combat various pathogens. Actinomycetes are biotechnologically valuable bacteria that are well exploited for secondary metabolite [2].

The result obtained from screening of actinomycetes isolates from 20 soil samples in four terrestrial places for the production of secondary metabolite indicated that many of them showed antimicrobial activity. Similarly, Raj and Chauhan [22] and Singh, Haque [33] also reported that soil microbes play a significant role in the extraction of novel drug and members of *Streptomyces spp* of actinomycetes were intensively utilized for the production of secondary metabolites.

In the present study, 74 actinomycetes isolates were screened to check whether isolates had the potential for the production of any antimicrobial compound against 8 test microorganisms using the cross-streak method in primary screening and a similar result was reported by Dholakiya, Kumar [34].

The present finding suggests that, when total isolates were subjected to primary screening, 28% were found to be active against the test microbes. The data obtained from the antimicrobial bioassay method during Smf, the ethyl-acetate crude extract showed different inhibition zones. The high zone of inhibition was observed by isolate DBUMU14 (18.33 ± 1.25^a) against *S.aureus* ATCC25923 and the smallest was shown by isolate DBUA2 (9.67 ± 1.25^c) against *E.coli* ATCC25922 and among ethyl-acetate crude extract of the isolates, the highest inhibition zone was showed by DBUA4 (13.67 ± 1.70^a); however, the crude extract of DBUA2 had not any antimicrobial activity against *C. albicans* ATCC25925.

This difference can be attributed to the different chemical compounds produced by isolates, their potential to produce more than one secondary metabolite, or the isolates might have diverse inhibition mechanisms. The result of this study may also confirm that some active strains have a broad spectrum of antimicrobial actions to Gram-positive and Gram-negative bacteria with diverse activities, as well as *C. albicans* which is supported by Yilmaz, Yavuz [35].

The active isolates show diverse activity against Gram-positive and Gram-negative test organisms during primary and secondary screening techniques. Similarly, Shrestha, Nath [36] revealed that the morphological difference between Gram-positive and Gram-negative bacteria showed distinct levels of sensitivity. As a result of this, Gram-negative bacteria have an exterior polysaccharide membrane that prevents the entering of lipophilic solutes into the cell wall; however, gram-positive bacteria only have an outer peptidoglycan which is ineffective as a permeability barrier.

The difference in the results between primary and secondary screening among isolates may be due to the appearance of actinomycetes as filamentous mycelia when growing in liquid and solid media respectively. During secondary screening, the broth culture flasks were subjected to a shaker incubator and there might be a significant breakage of the hyphae component of certain isolates rather than on the agar media plate. Most actinomycetes do not sporulate in liquid media [13]. Therefore, it is possible to say that, both solid and liquid media fermentation may affect the production of bioactive metabolites.

Interestingly, antimicrobial activity was exhibited in DBUMU14, DBUG7, DBUA4, DBUA2, DBUG11, and DBUMU17 in both solid and liquid cultivation media, suggesting that the extracellular antimicrobial compound secreted by the isolates was active in both solid and liquid fermentation media. A similar result was obtained by Almalki [37] both in solid or liquid media with promising antimicrobial metabolites.

The selected actinomycetes were grown in different media, and the cultural (macroscopic) characterization of aerial and substrate mycelium of isolates' color was varied according to the medium (Table 4). Zainal, Ser [38] have also reported that the color of aerial and substrate mycelium are media dependent. The three isolates were found to be positive for catalase, gelatin liquefaction, starch hydrolysis, and casein hydrolysis but negative for indole, urea hydrolysis, citrate, and TSI. The biochemical tests confirmed that the isolated strains belong to the actinomycetes [39].

The nutritional sources of carbon and nitrogen are known to have a profound effect on antimicrobial compound production by growth. DBUA4 preferred medium containing glucose as a sole carbon source compared to another carbon source. This is due to its easy degradability for the growth of the isolates during respiration [40].

DBUMU14 and DBUG7 showed the highest growth on the starch source. This phenomenon might be due to the occurrence of amylases in actinobacteria that commonly characterize the genus *Streptomyces*. Actinomycetes are known for the production of amylases, cellulase, chitinase, xylases, and pectinases; thus, amylase-producing microbes directly metabolize starch into disaccharides and monosaccharides [41].

Organic nitrogen sources were highly preferred for the maximum growth of the isolates to inorganic nitrogen sources [15]. However, two isolates (DBUG7 and

DBUA4) showed the highest growth in the ammonium sulfate-supplemented medium. Ammonium sulfate was a favorable ingredient for the production of bioactive compounds from *Streptomycetes* species [23]. The maximum growth of DBUMU14 was recorded on casein-supplemented media (Figure 6). Casein medium is an organic nitrogen source that also supplies vitamins, minerals, and accessory growth factors in the media [42].

Temperature is the other important parameter that influences the growth and development of microbes to produce the required metabolites. Therefore, different temperature gaps were studied in the present study and similar results were documented by Al Farraj, Varghese [27]. Although the isolates were able to grow at a temperature between 20-40^{0}c, the highest level of growth was noticed at 30^{0}c. Three isolates were found to be mesophilic.

The obstacle that microbes face below or above their optimal temperature is to retain the optimal functionality of their macromolecules (nucleic acids, proteins, and lipids), aiming for a balance between stability (robustness) and flexibility [43]. The growth and development of the microbes could be decreased when the temperature gets higher as well as much lower than the optimum temperature required.

In this study, the DBUMU14, DBUA4, and DBUG7 grew optimally at pH 7. Likewise, acidic conditions were not suitable for the growth and production of potential antimicrobial compounds [44]. This might be the isolates' need for the production of antimicrobial compounds in neutral pH.

The DBUMU14, DBUA4, and DBUG7 tolerated the salt concentration up to 7%, and no growth was detected beyond this limit (Figure 7) and 2% w/v concentration of NaCl was the optimum for the growth of the isolates. NaCl is also an important element for the microbes because of its water activity and the osmotic pressure of the surrounding environment [45].

The growth of actinomycetes was evaluated on 5 different culture mediums (ISSB, SCB, YEMB, GAB, NB) to select the best medium for the growth of actinomycetes. As a result of this, isolates DBUA4, DBUG7, and DBUMU14 showed good growth on SCB, and ISSB, and poor growth was observed on GAB, NB which indicates that medium has a visible effect on the growth of bacteria [46].

The column solvent fractions have shown a broad-spectrum antimicrobial activity. To achieve the best separation of the components of the ethyl acetate crude extract ethyl-acetate: hexane: methanol (3:2:1) solvent system was used for thin layer chromatography resulting in different R_f values confirming the presence of different compounds. The ethyl acetate crude extract of DBUMU14, DBUG7, and DBUA4 showed a mixture of different compounds and the component of the active fraction had a polar nature the R_f value is 0.73, 0.63, and 0.67 respectively [47].

TLC analysis of all column fractions indicated that DBUMU14 fraction F3 was relatively major and then subjected to NMR analysis. Structural elucidation of the sample using ^{1}H NMR, ^{13}C NMR, and DEPT-135 was not simple as its signals showed different intensities. The identification of the compound in the fraction was found difficult due to the presence of impurities of unresolved signals [48].

5. Conclusion

Menz-Guassa, Berehet, Mehal-Wenz, and Arbhara-Medihanealem contain potential actinomycetes capable of producing bioactive compounds to synthesize bioactive compounds. DBUMU14, DBUA4, and DBUG7 which were isolated Mehal-Wenz, Arbhara-Medihanealem, and Menz-Guassa, respectively are promising candidates for the production of bioactive compounds via submerged fermentation.

6. References

1. Sarika, K., et al., *Antimicrobial and antifungal activity of soil actinomycetes isolated from coal mine sites.* Saudi Journal of Biological Sciences, 2021. **28**(6): p. 3553-3558.
2. Shah, A.M., et al., *Antimicrobial investigation of selected soil actinomycetes isolated from unexplored regions of Kashmir Himalayas, India.* Microbial pathogenesis, 2017. **110**: p. 93-99.
3. Sudha, S. and R. Hemalatha, *Isolation and screening of antibiotic producing actinomycetes from garden soil of Sathyabama University, Chennai.* Asian J Pharm Clin Res, 2015. **8**(6): p. 10-4.
4. Kibret, M., et al., *Streptomyces spp. from Ethiopia producing antimicrobial compounds: Characterization via bioassays, genome analyses, and mass spectrometry.* Frontiers in microbiology, 2018. **9**: p. 1270.
5. Ayandiran, T. and S. Dahunsi, *Microbial evaluation and occurrence of antidrug multi-resistant organisms among the indigenous Clarias species in River Oluwa, Nigeria.* Journal of King Saud University-Science, 2017. **29**(1): p. 96-105.
6. Nandhini, S.U. and M.M. Selvam, *Bioactive compounds produced by Streptomyces strain.* International Journal of Pharmacy and Pharmaceutical Sciences, 2013. **5**(1): p. 176-178.
7. Rajaram, S.K., et al., *Extraction and purification of an antimicrobial bioactive element from lichen associated Streptomyces olivaceus LEP7 against wound inhabiting microbial pathogens.* Journal of King Saud University-Science, 2020. **32**(3): p. 2009-2015.
8. Ismail, S.A., et al., *Antimicrobial activity of isolated actinomycetes and optimization of*

bioactive metabolites production. Inventi Rapid: Pharm Biotechnol Microbiol, 2017. **2017**: p. 1-8.

9. Al-Ansari, M., et al., *Optimization of medium components for the production of antimicrobial and anticancer secondary metabolites from Streptomyces sp. AS11 isolated from the marine environment.* Journal of King Saud University-Science, 2020. **32**(3): p. 1993-1998.
10. Adeyemo, O., A. Onilude, and L. Babatola, *Effect of production parameters and inhibitory activity of antimicrobial compounds produced by co-cultured strains of Streptomyces xinghaiensis-OY62 and S. rimosus-OG95.* Journal of King Saud University-Science, 2020. **32**(1): p. 294-301.
11. Elbendary, A.A., et al., *Isolation of antimicrobial producing actinobacteria from soil samples.* Saudi journal of biological sciences, 2018. **25**(1): p. 44-46.
12. Ganesan, P., et al., *Antimicrobial activity of some actinomycetes from Western Ghats of Tamil Nadu, India.* Alexandria journal of medicine, 2017. **53**(2): p. 101-110.
13. Sapkota, A., et al., *Isolation, Characterization, and Screening of Antimicrobial-Producing Actinomycetes from Soil Samples.* International Journal of Microbiology, 2020. **2020**.
14. Yun, T.Y., et al., *Optimization of fermentation conditions through response surface methodology for enhanced antibacterial metabolite production by Streptomyces sp. 1-14 from cassava rhizosphere.* PloS one, 2018. **13**(11): p. e0206497.
15. Bawazir, A.M.A., et al., *Actinomycetes from Mountains of Hadhramout–Yemen.* Int. J. Curr. Microbiol. App. Sci, 2017. **8**: p. 3521-3530.
16. Gurung, T.D., et al., *Isolation and characterization of antibacterial actinomycetes from soil samples of Kalapatthar, Mount Everest Region.* Nepal Journal of science and Technology, 2009. **10**: p. 173-182.
17. Saraswathi, K., et al., *Isolation, characterization of bioinspired secondary metabolites producing actinomycetes from marine soil samples.* Int J Curr Microbiol Appl Sci, 2015. **4**: p. 107-119.
18. Mohanraj, R., et al., *Decolourisation efficiency of immobilized silica nanoparticles synthesized by actinomycetes.* Materials Today: Proceedings, 2020.
19. Begum, K., et al., *Isolation and characterization of bacteria with biochemical and pharmacological importance from soil samples of Dhaka City.* Dhaka University Journal of Pharmaceutical Sciences, 2017. **16**(1): p. 129-136.
20. Singh, C., et al., *Characterization of actinomycetes against phytopathogenic fungi of Glycine max.(L.).* Asian J Pharm Clin Res, 2016. **9**(Suppl 1): p. 216-9.
21. Salim, F.M., et al., *Isolation, molecular characterization and identification of antibiotic producing actinomycetes from soil samples.* J Appl Pharm Sci, 2017. **7**(9): p. 69-75.
22. Raj, Y. and V. Chauhan, *Isolation, characterization and screening of novel antibiotic producing bacteria from natural habitats of Western Himalayas and industrial waste soil samples.* International Journal of Computer Systems, 2019. **3**: p. 3282-3288.
23. Sholkamy, E.N., et al., *Antimicrobial and antinematicidal metabolites from Streptomyces cuspidosporus strain SA4 against selected pathogenic bacteria, fungi and nematode.* Saudi Journal of Biological Sciences, 2020. **27**(12): p. 3208-3220.
24. Pandey, A., et al., *Isolation and characterization of Actinomycetes from soil and evaluation of antibacterial activities of Actinomycetes against pathogens.* International journal of applied biology and pharmaceutical technology, 2011. **2**(4): p. 384-392.
25. Aliero, A.A., et al., *Molecular Characterization and optimization of Bioactive Compounds Production of three Actinomycetes spp Isolated from Waste Dump Soil from Western Uganda.* Current Trends in Biotechnology and Pharmacy, 2018. **12**(3): p. 230-244.
26. Singh, C., et al., *Optimization of Cultural Conditions for Production of Antifungal Bioactive Metabolites by Streptomyces spp. Isolated from Soil.* International Journal of Current Microbiology and Applied Sciences, 2017. **6**(2): p. 386-396.
27. Al Farraj, D.A., et al., *Antibiotics production in optimized culture condition using low cost substrates from Streptomyces sp. AS4 isolated from mangrove soil sediment.* Journal of King Saud University-Science, 2020. **32**(2): p. 1528-1535.
28. Singh, L., S. Mazumder, and T. Bora, *Optimisation of process parameters for growth and bioactive metabolite produced by a salt-tolerant and alkaliphilic actinomycete, Streptomyces tanashiensis strain A2D.* Journal de mycologie médicale, 2009. **19**(4): p. 225-233.
29. Jadon, P., et al., *Optimization of various physiochemical parameters to enhance production of secondary metabolite from soil actinomycetes against dermatophytes.* Environment Conservation Journal, 2019. **20**(1&2): p. 35-40.
30. Couillerot, O., et al., *Purification of antibiotics from the biocontrol agent Streptomyces anulatus*

S37 by centrifugal partition chromatography. Journal of Chromatography B, 2014. **944**: p. 30-34.

31. Ranjan, R. and V. Jadeja, *Isolation, characterization and chromatography based purification of antibacterial compound isolated from rare endophytic actinomycetes Micrococcus yunnanensis.* Journal of pharmaceutical analysis, 2017. **7**(5): p. 343-347.
32. Davies-Bolorunduro, O.F., et al., *Anticancer potential of metabolic compounds from marine actinomycetes isolated from Lagos Lagoon sediment.* Journal of pharmaceutical analysis, 2019. **9**(3): p. 201-208.
33. Singh, V., et al., *Isolation, screening, and identification of novel isolates of actinomycetes from India for antimicrobial applications.* Frontiers in microbiology, 2016. **7**: p. 1921.
34. Dholakiya, R.N., et al., *Antibacterial and antioxidant activities of novel actinobacteria strain isolated from Gulf of Khambhat, Gujarat.* Frontiers in microbiology, 2017. **8**: p. 2420.
35. Yilmaz, E.I., M. Yavuz, and M. Kizil, *Molecular characterization of rhizospheric soil streptomycetes isolated from indigenous Turkish plants and their antimicrobial activity.* World Journal of Microbiology and Biotechnology, 2008. **24**(8): p. 1461-1470.
36. Shrestha, B., et al., *Isolation and Characterization of Potential Antibiotic-Producing Actinomycetes from Water and Soil Sediments of Different Regions of Nepal.* International journal of microbiology, 2021. **2021**.
37. Almalki, M.A., *Isolation and characterization of polyketide drug molecule from Streptomyces species with antimicrobial activity against clinical pathogens.* Journal of infection and public health, 2020. **13**(1): p. 125-130.
38. Zainal, N., et al., *Streptomyces humi sp. nov., an actinobacterium isolated from soil of a mangrove forest.* Antonie van Leeuwenhoek, 2016. **109**(3): p. 467-474.
39. Sun, B., et al., *Streptomyces albicerus sp. nov., a novel actinomycete isolated from the sediments of the Tailan River in Xinjiang, China.* Archives of microbiology, 2020. **202**(7): p. 1639-1646.
40. Omran, R. and M.F. Kadhem, *Production, purification, and characterization of bioactive metabolites produced from rare actinobacteria Pseudonocardia alni.* Asian J Pharm Clin Res, 2016. **9**(3): p. 1-9.
41. Ashwini, K. and S. Kumar, *Partial-purification of alpha-Amylase from marine Streptomyces gancidicus-ASD_KT852565.* Research Journal of Pharmacy and Technology, 2016. **9**(6): p. 731.
42. Solihin, J., D.E. Waturangi, and T. Purwadaria, *Induction of amylase and protease as antibiofilm agents by starch, casein, and yeast extract in Arthrobacter sp. CW01.* BMC microbiology, 2021. **21**(1): p. 1-12.
43. Siliakus, M.F., J. van der Oost, and S.W. Kengen, *Adaptations of archaeal and bacterial membranes to variations in temperature, pH and pressure.* Extremophiles, 2017. **21**(4): p. 651-670.
44. Rajivgandhi, G., et al., *Molecular characterization and antibacterial effect of endophytic actinomycetes Nocardiopsis sp. GRG1 (KT235640) from brown algae against MDR strains of uropathogens.* Bioactive materials, 2016. **1**(2): p. 140-150.
45. Zhang, R., et al., *Streptomyces luozhongensis sp. nov., a novel actinomycete with antifungal activity and antibacterial activity.* Antonie Van Leeuwenhoek, 2017. **110**(2): p. 195-203.
46. Zhao, J., et al., *Streptomyces xiangluensis sp. nov., a novel actinomycete isolated from soil.* Antonie van Leeuwenhoek, 2018. **111**(12): p. 2249-2256.
47. Nandhini, S.U., et al., *Isolation, identification and extraction of antimicrobial compounds produced by Streptomyces sps from terrestrial soil.* Biocatalysis and agricultural biotechnology, 2018. **15**: p. 317-321.
48. Sebak, M., et al., *Isolation and optimized production of putative antimicrobial compounds from Egyptian soil isolate Streptomyces sp. MS. 10.* Beni-Suef University Journal of Basic and Applied Sciences, 2021. **10**(1): p. 1-12.