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Harnessing *Streptomyces werraensis* from Egypt's El Minia Region: Isolation, Screening, and Antimicrobial Evaluation

Ismail Gamal Ismail Ali[1], Amal W. Danial[2,*], Ahmed Shawky Mohamed Mousa[1], Maged Sayed Ahmed[1]

[1]Department of Botany and Microbiology, Faculty of Science, Beni-suef University, Egypt
[2]Department of Botany and Microbiology, Faculty of Science, Assiut University, Egypt

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Abstract The rise of multidrug-resistant (MDR) infections poses a significant threat to global health, challenging the efficacy of existing antibiotics and leading to increased morbidity and mortality. This alarming trend necessitates an urgent search for novel bioactive substances from environmental sources, particularly soil microorganisms, which have historically been a rich reservoir of antibiotics. *Streptomyces*, a genus of Gram-positive bacteria found predominantly in soil, has been a prolific source of antibiotics, contributing significantly to the arsenal of antimicrobial agents available today. Nine soil *Actinomycetes* were isolated from soil samples, purified, and then screened in the current investigation to determine the antibacterial potential of new *Actinomycetes*. *Escherichia coli, Bacillus subtilis, Staphylococcus aureus*, and other multidrug-resistant bacteria were the focus of both primary and secondary screening. The most active isolate, identified as *Streptomyces werraensis*, was selected for further investigation. Microbial strain was determined based on morphological, biochemical, and genetic characteristics. From our study, thirteen antibacterial substances were identified, and the most active compound was phenol, 2,4-bis (1,1-dimethylethyl), which demonstrated potent antibacterial properties against a range of MDR bacteria. Advanced analytical techniques, such as mass spectrometry are used to identify the bioactive compounds. This research addresses a critical need in the fight against MDR infections by exploring untapped environmental sources for new antibiotics. The findings could lead to the development of novel therapeutic agents, thereby contributing to global health and the management of antibiotic resistance.

Keywords Bioactive Compounds, Antimicrobial Screening, *Staphylococcus Aureus*, *Actinomycetes*-Derived Antibiotics

1. Introduction

Infectious diseases have plagued humans for millions of years. Infectious illness resurgence is a significant issue for people and the environment [1]. Over the past three decades, infectious diseases have become more prevalent. Pathogens are the primary cause of these illnesses [2]. The second most common cause of mortality globally [3], the primary cause of disability-adjusted life years (a year lost from a healthy life is called a disability-adjusted life year), and the third leading cause of death in the United States are infectious diseases [4]. Bacteria, viruses, prion disorders, and rickettsia are the causes of contagious diseases. These illnesses significantly negatively influence wildlife, agriculture, and public health [5]; [6]. Microbes like bacteria, fungi, viruses, etc., cause roughly 200 recognized human diseases [7].

Nowadays, drug- and multidrug-resistant organisms are the leading cause of many microbiological diseases. This poses a severe risk to public health and significantly raises morbidity and death rates [8]; [9]. Diseases resistant to several drugs are becoming more prevalent [10]. Therefore, many antibiotics are no longer effective in fighting microorganism diseases and infections, which is detrimental to the health of the community's large population [11]. The natural source of many microorganisms that create various biologically active natural products, such as clinically significant medications, is soil [12]. It is crucial to find new antimicrobial drugs to combat diseases that are resistant to medicines [13].

Today, there is a greater emphasis on screening soil *Actinomycetes* for antibacterial properties. Although some antibiotics are now made synthetically, some still come from microorganisms [14]. Green plants, lichens, *Actinomycetes*, bacteria, and fungi produce antibiotics [15]. Among the significant organisms required for the synthesis of bioactive chemicals are *Actinomycetes*. They specifically create anticancer medications, immunosuppressive compounds, enzymes [16], and particularly antibiotics—roughly half of all bioactive secondary metabolites ever discovered [17]. *Actinomycetes* are the leading candidate for becoming an antibiotic source in industrial microbiology [18].

Actinomycetes are a diverse collection of aerobic, filamentous, gram-positive microorganisms that produce spores. Their cell walls contain L-L diaminopimelic acid, and their DNA has a high G+C content (57–75%) [19]; [20]. They have a prominent role in various biological and metabolic processes, such as the production of extracellular enzymes, and are widely distributed in the natural environment [21]. Most significant metabolites were found in soil *Streptomyces* [22]; [23]. Seventh to eighty percent of known antibiotics originate from *Streptomyces* sp. [24]. Additionally, natural pigments in the colors of red, green, yellow, brown, and black are produced by *Actinomycetes* on various media [25]. The natural ability of Nocardia and *Micromonospora* to produce antibiotics is lower than that of *Streptomyces, which* are thought to follow *Streptomyces* as possible species that encompass the synthesis of antibiotics [26]. Many studies have been carried out on isolating and analyzing *Actinomycetes* that produce antibiotics. According to a belief, most new antibiotic discoveries have come from screening soil isolates [27].

The rise of multidrug-resistant (MDR) infections poses a severe and growing threat to global health, challenging the efficacy of existing antibiotics and contributing to increased morbidity and mortality rates. The World Health Organization has identified antibiotic resistance as one of the top global health challenges, necessitating urgent action to address the dwindling pipeline of effective antimicrobial agents. MDR pathogens, such as *Staphylococcus aureus* and *Klebsiella pneumoniae,* have rendered many commonly used antibiotics ineffective, leaving healthcare systems struggling to combat once-treatable infections. This crisis is further compounded by the slow pace of antibiotic discovery and development, driven partly by economic disincentives and the technical challenges of identifying new compounds with novel modes of action [28], [29]. However, despite this rich history, the potential of soil *Actinomycetes* as sources of novel antibiotics remains underexplored [30], particularly in unique or under-sampled environments such as the soils of Egypt's El Minia region.

Therefore, this study focuses on the isolation and characterization of *Actinomycetes* from agricultural soils in El-Minia, Egypt, intending to identify new antimicrobial compounds. This research aims to address the urgent need for novel antibiotics capable of combating MDR pathogens by targeting an unexploited environmental niche. The investigation emphasizes the importance of revisiting natural sources for drug discovery, leveraging modern analytical techniques to uncover bioactive compounds with potential therapeutic applications.

2. Materials and Methods

2.1. Samples Collection

Samalut, Minia, Maghagha, Bani Mazar, Abu Qurqas, Malawy, and Matay are among the cities where agricultural soil samples were taken in the Minya governorate in 2019, between March and September. The soil's top layer was eliminated, and the core was gathered in sterile plastic bags. Soil mixtures from three holes ranging in depth from 10 to 30 cm comprise each sample. Samples were refrigerated until they were processed in the laboratory [31]. Below is a table showing the details of the collected soil samples with GPS coordinates of each location **(Table 1)**.

Table 1. Details of the collected soil samples with GPS coordinates of each of these locations

Sample No.	Area	Site (GPS)
1	Samalut	28°19'02"N, 30°42'10"E
2	Minia	28°07'45"N, 30°44'27"E
3	Maghagha	28°38'25"N, 30°49'51"E
4	Bani Mazar	28°28'59"N, 30°48'34"E
5	Abu Qurqas	27°55'52"N, 30°50'20"E
6	Mallawy	27°44'45"N, 30°50'50"E
7	Matay	28°25'55"N, 30°46'20"E

2.2. Isolation of *Streptomyces*

The Hayakawa and Nonomura serial dilution method (1987) has been used to isolate *Streptomyces* from soil samples [32]. *Streptomyces* were isolated using plated suspensions. To isolate *Streptomyces*, each Petri plate was filled with 0.1 ml of the suitable inoculum dilution, which was then disseminated using a sterilized glass rod and starch-nitrate agar media. This medium is composed of the following ingredients: Agar, 20.0; starch, 20.0; KNO_3, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; NaCl, 0.5; $FeSO_4.7H_2O$, 0.01; $CaCO_3$, 3.0, and 1000 milliliters of distilled water (g/l). Nystatin (50 micrograms per milliliter) and Rifampicin (10 micrograms per milliliter) were used as supplements to prevent bacterial or fungal contamination. The pH was adjusted to 7.2 following a 7–10-day incubation period at 28 ℃. After the Petri dishes were prepared the day before plating and left overnight at room temperature to remove moisture films from the agar surface, the inoculation plates were examined for *Streptomyces* growth. The classification of *Streptomyces* is based on their specific physical characteristics (e.g., colonies that sit deeply, sporulation, and color). The streak plate method selected and purified plates containing countable single colonies.

2.3. Physiological Characterization of *Streptomyces* Isolates

The *Streptomyces* isolate with increased antibacterial activity was evaluated for its physiological, biochemical, and cultural traits. The color was picked out by sight, production of the enzyme's catalase, hydrogen sulfide, nitrate reductase, and urease, casein hydrolysis, triple sugar iron agar, gelatin hydrolysis, anaerobic growth, production of indole, methyl red, Voges Proskauer, and citrate utilization [33].

2.4. Antagonistic Activity of the Isolated Strains

Actinomycetes were isolated from several soil samples, identified, and then their antimicrobial spectra were evaluated. The bacterial strains used in this study *(Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae, and Bacillus cereus)* were obtained from the Microbiology Laboratory at Assiut University Hospital, Assiut, Egypt. These strains were selected due to their clinical significance and known multidrug resistance profiles, allowing for a comprehensive evaluation of the antimicrobial activity of the isolated *Streptomyces werraensis*. Nutrient agar was used to assess activities. Each isolate was placed in the middle of a plate and streaked across each plate before being incubated at 37 ℃ for a week. This step was performed to promote the *Streptomyces werraensis* isolates' growth and ensure adequate interaction with the test organisms during subsequent screening. By placing the isolate in the center and streaking across the plate, we aimed to enhance the diffusion of bioactive compounds produced by the *Streptomyces werraensis* isolate throughout the agar medium. This facilitates the evaluation of antimicrobial activity against the test organisms. Then, freshly sub-cultured test organisms were streaked parallel to the actinomycete isolate [34]; [35]. The plates were left at 37 ℃ for a day to allow the bacteria to develop. The zone of inhibition was assessed and noted following incubation.

2.5. Extraction of a Crude Secondary Metabolite from *Actinomycetes*

For fermentation and extraction, isolates demonstrating possible antibacterial activity were chosen based on the initial screening's zone of inhibition. 200 mL of starch nitrate broth was contained in a 500 mL conical flask. It was inoculated with the prospective actinomycete strain and then maintained in a rotary shaker set at 200 rpm for three days at 28 ℃. The pH of the medium was adjusted to 7.0 [36], This culture was inoculated with 50 ml into each of the four fermentation flasks that held 1000 ml of the production medium (g/l) soluble starch, 20.0; $NaNO_3$, 2.0; K_2HPO_4, 0.1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; and $FeCl.5H_2O$, 0.01; and 7.0 was established as the medium's pH. Flasks with the inoculation were incubated for five days at 30 ℃ and 250 rpm in a shaker [37] [38]. After the incubation period, a total volume of four liters was filtered. The active component-containing supernatant was separated and saved after centrifuging the filtrate for 20 minutes at 5,000 rpm. After that, the extract was made with ethyl acetate diluted 1:1 with water. The resultant ethyl acetate extract was then utilized as a stock concentration for assessing antibacterial activity after being forced to evaporate under vacuum in a rotating evaporator at 40 ℃.

2.6. Secondary Screening

Testing the antibacterial effectiveness against test microorganisms employed ethyl acetate as a reference. The six mm-diameter wells were drilled on Muller-Hinton agar using a sterile cork borer. Young cultures of *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, Acinetobacter,* and *Klebsiella pneumonia* were swabbed for twenty-four hours on the Muller-Hinton agar surface for microorganisms. Five microliters of dissolved crude extract were added to each well, and the mixture was left to settle for 30 minutes to allow the metabolite to spread, utilizing tetracycline (30 µg) and penicillin (10 µg) as standard. Afterward, the plates were cultured for microorganisms for a full day at 37 ℃. The zone of inhibition was measured and recorded after incubation.

2.7. Identification of *Streptomyces* Isolates Using Molecular Sequencing

In sterile test tubes, DNA was extracted from the bacterial isolate using the Patho-gene-spin DNA/RNA extraction kit (Intron Biotechnology, Korea) following the manufacturer's protocol. For gene sequencing and polymerase chain reaction (PCR), the extracted DNA from each sample was sent to SolGent Company in Daejeon, South Korea. The PCR amplification was performed using two universal primers: 27F (5'-AGAGTTTGATCCTGGC TCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The amplified products (amplicons) were confirmed by electrophoresis on a 1% agarose gel, using a 100 base pair size nucleotide marker. Dideoxynucleotides (ddNTPs) were incorporated into the sequencing reaction mixture. Sequencing was performed in both the sense and antisense directions using the 27F and 1492R primers. The generated sequences were aligned and analyzed using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI). Phylogenetic analysis was conducted using MUSCLE (Phylogeny.fr) to construct phylogenetic trees. The sequences of the detected strains were submitted to NCBI for accession number assignment [39].

2.8. Thin-Layer Chromatography

Thin-layer chromatography was utilized to observe the chemical components in isolated bacteria's crude ethyl acetate extract [40]. The crude extract of ethyl acetate was spotted 1.5 cm from the bottom of aluminum plates used for silica gel thin-layer chromatography (TLC), Merck, Darmstadt, Germany, 20 cm × 20 cm; thickness 0.25 mm and the solvent was then evaporated until the material was dry. A 15-ml solvent (mobile phase) containing a 6:4 ratio of ethyl acetate to methanol was poured into the container, allowing the solvent vapor to saturate the glass beaker thoroughly. The beaker was then covered, and it was left for 30 minutes. Afterward, the blotted sheet was placed into the beaker. Until the sample was 2.5 cm from the top of the TLC plate, it took 30 minutes for the solvent to move with it. After that, the plate for TLC was removed from the beaker and let air dry for five to ten minutes. Under ultraviolet light, the dried TLC plate was inspected, and the retention factor Rf value was computed.

Concentrated ethyl acetate extracts produced 1.9 g of crude extract with a reddish-brown color. The distinct extract components were separated via purification and structural elucidation of bioactive substances. In the stationary phase, the components were distributed and separated. A successful thin-layer chromatography analysis requires only a few micrograms of material, making it a quick, sensitive, and affordable method. The number of components in a combination and the purity of the components are routinely determined using TLC [42]. TLC of the isolated bacteria's ethyl acetate crude extract was performed in triplicate to identify its components and their chemical nature.

2.9. Determination of Antimicrobial Activity

The resulting extract was diluted at a concentration of 1 mg/ml in 10% dichloromethane (CH_2Cl_2) in methanol (MeOH) [41], and 50 l aliquots were soaked on Whatman No. 1 filter paper discs measuring 5 mm, sterilized, and permitted room temperature drying. Test bacteria were sown on the agar plates holding the paper discs. They were incubated for 24 hours at each test organism's ideal temperature. Nutrient agar media were used to cultivate bacterial microorganisms. Each microorganism's culture was diluted to 10^7–10^9 CFU/ml using sterile distilled water. The inoculated agar plates were initially placed in the freezer for two hours before an incubation period of twenty-four hours at 37 ℃. Following incubation, various test microorganisms were used to gauge the diameter of inhibitory zones.

2.10. Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

At the analytical chemical department of the Faculty of Science, Assiut University, the antimicrobial agents isolated from *Streptomyces* spp. were twice examined by GC-MS, first to identify the composition of all components and the second time to determine only the most active molecule after doing thin layer chromatography and determination the antimicrobial activity. The chemical composition of the extracted samples was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS) on model Agilent 7890A GC coupled with 5975C inert MSD. The instrument had an HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm film thickness). Helium was used as the carrier gas at a 1 mL/min flow rate. The injection volume was 1 μL, with the injector temperature set to 250 ℃ in split mode. The oven temperature was programmed to start at 50 ℃, held for 2 minutes, then increased at a rate of 10 ℃/min to 300 ℃, where it was held for 10 minutes. The MS was operated in electron ionization (EI) mode at 70 eV. The scan range was set from 50 to 500 m/z to detect and identify a broad spectrum of compounds. The detected peaks were identified by comparing their mass spectra with the National Institute of Standards and Technology (NIST) library or other reference databases. The relative abundance of each compound was determined by calculating its percentage area relative to the total chromatogram area. The analysis provided qualitative and semi-quantitative data on the volatile compounds present in the sample [40].

2.11. Optimization of Carbon, Nitrogen Sources, and pH Levels

For obtaining the optimum carbon and nitrogen sources, three carbon sources: mannitol, glucose, sucrose of 20 g/L and three nitrogen sources: $NaNO_3$, yeast extract, and peptone of 2 g/L were studied. Different pHs (6, 7, and 8) were also studied. All of the experiments were run in triplicate.

3. Results

3.1. Isolation and Morphological Analysis of *Actinomycetes*

Egypt's El-Menia governorate's agricultural area in various districts with different geographical regions **(Table 1)** was isolated for its soil *Actinomycetes*. *Actinomycetes'* development on the plate was noticed after 7 days of incubation. Starch-nitrate agar was used to develop and maintain the isolates. Individual isolates can be white, brown, gray, nucleated black, or exhibit some variations in these colors, which are visible on the substrate and aerial mycelium.

3.2. Biochemical Characteristics of *Actinomycetes*

Actinomycetes are identified based on their biochemical properties. Gram stain, catalase, oxidase, Casein hydrolysis, Triple sugar iron agar, Gelatin hydrolysis, amylase, and urea hydrolysis tests revealed positive isolates obtained through serial dilution and direct inoculation. However, their tests for anaerobic growth, lipase, Methyl red Voges Proskauer, citrate utilization, nitrate reduction, indole synthesis, and H_2S were negative **(Table 2).**

Table 2. The biochemical and physiological properties of the isolates of *Streptomyces werraensis*

Characteristics	***Streptomyces werraensis***
Gram stain	+
Pigmentation	Grey to light brown aerial mycelia
Anaerobic growth	-
Catalase	+
H_2S	-
Urease	+
Amylase	+
Lipase	-
Nitrate reductase	-
Casein hydrolysis	+
Triple sugar iron agar	+
Indole production	-
Methyl red	-
Voges Proskauer	-
Citrate utilization	-
Gelatin hydrolysis	+
Carbon sources:	
Starch	+
Glucose	+
Sucrose	+
Fructose	+
Mannitol	+
Nitrogen sources:	
Sodium nitrate	+
Peptone	+
Potassium nitrate	+
Yeast extract	+
Ammonium sulfate	+

3.3. Antimicrobial Screening

The cross-stripping method was used to calculate the value of isolates' minimal inhibitory concentration (MIC) against several human diseases. The tested Pathogens included *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Escherichia coli.*, and *Bacillus cereus.* For 24 hours, the *Actinomycetes* isolates were incubated to see if they had any antibacterial effects. The pathogen strains were grown perpendicular to the *Actinomycetes* isolates in the middle of the plates. The preliminary screening identified *Streptomyces werraensis* as the most effective isolate against *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae*, with 23–29 mm inhibition zones. Secondary screening confirmed the isolate's superior activity compared to tetracycline and penicillin **(Table 3)**. According to the screening results, more isolates proved more effective against gram-positive bacteria than gram-negative ones. This suggests that additional research into multi-drug-resistant *Staphylococcus aureus* may be prompted by the ability of this strain to combat *S. aureus* (MRSA).

3.4. Fermentation and the *Actinomycetes'* Crude Secondary Metabolite Extraction

The production medium for the fermentation process, starch nitrate broth, was used for five days at 30 ℃. The entire volume filtered was four liters, and then the filtrate was collected and centrifuged the clear filtrate with the active metabolite for 20 minutes at 5,000 rpm. Next, the filtrate's pH was raised to 7.0. The procedure for extraction was completed using an equal volume of ethyl acetate that had been saturated with water at a ratio of 1:1 after vaporization until it was dried in a 40 ℃ rotating evaporator under vacuum to the smallest volume.

3.5. Secondary Screening

The zone of inhibition (ZOI) against the pathogens was determined by employing the agar-well diffusion technique. Further use was made of the isolates that passed the initial screening. It has been demonstrated that the isolates' crude extracts significantly affect human pathogens, including *S. aureus* and *Klebsiella pneumoniae*. There is a table showing the results of measuring and comparing the statistically significant zone of inhibition with penicillin and tetracycline standards **(Table 3)**. The zone of inhibition has been seen to grow along with the concentration of crude oil in the well. This is an example of a concentration-dependent crude extract activity. Compared to regular tetracycline and penicillin, the crude extract from strain S6 exhibited antibacterial solid activity against *S. aureus*. Antimicrobial screening was performed on a total of nine isolated strains. One of the nine strains was chosen for thorough microbial characterization research after one revealed high antibacterial activity. Additionally, one strain was identified as *Streptomyces* sp. when nucleotide sequencing was used to characterize it.

3.6. Molecular Identification of the Most Potent Strains of *Streptomyces* sp.

A high degree of similarity (99%) with *Streptomyces* spp. was found in the sequence of the soil isolate *Streptomyces*. The *Streptomyces*-specific sequence that was given was deposited in the Gene Bank under the name *Streptomyces* sp. **Figure 1** revealed the phylogenetic tree of *Streptomyces* sp. using the neighbor-joining method, and according to biochemical and morphological characteristics **(Table 2, Figure 2)**, our isolate may be identified as *Streptomyces werraensis*.

3.7. Thin-Layer Chromatography

According to the results, the crude extract was divided into four fractions (bands). Bands 2, 3, 4, and 5 have retention factors Rf of 0.157, 0.276, 0.434, and 0.921, respectively. Additionally, the conclusion that this extract contains UV-absorbing components was aided by separating bands in a TLC plate under ultraviolet light **(Figure 3)**. In that order, the five separated compounds were measured at RF 1.8, 3.2, 5.1, 10, and 11.5. After finding the most active molecule at Rf 5, a GC-MS analysis was done.

Table 3. Secondary screening of nine isolates of *Actinomycetes* compared with two antibiotics

Bacterial pathogens	**Zone (mm)**										
	S1	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**	**S8**	**S9**	**Tetracycline**	**Penicillin**
E. coli	21^{cd}	14^{b}	24^{d}	8^{a}	25^{d}	29^{ef}	-	-	22^{cd}	45^{g}	27^{e}
Staph. aureus	20^{c}	12^{ab}	18^{c}	8a	22^{cd}	23^{d}	-	-	24^{d}	22^{cd}	31^{f}
Klebsiella. pneumoniae	27^{e}	-	10a	-	31	28^{e}	-	-	16b	20^{c}	29^{ef}
Pseudomonas aeruginosa	15^{b}	-	-	-	27^{e}	18^{c}	-	-	12^{ab}	18^{c}	22^{cd}
Acinetobacter	10^{a}	-	22^{cd}	12^{ab}	20^{c}	12^{ab}	-	-	10^{a}	24^{d}	18^{c}
Bacillus cereus	23^{ab}	17^{ab}	25^{cd}	18^{ab}	20^{c}	22^{ab}	-	-	18^{a}	29^{d}	12^{d}

Different letters revealed significance at $p < 0.01$ level based on Duncan's multiple range test.

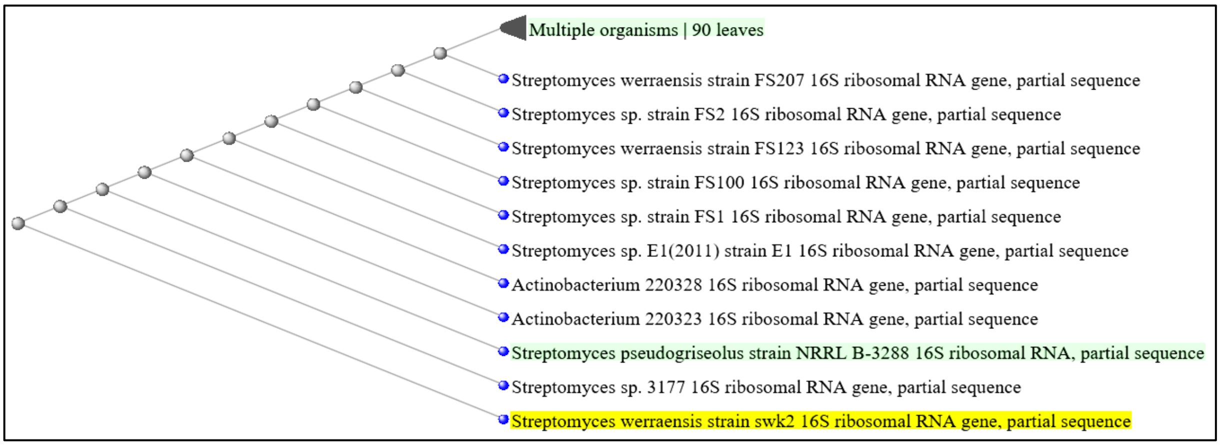


Figure 1. Phylogenetic tree of *Streptomyces werraensis*

Figure 2. *Streptomyces werraensis* isolates

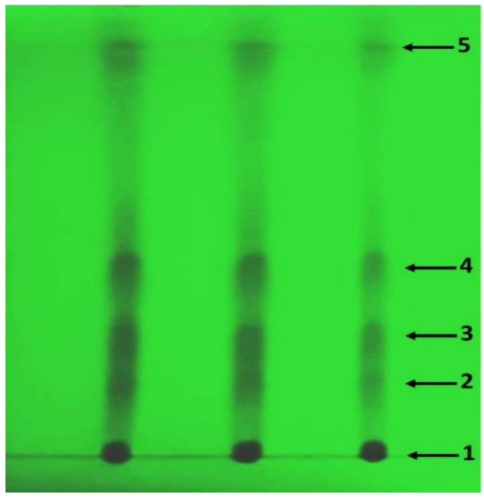


Figure 3. TLC plate of extract of the isolated bacteria showing 1: crude extract, 2, 3, 4, and 5: separated bands representing different components

3.8. GC-MS Analysis

To identify the active components in the antibacterial agents isolated from *Streptomyces* spp., GC-MS analysis was performed **(Table 4)**. Several peaks were detected with RT values of 3.96, 4.16, 6.98, 11.77, 20.15, 23.63, 26.04, 40.51, 41.00, 43.82, 56.66, and 60.23 min, corresponding to hexadecane, 1,1-bis(dodecyloxy)-cyclop ropane dodecanoic acid, 2-octyl-methyl ester, and N, N'-Bis(carbobenzyloxy)-lysine methyl ester, 2-Hydroxy-2-methyl-succinic acid, bis-(2-oxo-2-phenyl-et hyl ester, 1-Hexadecanol, 2-methyl Hexadecane, 1,1-bis(dodecyloxy), Phenol, 2,4-bis(1,1-dimethylethyl), Ethyl iso-allocholate, 9,12,15-Octadecatrienoic acid, 2,3-bis(trimethylsilyl)oxy propyl ester (Z, Z, Z) 17-Pentatriacontene, 8,14-Seco-3,19-epoxyandrostane-8,1 4-dione, 17-acetoxy-3-méthoxy-4,4-dimethyl Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl)-4-methyl, 1-monolinoleoylglycerol trimethylsilyl ether, respectively.

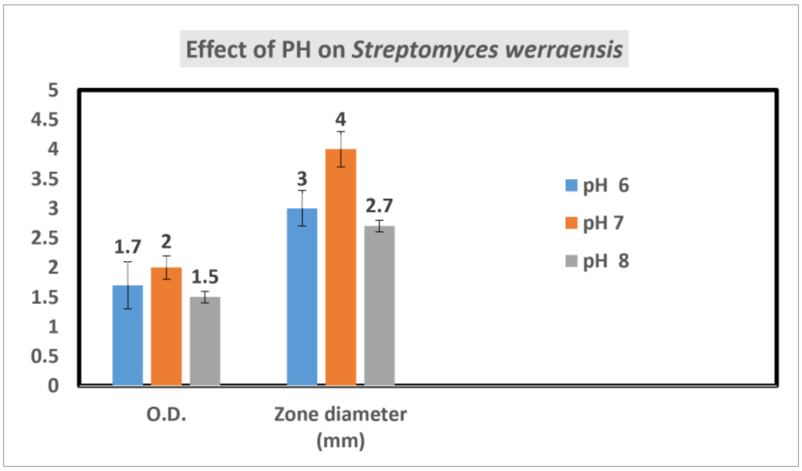
After TLC and the determination of the active compound, the TLC bands were analyzed using GC mass for the second time, and the most active molecule was phenol, 2,4-bis (1,1-dimethylethyl).

3.9. Optimal Carbon, Nitrogen Sources, and pH Levels

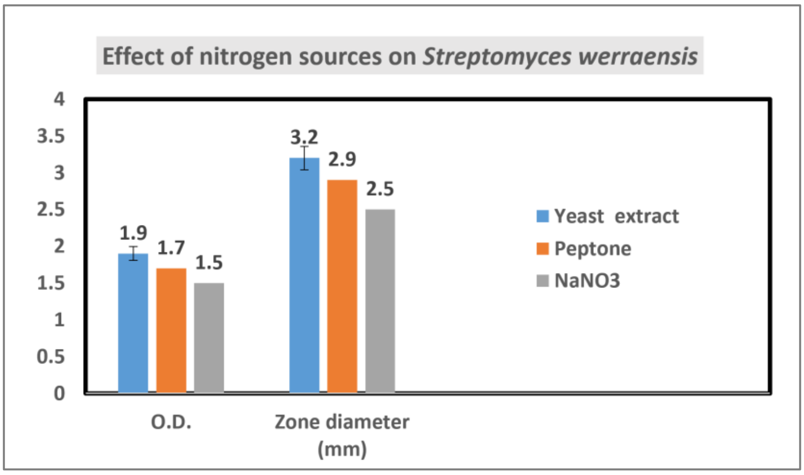
Diverse nitrogen and carbon sources, as well as various pH levels, were used during optimization. Our research revealed that mannitol and yeast extract were the best sources of N_2 and C, respectively, and a pH of 7 was ideal **(Figure 4A-C)**. The results mentioned indicated that the following ingredients made up the optimal medium: (g/l) 20.0 for starch; 3.2 for yeast extract; K_2HPO_4, 1.0 for $MgSO_4$, 0.5 for NaCl; 0.01 for $FeSO_4.7H_2O$; 3.0 for $CaCO_3$; 20.0 for agar; together with a thousand milliliters of distilled water.

Table 4. GC-MS data for antimicrobial components in *Streptomyces werraensis*

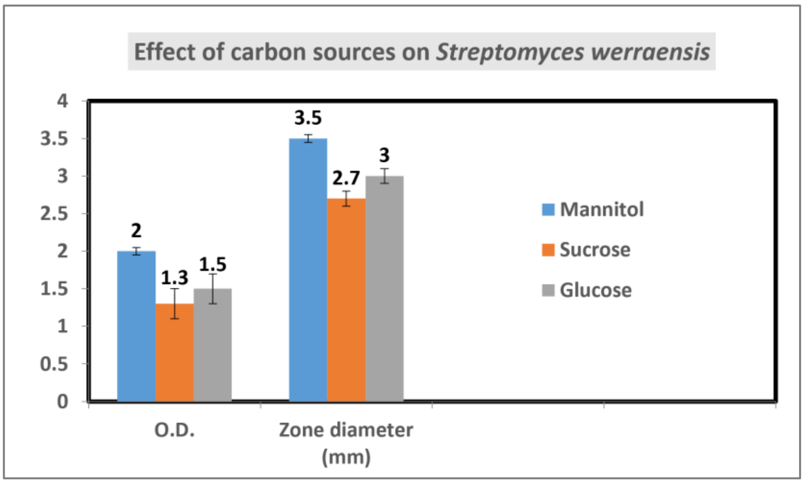
Chemical name	Retention time	area %	Molecular formulae	Molecular weight
Hexadecane, 1,1-bis(dodecyloxy)-	3.96	2.98	$C_{40}H_{82}O_2$	594
Cyclopropane dodecanoic acid, 2-octyl-, methyl ester	3.96	2.98	$C_{24}H_{46}O_2$	366
N,N'-Bis(Carbobenzyloxy)-lysine methyl(ester)	4.16	1.76	$C_{23}H_{28}N_2O_6$	428
2-Hydroxy-2-methyl-succinic acid, bis-(2-oxo-2-phenyl-ethyl ester	6.98	3.02	$C_{21}H_{20}O_7$	384
1-Hexadecanol, 2-methyl-	11.77	1.67	$C_{17}H_{36}O$	256
Hexadecane, 1,1-bis(dodecyloxy)-	20.15	3.32	$C_{40}H_{82}O_2$	594
Phenol, 2,4-bis(1,1-dimethylethyl)-	23.63	23.53	$C_{14}H_{22}O$	206
Ethyl iso-allocholate	26.04	1.00	$C_{26}H_{44}O_5$	436
9,12,15-Octadecatrienoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester, (Z,Z,Z)-	40.51	1.39	$C_{27}H_{52}O_4Si_2$	496
17-Pentatriacontene	41.00	3.15	$C_{35}H_{70}$	490
8,14-Seco-3,19-epoxyandrostane-8,14- dione, 17-acetoxy-3á-methoxy-4,4-dimethyl	43.82	1.51	$C_{24}H_{36}O_6$	420
Phenol, 2,2'-methylenebis[6-(1,1-dimethylethyl) -4-methyl	56.66	5.95	$C_{23}H_{32}O_2$	340
1-Monolinoleoylglyceroltrimethylsilyl ether	60.23	2.45	$C_{27}H_{54}O_4Si_2$	498



(a)



(b)



(c)

Figure 4. Effect of different pH **(a)**, nitrogen **(b)**, and carbon sources **(c)** on growth and antibacterial activity of *Streptomyces werraensis* on E. coli.

4. Discussion

The discovery of new types of antimicrobials that contribute to treating diseases resulting from bacteria, especially those resulting from multidrug-resistant bacteria, has become an absolute necessity with the increasing risk of this disease [30]. This occurs as *Actinomycetes* actively participate in the fight against numerous diseases. According to earlier research, *Actinomycetes* have the great potential to inhibit the proliferation of infections [43]. Therefore, this study was carried out, which aimed to examine nine *Actinomycetes* in total that were separated and analyzed for antibacterial activity from different regions of Minia Governorate, Egypt. The outcomes of morphological and biochemical characterization, as well as the nature of the substances made by the bacteria, established the variety among the *Actinomycetes* members.

In the current study, the primary standard used to check antibacterial activity against microorganisms in the preliminary and secondary stages is the size of the inhibitory zone. The pathogens were tested using bacteria of various kinds, both gram-positive and gram-negative, including *Acinetobacter* sp., *Bacillus cereus, Escherichia coli, Staphylococcus aureus*, and *Klebsiella pneumoniae*. The outcomes were statistically compared to tetracycline and penicillin zone of inhibition standards.

Seven active isolates from nine isolates of *Actinomycetes* have antibacterial activity, and most of these isolates were efficient against Gram-positive bacteria, demonstrating a reasonable zone of inhibition. The enhanced activity of *S. werraensis* against Gram-positive bacteria may stem from differences in cell wall structure. With their lipopolysaccharide barrier, Gram-negative bacteria often exhibit reduced susceptibility to hydrophobic compounds. This highlights the need for targeted screening to identify broad-spectrum activity [44, 45].

Among the seven active isolates, isolate No. 6 was chosen to be studied because it significantly opposes human pathogens such as *S. aureus, E. coli, Klebsiella pneumoniae*, and *Pseudomonas aeruginosa.* Compared to regular tetracycline and penicillin, crude is more potent than penicillin against *E. coli* and more powerful than tetracycline against *S. aureus* and *Klebsiella pneumoniae* and has the same efficacy against *Pseudomonas aeruginosa*.

The strain's biochemical characterization indicates that it is an actinomycete. Additionally, the results of the phylogenetic tree and 16S rRNA analysis verify that this strain belonged to the genus *Streptomyces*, exhibiting high sequence similarity (99%) and close phylogenetic affinity to *Streptomyces* sp. and according to morphological characteristics; our isolate may be identified as *Streptomyces werraensis* via global transcription factors that act as mediators for environmental signals such as carbon (CreA) [46], nitrogen (AreA) [47], and pH (PacC) [48], responses to environmental signals are transmitted in this way, converting stimuli from the environment into secondary metabolites and the corresponding physiological reactions. Because of that, the culture conditions were optimized to increase the process by which a selected isolate produces antibacterial metabolites, including carbon, nitrogen, and pH.

The composition of carbon sources, including glucose, sucrose, and mannitol, is essential for developing and generating secondary metabolites. They are crucial for gene regulation, signal transduction, and development. The maximum growth was observed with mannitol; this outcome is in agreement with studies by many authors (Kreig and Holt, 1989; Thakur and Bora, (2009) [49];[50] who discovered that one of the best fermentable sources of carbon for Streptomycetes to utilize for growth and the synthesis of antibiotics is mannitol. These findings underscore the importance of optimizing growth conditions to maximize secondary metabolite production. The preference for mannitol as a carbon source aligns with previous reports on *Streptomyces* fermentation processes, emphasizing its role in supporting robust growth and biosynthetic pathways.

A source of nitrogen is required to synthesize cells, proteins, and their energy requirements.

The maximum growth was observed with yeast extract from 3 different nitrogen sources, including sodium nitrate, peptone, and yeast extract. The outcome is consistent with the findings of Veilumuthu and Christopher (2022), who revealed that yeast extract is the most fermentable nitrogen source for *Streptomyces* sp. VITGV01 growth. Microorganisms may grow in a large variety of pH values. Additionally, the third important factor influencing microbial development is pH. A pH shift can also impact the secondary metabolite's stimulation or inhibition in the growth of bacteria [51]; [52].

This study reported that pH 7 is the ideal pH for maximal growth. This is comparable to the research done in 2022 on *Streptomyces* sp. VITGV01 by Veilumuthu and Christopher (2022) found that the ideal pH for maximal growth is 7.0.

GC-MS analysis has significantly contributed to bioprospect natural chemicals isolated from *Streptomyces* bacteria [53]; [54]. There are numerous publications on the actinobacterial secondary metabolite that was chemically analyzed using GC-MS. This investigation's GC-MS analysis reveals thirteen main chemical components with various retention periods. The TLC technique is used to determine the most active element from them. A GC-MS analysis was then performed after finding the most active molecule at Rf 5, and the most effective compound was phenol, 2,4-bis(1,1-dimethylethyl), which has antimicrobial activity. Similar to this, the compound was reported from *Streptomyces* sp. QUS7 [55] must be thoroughly investigated given the antibacterial capabilities attained in this work, which may be due to the use of the active component phenol, 2,4-bis(1,1-dimethylethyl) [PD], which inhibits the formation of biofilm; it is based on the fact that quorum sensing, a fundamental phenomenon in the pathogenesis and spread of disease, occurs between bacteria. Phenol derivatives have been widely reported for their antimicrobial properties, particularly against biofilm-forming pathogens. The activity of phenol, 2,4-bis(1,1-dimethylethyl), highlights its potential as a lead compound for developing new antibiotics [56].

To enhance the study and the potential application of *Streptomyces werraensis*, several strategies can be implemented to optimize its utility and impact. First, improving isolation and cultivation methods by experimenting with diverse environmental conditions, growth media, and stress factors may induce the production of bioactive compounds that were previously undetectable. This approach could uncover a broader range of antimicrobial agents and enhance yields. Additionally, conducting genomic and biochemical profiling of *S. werraensis* can provide insights into the genetic and metabolic pathways responsible for antimicrobial compound synthesis. Such knowledge may enable metabolic engineering or synthetic biology techniques to optimize the production and potency of these compounds.

Expanding the screening process to include a broader spectrum of pathogens, particularly antibiotic-resistant strains, will provide a more comprehensive understanding of the antimicrobial capabilities of *S. werraensis*. This step is critical for assessing its potential clinical applications and addressing the urgent need for novel treatments against multidrug-resistant infections. Furthermore, sustainable practices should be adopted to ensure minimal environmental impact during sourcing. Utilizing bioreactor systems or advanced fermentation techniques for large-scale production of bioactive compounds can support industrial applications while conserving natural resources. Collaboration with local communities, including researchers, healthcare professionals, and policymakers, is essential to ensure ethical bioprospecting and equitable distribution of benefits from potential therapeutic discoveries. Ensuring safety in human applications will pave the way for successfully translating *S. werraensis* metabolites into clinical use. By adopting these strategies, this study can significantly contribute to developing innovative solutions for combating antibiotic-resistant infections and advancing global healthcare.

5. Conclusions

This study highlights the urgent need for new antimicrobials to combat multidrug-resistant bacteria, focusing on *Streptomyces werraensis*, a promising isolate with stronger antibacterial properties than penicillin and tetracycline against key pathogens. Optimal growth conditions were identified, with GC-MS analysis revealing phenol 2,4-bis(1,1-dimethylethyl) as the most active compound, showing potential for combinatorial therapy. Future research will optimize isolation and cultivation methods, expand pathogen screening to antibiotic-resistant strains, and explore genomic and biochemical profiling to enhance bioactive compound production. Sustainable practices and large-scale bioreactor systems will be prioritized alongside ethical bioprospecting. Preclinical and clinical evaluations will assess safety and efficacy, paving the way for therapeutic applications of *S. werraensis* metabolites, either alone or in combination with existing treatments.

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