Exploring the Microbial Diversity of Aswan Soil: Isolation and Characterization of a Potent Antimicrobial Compound from *streptomyces thermolilacinus* AZHD22

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ABSTRACT

The selection of natural environments, like soil with diverse climates, can increase the chances of discovering unique isolates and potentially novel compounds. The region of Aswan in Egypt, known for its geographical and climatic diversity, was investigated for actinomycete isolates. One particular isolate, designated as *Streptomyces thermolilacinus* AZHD22, demonstrated the production of bioactive substances with antimicrobial properties against microbes. The objectives of this work were to discover the generating actinomycete isolate, describe the bioactive molecule, and optimize the growth conditions for the synthesis of antimicrobials. The best conditions for antimicrobial production were observed in sterile starch-nitrate broth, incubated at 45 °C with an adjusted pH of 8 and 1% NaCl concentration. Glucose at 1% concentration was determined as the optimal carbon source, while peptone at 0.2% concentration provided the best nitrogen source. Additional components, such as 2.5 g/l $K_2HPO_4 \cdot 3H_2O$ and 0.5 g/l $MgSO_4$, were added to the broth media, followed by incubation in a shaker at 150 rpm for 9 days. The bioactive substances were extracted using ethyl acetate as the solvent, and purification revealed that fraction 9 exhibited potent antimicrobial activity against selected test microorganisms. Through characterizations including UV, IR, HNMR, and GC-Mass spectrum analysis, the bioactive compound was identified as (9,12-Octadecadienoic acid (Z, Z)-, methyl ester) with the chemical formula $C_{19}H_{34}O_2$. The compound exhibited non-lethal effects on Vero cells up to a concentration of 250 µg/mL.

Keywords: *Streptomyces thermolilacinus*; 9,12-Octadecadienoic acid (Z, Z)-; methyl ester; antimicrobial activity.

INTRODUCTION

There is currently little probability of isolating unidentified strains from terrestrial environments, hence searching for novel products is restricted to uncommon actinomycetes from typical surroundings habitats or probably the identification of new species or species found in uncommon habitats. These methods are justified by the possibility that these strains can produce novel bioactive chemicals (Khanna et al. 2011). Actinomycetes, which were previously believed to be rich sources of new chemicals, are potentially found in unfamiliar and underexplored settings (Subramani and Sipkema, 2019). Furthermore, due to frequent genetic exchange, many streptomycetes synthesize the same recognized chemicals even though they are isolated from diverse environments (Subramani and Aalbersberg, 2013). The richness and diversity of actinomycetes present in any given soil are prompted by influences such as soil type, pH, temperature, geographical location, cultivation, and organic matter, as well as aeration and moisture content. Scientists have conducted numerous studies to isolate actinomycetes as antibiotic sources. The ability of filamentous actinomycetes to produce a variety of chemically unique secondary metabolites is largely responsible for their significance as the most potent source of antibiotics and other bioactive secondary metabolites. The genetic potential of each actinomycete strain allows it to produce ten to twenty different secondary metabolites. (Sosio et al. 2000; Bent ley et al. 2002).

The capital of the Aswan Governorate is Aswan, a city in southern Egypt that is situated between N 32º.53'59"E" 20' 05º 24 (Smith, 2016). Because soil is a good medium for a vast array of creatures and interacts actively with the larger biosphere, biodiversity and soil are closely intertwined. Similarly, biological activity is thought to be a major component in the chemical and physical makeup of soils. (Bardgett et al., 2005). One of the planet's most varied and densely populated microbial environments is soil, which is home to a wide range of fungal and bacterial species, both functional and taxonomic. (Fierer et al. 2012).

A few investigations concentrated on employing biocontrol agents against deteriorating fungus and bacteria rather than utilizing chemical tests that leave residues that cause human toxicity and environmental pollution, notwithstanding the threats they

posed to the Egyptian stone structures. (Basma et al. 2023)

The goal was to rediscover new natural bioactive compounds that can combat pathogens. Many actinomycetes in soil, which are potential sources of drugs, remain uncultivable and inaccessible for novel antimicrobial discovery. Given the problem of antibiotic resistance, it is crucial to discover new antimicrobial agents effective against bacteria and fungi. Therefore, the study focused on screening Actinomycetes from unusual habitats for antimicrobial activity, with the hope of finding strains that produce undiscovered antimicrobial agents active against a wide range of microbes.

MATERIALS AND METHODS:

Selection and Collection of Sample Area

The botanical garden situated on an island in the Nile River was chosen as the sampling location due to its unique location and composition. The island is formed by the deposition of silt from the Nile River on granite rocks. Soil samples were collected from different areas of the island and carefully transported in specialized plastic bags to the laboratory at the Faculty of Science for Girls, Al-Azhar University, for subsequent analysis.

Soil sample screening using the crowded plate technique

Following the method described by (Narendra et al. 2010), the soil samples were subjected to screening using the crowded plate technique. A suspension of 1g of soil in 100 ml of sterilized water was prepared and incubated at 28°C, with shaking at 200 rpm for 30 minutes in an incubator shaker. After allowing the mixtures to settle, serial dilutions were prepared using sterilized water and vortexed vigorously. From dilutions ranging from 10-2 to 10-5, 0.1 ml aliquots were spread evenly onto starch nitrate agar (SNA) medium, which is a recommended complex and nutrient-rich medium. The pH of the medium was adjusted to an alkaline level, and 25 µg/mL of Amphotericin B was added to prevent fungal contamination and promote the growth of actinomycetes strains. The plates were incubated at 37°C for 48 hours, with periodic observations made during the incubation period. After 72 hours, pinpoint colonies displaying whitish, greyish, or other pigmented appearances were selected and purified using multiple streaking.

Screening of the isolated microorganisms for antimicrobial activity

Test microorganisms

The microbial strains were politely provided by the Faculty of Agriculture at Ain Shams University in Cairo, Egypt, and the Central Water Quality Laboratory of the Greater Cairo Water Company in Cairo, Egypt. The antibacterial activity of the actinomycetes isolates was assessed using test microorganisms as following, Gram-positive bacteria [*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538 and *Streptococcus pyogenes* MTCC 655]. Gram-negative bacteria [*Proteus vulgaris* ATTCC 13315, *Salmonella typhi* ATTC14028, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumonia* ATCC53637, *Escherichia coli* ATCC 25922 and *Enterobacter cloacae* LMG 2683] and test fungal strains and yeast, [*Fusarium chlamydosporum* F25, *Aspergillus terrus* SQU14026, *Alternaria alternata* Te19, and *Candida albicans* ATTC90028].

Antimicrobial Activity Assessment

The cork-borer method was utilized for determining the actinomycetes isolates' antimicrobial properties (Muharram et al. 2013). The plates containing the test microbes were refrigerated for two hours to ensure uniform diffusion of the antimicrobial agent before the growth of the test organisms. Subsequently, the plates were incubated at specific temperatures: 28°C for 3 to 5 days for fungi, 37°C for 24 hours for bacteria and 30°C for 24 hours for yeast. After incubation, the inhibition zones (measured in millimeters) were recorded. In the secondary screening, the agar well diffusion method with a submerged fermentation system was employed. The test organisms were seeded onto agar plates, and wells were filled with 0.1 ml of actinomycetes filtrate obtained from previous growth in starch nitrate broth medium. Following the incubation period, the antimicrobial activities in the filtrates were evaluated to identify the most potent actinomycetes isolate for further analysis.

Identification of the most potent actinomycetes isolates

The selected actinomycete isolate underwent morphological, physiological, and biochemical studies using media and methods outlined in the International *Streptomyces* Project (ISP) guidelines. The observation of mycelium, recording of colors, and melanoid pigments followed the ISP methods, with

reference to a universal color language and dictionary of names (Kenneth, 1979). Cell wall analysis was conducted based on the procedures described by (Becker et al. 1964 and Lechevalier and Lechevalier 1968). Subsequently, molecular identification was performed at the Sigma Company for Genetics and Scientific Service, Cairo, Egypt. Genomic DNA purification (Qiagen, USA) was carried out to isolate the DNA, Beck and Pohl (1984). PCR amplification of the 16S rRNA gene was accomplished using a multigene gradient thermocycler (Labnet, USA). The purified PCR product was subjected to bi-directional sequencing at GATC Biotech Company, Germany. The obtained sequencing products were aligned with similar sequences from the Gene Bank database using Cluster X (Thompson et al.1997) to determine their uniqueness or similarity. The resulting sequences were compared to the 16S rRNA gene database offered by NCBI/BLAST (Altschul et al.1997) to identify the closest match.

Optimization of Culture Conditions for Antimicrobial Metabolite Production

The production of antimicrobial metabolites by the most potent actinomycetes isolate was subjected to optimization studies to determine the effects of various environmental and cultural conditions. Incubation time, temperature, and pH were optimized based on previous studies by (Suthindhiran et al. 2009, Saadoun et al. 2008, and Ahmed et al. 2016). Additionally, optimization experiments focused on biochemical parameters such as different concentrations of sodium chloride, carbon and nitrogen sources, different concentrations of the best carbon and nitrogenous compounds, different amino acids, varying concentrations of $MgSO_4.7H_2O$ and K_2HPO_4, and the impact of different heavy metals on the bioactive compounds. These optimization studies were conducted according to (Ahmed et al. 2016, Singh et al. 2009, Usha et al. 2010, and Li et al. 2010). The production medium used for selecting the best bioactive compounds was starch nitrate broth medium (SNB), and after a 24-hours, the inhibition zones were measured to assess the antimicrobial activity.

Production and Extraction of Active Antimicrobial Compounds

The most potent actinomycetes isolate, identified through the optimization studies, was cultured at a large scale (15 L) in 50 mL volumes in 250 mL conical flasks containing an optimized production medium, such as starch-nitrate broth. The fermentation broth was prepared and subjected to centrifugation at 5000 rpm for 20 minutes. The resulting supernatant was filtered, and the filtrate was extracted using different solvents to obtain the antimicrobial metabolites in a pure form. Organic solvents such as chloroform, ethyl acetate, and a mixture of chloroform and ethyl acetate were used for extraction. The solvents were added to the filtrate in different ratios (1:1 v/v or 1:4 v/v) and vigorously shaken for an hour to facilitate extraction (Zeinab, 1987). Using the agar well diffusion method, the antimicrobial activity of the crude extract was assessed against the selected microorganisms. During the test, ethyl acetate and chloroform were employed as negative controls. (Noura *et al.* 2017). The bioactive ethyl acetate phase was separated from the aqueous phase using a rotary evaporator operating at 40°C. Thin layer chromatography (TLC) was performed on the crude ethyl acetate extract to analyze the number of compounds present and assess their purity.

Purification of the antimicrobial active compounds obtained from actinomycete isolate crude extract TLC analysis

According to the methods of (Attimarad *et al.* 2012) followed for purification of the antimicrobial active compounds obtained from the crude extract of the actinomycete isolate. To determine the quantity of chemicals, present and evaluate the crude ethyl acetate extract's purity, TLC analysis was carried out. Multiple mobile phases were tested to select the optimal solvent system for further separation through column chromatography (Atta *et al.* 2009). The bioactive fractions obtained from the chromatography were tested for antimicrobial activity using the agar well diffusion method. TLC was used to examine the homogeneity of individual fractions, and the compounds were visualized by spraying with a vanillin/sulfuric reagent, 0.5 g vanillin in 100 ml sulphuric acid/ ethanol (40: 10). At Nawah-Scientific Company in Cairo, Egypt, this operation was done by collecting the purified substance and evaporating it using a rotary evaporator for characterization.

Characterization and Identification of active pure compounds

The purified substances obtained from the most active actinomycete isolate were subjected to physiochemical characterization. This involved studying their solubility,

elemental content, empirical formula, and spectroscopic characteristics using techniques such as UV-analysis, IR spectroscopy, ^{1}H NMR spectroscopy, and GC-MS analysis. This work was conducted at Nawah-Scientific Company in Cairo, Egypt.

Assessing the ethyl acetate strain extract for cytotoxicity

The cytotoxic activity of the purified active substances obtained from the most active actinomycete isolate was evaluated on Vero cells (ATCC CCL-81), which are a type of viral host cells. Different concentrations of the ethyl acetate actinomycetes extract were tested (0, 3.9, 7.8, 15.62, 31.25, 62.5, 125, 150, 250, and 500 μg/mL), In order to prevent toxicity or morphological changes to the Vero cells, the highest tolerable concentration was identified. (Todorov et al. 2015 and Andrighetti-Fröhner et al. 2003). The percentage of cytotoxicity was calculated based on absorbance values, and the 50% inhibitory concentration (IC50) was estimated using dose-response curves using GraphPad prism software (San Diego, CA.USA).

RESULTS:

Screening of isolates for antimicrobial activity

Six actinomycetes isolates, designated as SS 1 to SS 6, were obtained from the soil of a botany garden in Aswan. The antagonistic properties of these isolates against a panel of three gram-positive bacteria, six gram-negative bacteria, one yeast species, and three fungi were assessed using the classical diffusion agar plug discs method. However, during the secondary screening under shaking conditions, isolate SS 2 exhibited the most promising antimicrobial activity compared to the other isolates. Table 1 presents the results, showing that SS 2 displayed a strong antimicrobial response across all test microorganisms, while the other isolates exhibited weaker activity.

Identification of the most potent actinomycete isolate SS 2

The most potent actinomycete isolate SS 2 producing antibacterial and antifungal bioactive compounds was selected for further characterization summarized in table (2a.). The isolate showed excellent growth on starch nitrate agar medium, with white substrate mycelium, light greyish yellowish brown aerial mycelium, and the production of dark yellowish brown diffusible pigments (Fig. 1a). Electron micrographs and phase contrast microscopy revealed that the spore chain of isolate SS 2 belonged to the Rectiflexibiles type, with an irregular rugose spore surface (Fig. 1b). Table 2b provides detailed information on the physiological and biochemical characteristics of isolate SS 2.

Molecular identification and Phylogenic tree of isolate SS 2

Through 16S rRNA sequencing at Sigma Company in Cairo, Egypt, the most effective antimicrobial-producing isolate, SS 2, was molecularly identified. A public database comparison was performed with the obtained partial 16S rRNA sequence at the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) to identify the closest match. SS 2 showed 100% similarity to Streptomyces thermolilacinus strain NBRC 14274. A phylogenetic tree (Fig. 1c) was constructed to illustrate the relationship between SS 2 and other related strains. The selected isolate was officially named Streptomyces thermolilacinus AZHD22 and deposited in the NCBI GenBank with the accession number OR632436.1.

Optimization of cultural and environmental conditions for bioactive compounds production

To maximize *Streptomyces thermolilacinus* AZHD22's capacity to produce bioactive chemicals, various cultural and environmental conditions were investigated. Figure 2a demonstrates that the optimal pH for promoting active metabolite biosynthesis and achieving maximum inhibition zone was alkaline, specifically at pH 8. The antimicrobial effects against test microorganisms such as *B. subtilis, S. aureus, E. coli, S. typhi, C. albicans,* and *A. terrus* were observed to be 45, 37, 42, 23, 22, and 41 mm, respectively.

Furthermore, figure 2b shows that the maximum production of antimicrobial secondary metabolites occurred at an incubation temperature of 45°C, with inhibition zones reaching up to 45 mm against test organisms. The antimicrobial activity decreased significantly above 45°C and below 20°C for Streptomyces thermolilacinus AZHD22.

The suitable incubation period for achieving the highest yield of active metabolites by Streptomyces thermolilacinus AZHD22 was determined to be 9 days (Fig. 2c). The zones of inhibition against test microorganisms were measured at 45, 39, 42, 25, 22, and 39 mm, respectively.

Figures 2d to 2k depict the influence of different factors on antimicrobial production by Streptomyces thermolilacinus AZHD22. These factors include the addition of 1% NaCl (Fig. 2d), the presence of 1% D-glucose (Figs. 2e and 2f), the use of 0.2% peptone as a nitrogen source (Figs. 2g and 2h), the concentration of K2HPO4 (Fig. 2i), the concentration of MgSO4.7H2O (Fig. 2j), and the presence of glycine (Fig. 2k). Optimal antimicrobial production was observed under specific conditions for each factor.

Finally, the addition of zinc sulfate at a concentration of 50 mg/L (Fig. 2l) induced maximum biosynthesis of bioactive compounds, resulting in inhibition zones of 42, 34, 40, 19, 19, and 39 mm against the tested microorganisms.

Production, extraction, and purification of bioactive substances from *Streptomyces thermolilacinus* AZHD22

For the production, extraction, and purification of bioactive substances from Streptomyces thermolilacinus AZHD22, a large-scale culture was conducted using 15 L of starch-nitrate broth (SNB) in 250 mL conical flasks under sterile conditions. The culture was incubated under optimized conditions as mentioned earlier. After incubation, the clear filtrate was obtained and subjected to extraction.

The clear filtrate was treated with a 1:1 (v/v) mixture of ethyl acetate and chloroform solvents in order to extract the bioactive compounds. After collecting the organic phase, the solvent was eliminated at 40°C using a rotary evaporator operating at a reduced vacuum. Until a viscous deep yellow syrup was achieved, this evaporation procedure was repeated. After that, the resultant syrup was filtered through Whatman No. 1 filter paper after being dissolved in the least amount of solvent possible. By adding petroleum ether (60–80°C) and centrifuging the mixture for ten minutes at 4000 rpm, the active ingredients were precipitated.

Among the extraction solvents tested, ethyl acetate (1:1, v/v) was found to be the most effective in extracting all the antimicrobial agents from the fermentation broth (Figure 3). The production of various secondary metabolites was observed, indicating the presence of a diverse range of bioactive compounds.

The active metabolites obtained from *Streptomyces thermolilacinus* AZHD22 were purified using thin-layer chromatography (TLC) with different solvent systems. The most effective solvent system for eluting the active metabolites was found to be dichloromethane: ethyl acetate: methanol (70:12:10 v/v). Silica gel column chromatography was then employed for further purification using the same solvent system. Fractions (5 mL each) were collected and tested for antimicrobial activity using agar well diffusion. Among the eleven fractions obtained, Fraction 9 (F9) exhibited significant antimicrobial activity based on the inhibition zones. To confirm the purity of the compound, TLC analysis was performed, revealing a single spot.

Physicochemical Characteristics of the F9

The physicochemical characteristics of Fraction 9 were investigated. It was found to be soluble in various solvents including chloroform, ethyl acetate, acetone, benzene, toluene, butanol, diethyl ether, methanol, and ethanol when dissolved in a concentration of 1 mg in 5 mL of solvent and precipitated with petroleum ether.

The spectroscopic characteristics of F9

The spectroscopic characteristics of Fraction 9 were also examined. The UV spectrum (fig. 4a) showed a maximum absorption (λmax) at 260 nm. The IR spectrum displayed characteristic peaks, including a C=O stretch (Ester Carbonyl) around 1735-1750 cm-1, C-H stretches (Alkane) in the range of 2800-3000 cm-1, C-H bends (Alkane) in the range of 1350-1450 cm-1, and C=C stretches (Alkene) (fig.4b). The 1H-NMR spectrum at fig.4c exhibited proton peaks in regions corresponding to aliphatic CH2 groups (between 1.5 and 2.5 ppm), aliphatic CH3 groups (around 5-6 ppm), protons near unsaturated bonds (around 5.3-5.4 ppm for cis double bonds), and protons near the carboxylic acid group (around 7-8 ppm).

Elemental analysis of Fraction 9 revealed the following composition (%w/w): C=77.5%, H=11.64%, and O=10.86%. Based on these results, the empirical formula of the active substance from *S. thermolilacinus* AZHD22 was determined to be $C_{19}H_{34}O_2$. GC-MS analysis (fig.4d) further confirmed the presence of a bioactive compound, specifically 9,12-Octadecadienoic acid (Z, Z)-, methyl ester, with a chemical formula of $C_{19}H_{34}O_2$ and a molecular weight of 294. This compound constituted the major peak area, accounting for 67% of the total peaks observed. (fig.4e).

Cytotoxic assay of 9,12-Octadecadienoic acid (Z, Z)-, methyl ester

The cytotoxicity assay was conducted using Vero cells. The cells were exposed to various dilutions of the purified compound, 9,12-Octadecadienoic acid (Z, Z)-, methyl ester, for 24 hours at 37°C. The results indicated that besides demonstrating antibacterial and antifungal activities, Fraction 9 also exhibited antiviral activity at non-toxic concentrations ranging from 0 to 500 μg/mL. Figure (5) displayed the results, revealing that the IC50 for the purified compound was 250 μg/mL at a non-lethal dose.

DISCUSSION:

The study's goal was to investigate several actinomycetes species, particularly in hot environments like Aswan, known for its high temperatures throughout the year. Actinomycetes are known to thrive in such conditions and produce various bioactive substances. The objective was to discover novel antimicrobial compounds against test microorganisms that have become resistant to many antibiotics in recent decades. Six different actinomycete isolates were obtained from a botanical garden on an island in the river Nile. Among these isolates, one showed promising antimicrobial activity against bacteria, fungi, and yeast. The accurate morphological characterization and identification of this isolate, previously referred to as SS 2, were conducted using standard media according to the ISP protocol (Shirling and Gottlieb, 1966).and international keys for identification (Williams et al. 1989; Hensly, 1994 and Whitman et al. 2012).

Further molecular and genetic identification revealed that the isolate had 100% similarity to Streptomyces thermolilacinus. It was named *Streptomyces thermolilacinus* AZHD22 and deposited in the NCBI GenBank with accession number OR632436.1. The effect of different cultivation methods on the production of bioactive metabolites by the isolated strain was investigated. Submerged cultivation under optimized conditions significantly increased the antimicrobial activity, as observed by the range of inhibition zones. This finding is supported by (Dharmawan et al. 2009) who emphasized how the content, temperature, pH, and agitation rate of fermentation media affect the generation of antimicrobial metabolites. Optimizing these parameters is crucial for enhancing the production of microbial products, such as antimicrobial substances. In order to promote overproduction from microorganisms such as actinobacterial species, it is important to optimize the production conditions of any microbial product. The simplest method to increase output is to optimize fermentation settings since distinct routes in microbial cells create most secondary metabolites, including antimicrobial compounds, in different ways. (Linda and Rabab, 2017).

In this study, the suitable pH for antimicrobial production from *S. thermolilacinus* AZHD22 was found to be 8. This aligns with (Guimaraes *et al.* 2004), who demonstrated the highest growth and antimicrobial activity of *S. sannanensis* SU118 at pH 8. However, this strain exhibited no activity at pH 9.

On the contrary, the growth and production of bioactive metabolites, specifically antimicrobial agents, were found to be influenced by the temperature during incubation. Increasing the temperature up to 45°C did not significantly affect the production of most antimicrobial substances by the *S. thermolilacinus* AZHD22 isolate. This finding is in agreement with a study by (Ahmed et al. 2020). The duration of the incubation period also had an impact on the production of antimicrobial compounds. On the 9th day, the *S. thermolilacinus* AZHD22 isolate exhibited strong activity against test microorganisms. Our results for isolate *S. thermolilacinus* AZHD22 match by (Sunita et al. 2015), who suggested that though the stationary phase must have begun on the 9th day, maximum antibiotic production had started in the mid log and late log phase and continued in the stationary phase.

In terms of other factors, maintaining the NaCl concentration at 1% g/L was found to be optimal for the production of antimicrobial compounds. This finding supports previous studies that investigated Streptomyces sp. NMF76, which grew well in ISP5 medium supplemented with 1-10% NaCl, but failed to grow in the absence of NaCl, even when complex media were prepared using deionized water. Additionally, NaCl concentrations of 1% and 3% were identified as optimal for growth and antimicrobial activity, respectively, in a study by (Nayer, 2020).

As a part of the growing medium, glucose was essential in giving the spores the carbon atoms it needed to create generations. When glucose was utilized, the highest levels of antibacterial compound synthesis and

mycelium development were seen. Our study investigating the effect of carbon sources on antimicrobial secondary metabolite production, similar to the findings of (Nayera et al. 2012), revealed that maximum antimicrobial compound production from two *streptomyces* sp. Occurred with glucose which was the most effective carbon source, followed by lactose, sucrose, mannose, fructose, and raffinose, in descending order of effectiveness.

Among the minerals tested, K_2HPo_4 was found to yield a high quantity of bioactive compounds, which aligns with the results reported by (Bindu et al. 2018 and Magamuri et al. 2012). Additionally, magnesium sulfate was found to be important for the production of antimicrobial agents, as supported by (Sweetline and Usha 2021), who reported that an optimum concentration of 0.5 g/L of $MgSo_4.7H_2O$ was required for the production of antimicrobial compounds.

Glycine showed the most significant impact on the production of antimicrobial agents by the *S. thermolilacinus* AZHD22 strain. Other amino acids, including arginine, alanine, proline, tyrosine, and tryptophan, also produced antimicrobial compounds, although not to the same extent as glycine. Amino acids, such as L-tyrosine and glycine, have been suggested by several researchers to play a crucial role in the biosynthesis of certain antibiotics and bioactive agents. The presence of tryptophan and glycine has been associated with the production of antimicrobial substances. This is supported by the findings of (McCormick and Flardh 2012).

Zinc sulfate was found to have a maximum inducing effect on antimicrobial biosynthesis, confirming the results reported by (Haferburg et al. 2008) and Haferburg and Kothe 2013). These investigations showed that because *Streptomyces* sp. can grow in conditions with high metal ion concentrations and low nutrition levels, it is a good option for the synthesis of bioactive chemicals.

Crude extracts from actinomycete were mostly extracted using ethyl acetate as the extraction solvent. This finding associates with the research oversaw by (Vijayakumar et al. 2012), they stated that maximal inhibitory zones of 20 and 19 mm were seen in the ethyl acetate extract of *Streptomyces afghaniensis* strain VPTS3-1, which demonstrated strong antibacterial activity against *Candida mirabilis* and *Candida albicans*.

UV spectral data analysis of the ethyl acetate extract of the fermentation broth revealed a peak at 260 nm. This finding is consistent with the study by (Slavica et al. 2005), which reported that UV spectral data peaks of *Streptomyces* isolates from soil samples ranged between 215 and 270 nm in Southeastern Serbia.

FTIR analysis indicated the presence of functional groups such as C-H bending Alkane and =CH bending Alkene at specific wavelengths, suggesting the presence of bioactive compounds like Linoleic acid and palmitic acid, which have antimicrobial activity. These results agree with the research of (Nivetha and Prasanna 2016), who identified multiple functional groups in their analysis of bioactive compounds using FT-IR.

The antimicrobial activity of the bioactive pure fraction 9 may be attributed to the presence of methyl ester groups. This finding contradicts the report by (Chifu et al. 2010), which stated that compounds with methyl esters exhibited significantly higher antimicrobial activity. Additionally, the functional groups found in fraction 9 indicated a strong antimicrobial effect, which is consistent with the work of (Jorge et *al.* 2017), who reported that compounds with aliphatic carboxylic acid groups display antimicrobial activity.

Mona et al. (2022), who investigated the NMR analysis of the *Streptomyces* sp. MMM2 ethyl acetate extract, revealing bands that corresponded to the methyl ester of 9,12-octadecadienoic acid. This discovery aligns with our findings that indicated essential fatty acids called linoleic and linolenic acids are found in plants and some microorganisms, such as actinomycetes (gram-positive bacteria) and algae (cyanobacteria), These fatty acids cannot be produced by humans.

Our findings support by the results of (Bidhayak et al. 2022), who reported that 42 bioactive substances, including fatty acid esters such 9,12-octadecadienoic acid (Z,Z)-methyl ester was found in the ethyl acetate extract of *Streptomyces levis* strain KS46. And most *Streptomyces* sp. strain KS46 secondary metabolites showed antibacterial, antifungal, insecticidal, and other antimicrobial characteristics.

Furthermore, (Noura et al. 2017), discovered that ethyl acetate extract of *Streptomyces anulatus* NEAE-94 contained Octadecanoic acid methyl ester, a fatty acid that showed activity against multidrug-resistant Staphylococcus aureus. In our study, we found that the pure bioactive compound

9,12-Octadecadienoic acid (Z, Z) exhibited antibacterial and antifungal effects, which is consistent with (Mohammed et al. 2016), who reported its antifungal activity targets *Asp. niger, Asp. terreus, Asp. flavus,* and *Asp. fumigatus*, whereas its antibacterial activity targets *Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus,* and *E. coli*

Regarding cytotoxicity, our compound 9,12-Octadecadienoic acid (Z, Z) methyl ester demonstrated a non-lethal effect on Vero cells at a concentration of 150. This preliminary finding is crucial for virology researchers evaluating bioactive compounds as potential antivirals. The cytotoxicity data in our study agrees with the search of (Bolger et al. 2006), They showed that the substance had over 90% cell viability in all treatments and very low cytotoxicity against the Vero cell line. It is crucial to evaluate cytotoxicity in order to ascertain the hazardous concentrations of investigated substances that cause necrosis or apoptosis in cells. Furthermore, the tested drug needs to show antiviral activity at low cytotoxicity thresholds; otherwise, testing at low concentrations without accounting for cytotoxicity may result in negligible antiviral activity.

CONCLUSION:

In conclusion, natural products are valuable sources of potential pharmaceuticals, and there is a need to continue exploring and discovering new bioactive compounds. While many microbes, especially actinomycetes, have been studied, more research is necessary, particularly in natural and wild habitats and under unique conditions like high-temperature environments. Additionally, the development of antibiotic resistance highlights the importance of ongoing research in this area. Chemotherapy has shown effectiveness in treating various types of cancer, underscoring the need for further investigation into the chemistry of natural products. Based on our findings, the bioactive compound demonstrated a non-lethal dose, suggesting its potential application as an antiviral agent in future testing and development.

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Table 1: Secondary screening of actinomycetes isolates for antimicrobial metabolite production.

No.	Actinomycetes code.	Antimicrobial activity (mm.)												
		Gram positive bacteria			Gram negative bacteria						Fungi and yeast			
		B. subtilis	*S. aureus*	*S. pyogenes*	*E. coli*	*S. typhi*	*P. aeruginosa*	*K. pneumonia*	*P. vulgaris*	*E. cloacae*	*F. chlamydosporum*	*A. terrus*	*A. alternata*	*C. albicans*
1	SS 1	0	12	8	0	9	0	11	0	9	7	0	8	0
2	SS 2	38	30	33	37	17	21	19	26	18	36	38	12	16
3	SS 3	11	0	15	7	0	7	9	10	0	11	11	8	0
4	SS 4	0	0	8	0	0	8	0	8	0	14	0	8	10
5	SS 5	12	8	0	11	0	12	0	7	10	10	6	0	12
6	SS 6	0	6	9	0	10	0	8	9	12	0	12	9	0

Table 2a: Actinomycetes isolate SS 2 showing different cultural characterization on different media.

Types of media	Growth	Substrate mycelium	Aerial mycelium	Diffusible pigment
Starch nitrate agar	excellent	White (ISCC-NBS-263)	Light grayish yellowish brown (ISCC-NBS-79)	Dark yellowish brown (ISCC-NBS-78)
Yeast- malt extract agar (ISP2)	Good	White (ISCC-NBS-263)	White (ISCC-NBS-263)	Moderate yellowish brown (ISCC-NBS-77)
Oatmeal agar (ISP3)	Good	White (ISCC-NBS-263)	White (ISCC-NBS-263)	Dark yellowish brown (ISCC-NBS-78)
Inorganic salts-starch agar (ISP4)	No growth	-	-	-
Glycerol- asparagine agar (ISP5)	No growth	-	-	-
<u>Melanin production medium</u> 1-Tryptone-yeast extract broth (ISP 1)	Good	Deep orange yellow (ISCC-NBS-69)	Deep orange yellow (ISCC-NBS-69)	Deep orange yellow (ISCC-NBS-69)
2- Peptone iron agar (ISP 6)	No growth	-	-	-
3- Tyrosine agar medium (ISP7)	Good	Deep yellowish brown (ISCC-NBS-75)	Dark yellowish brown (ISCC-NBS-78)	Deep yellowish brown (ISCC-NBS-75)

Table 2b: Actinomycete isolate SS 2 showing morphological, physiological, and biochemical characteristics.

Character	Results
1- morphological characteristics: Spore surface Spore chain Spore mass	Irregular rugose Rectiflexibiles type White
2- Cell wall hydrolysis:	LL- DAP
3-Physiological characteristic:	
a- Melanin pigment:	
Tryptone-yeast extract broth (ISP 1)	+
Peptone iron agar (ISP 6)	-
Tyrosine agar medium (ISP7)	+
b-Carbon utilization:	
starch,	+++
D-glucose,	+++
dextrose,	+++
sucrose,	++
D-fructose,	+++
Maltose,	+++
Galactose,	++
L-arabinose,	-
D-xylose,	-
cellulose	-
D-mannitol	-
c-nitrogen utilization	
Tryptophan	++
Arginine	++
Alanine	++
Proline	++
Tyrosine	++
Glycine	+
Growth pH	
4-9	+
10-12	WG
Growth temperatures °C:	
20-55	+
Growth in presence of Na Cl conc. (%) 1-5	+

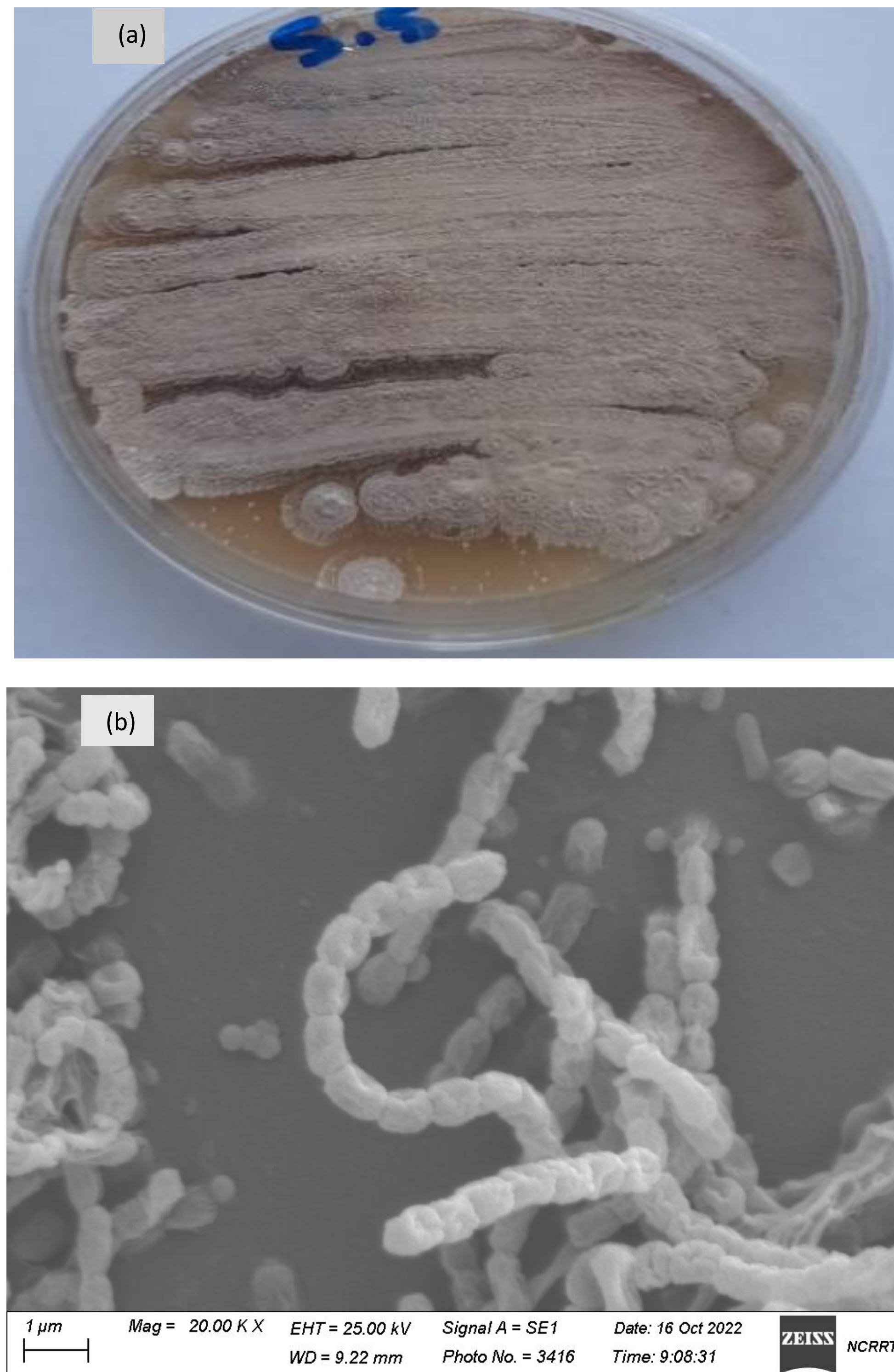


Figure 1: (a.) Isolate (SS 2) on starch nitrate agar medium after 14 days showing aerial and substrate color with diffusible pigment. **(b.)** Scanning electron micrograph of isolate SS 2 showing (Rectiflexibiles type) Shaped mycelia and Irregular rugose spore surface.

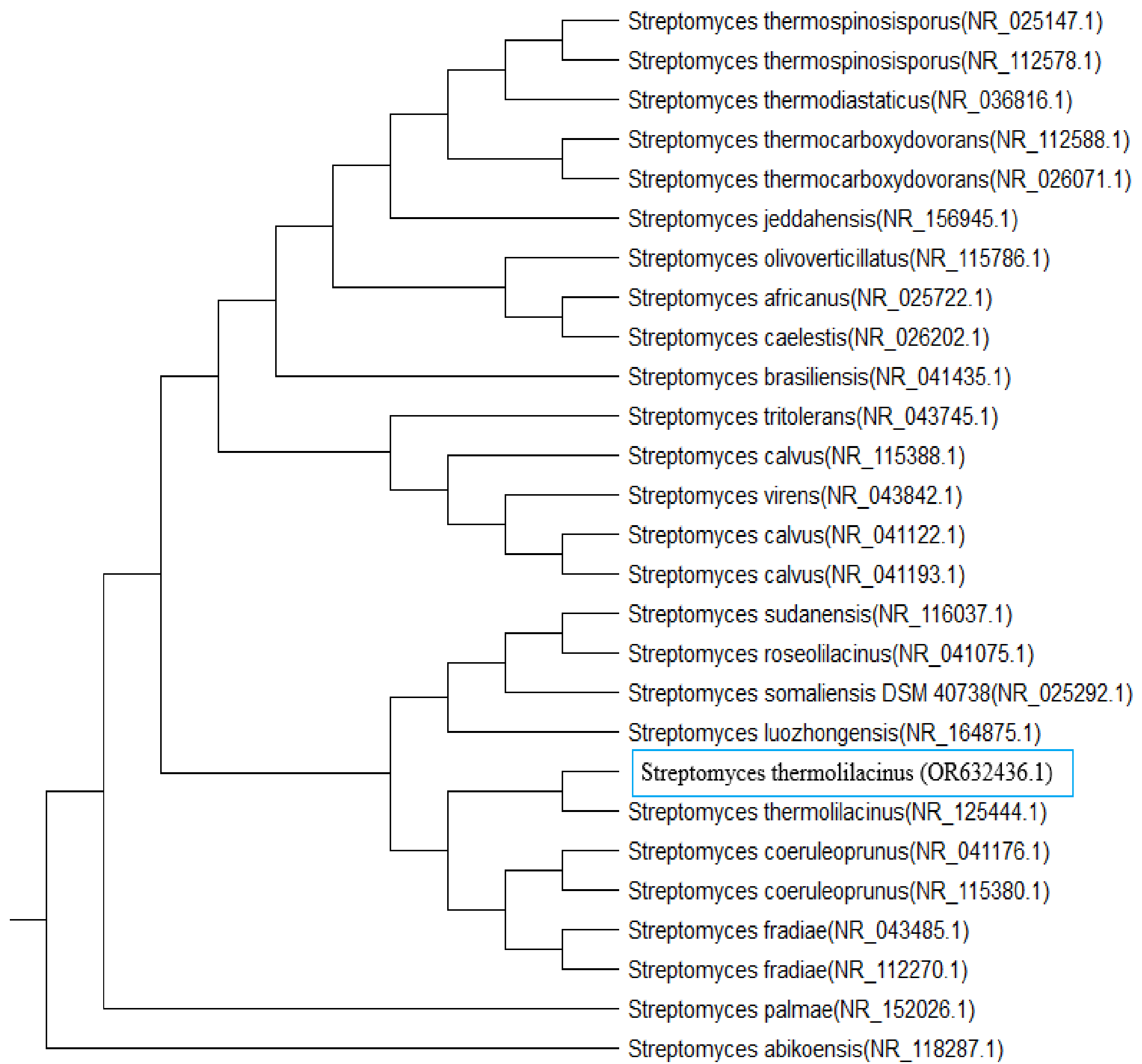
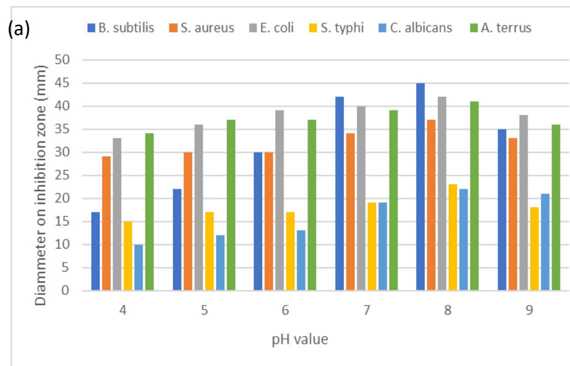
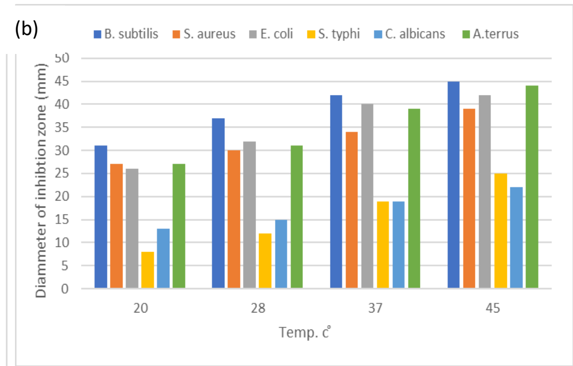
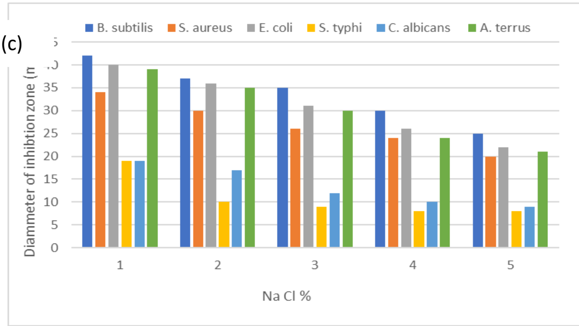
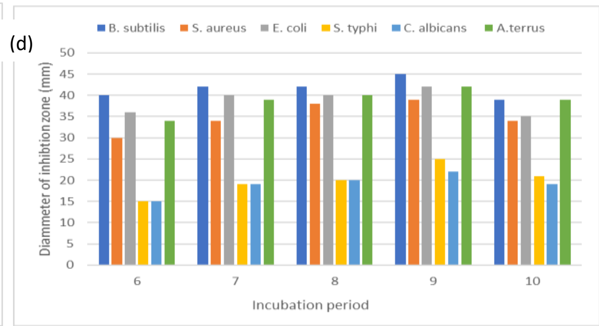


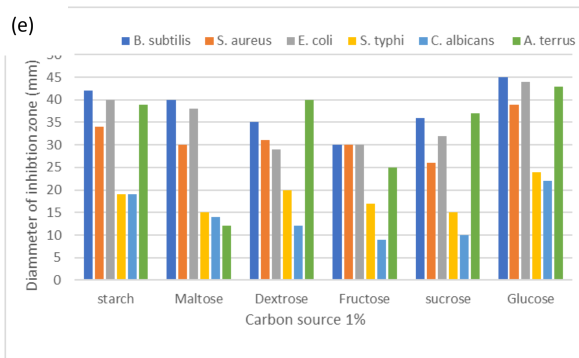
Figure (1c): The 16S rRNA gene sequences showing relationships between isolate SS 2 strain and other *Streptomyces* sp. related strains in a phylogenetic tree (dendrogram).

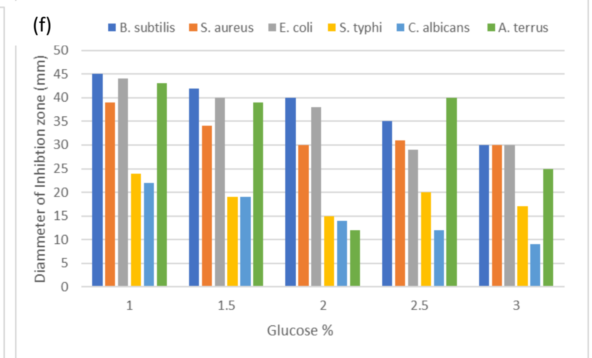

(a)
B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A. terrus
Diammeter on inhibition zone (mm)
pH value

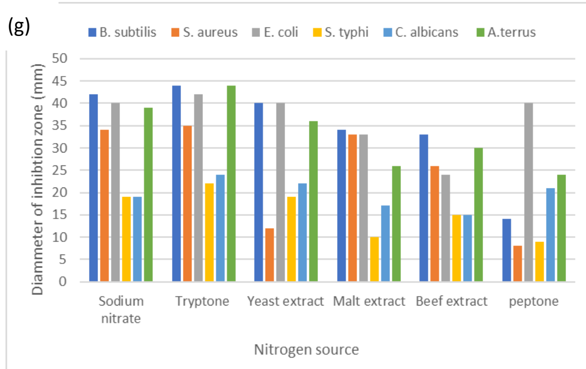

(b)
B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A.terrus
Diammeter of inhibition zone (mm)
Temp. c°

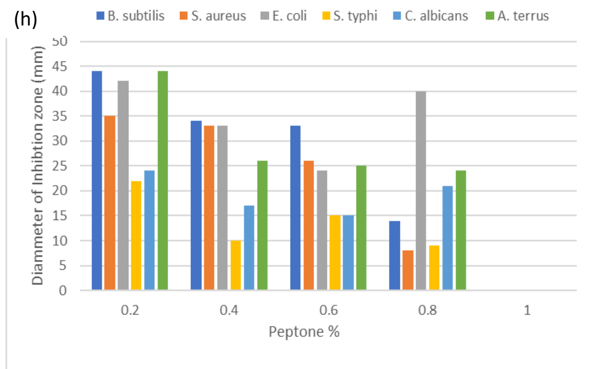

(c)
B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A. terrus
Diammeter of inhibition zone
Na Cl %


(d)
B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A.terrus
Diammeter of inhibtion zone (mm)
Incubation period


(e)
B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A. terrus
Diammeter of inhibtion zone (mm)
starch
Maltose
Dextrose
Fructose
sucrose
Glucose
Carbon source 1%


(f)
B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A. terrus
Diammeter of Inhibtion zone (mm)
Glucose %


(g)
B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A.terrus
Diammeter of inhibtion zone (mm)
Sodium nitrate
Tryptone
Yeast extract
Malt extract
Beef extract
peptone
Nitrogen source


(h)
B. subtilis
S. aureus
E. coli
S. typhi
C. albicans
A. terrus
Diammeter of Inhibtion zone (mm)
Peptone %

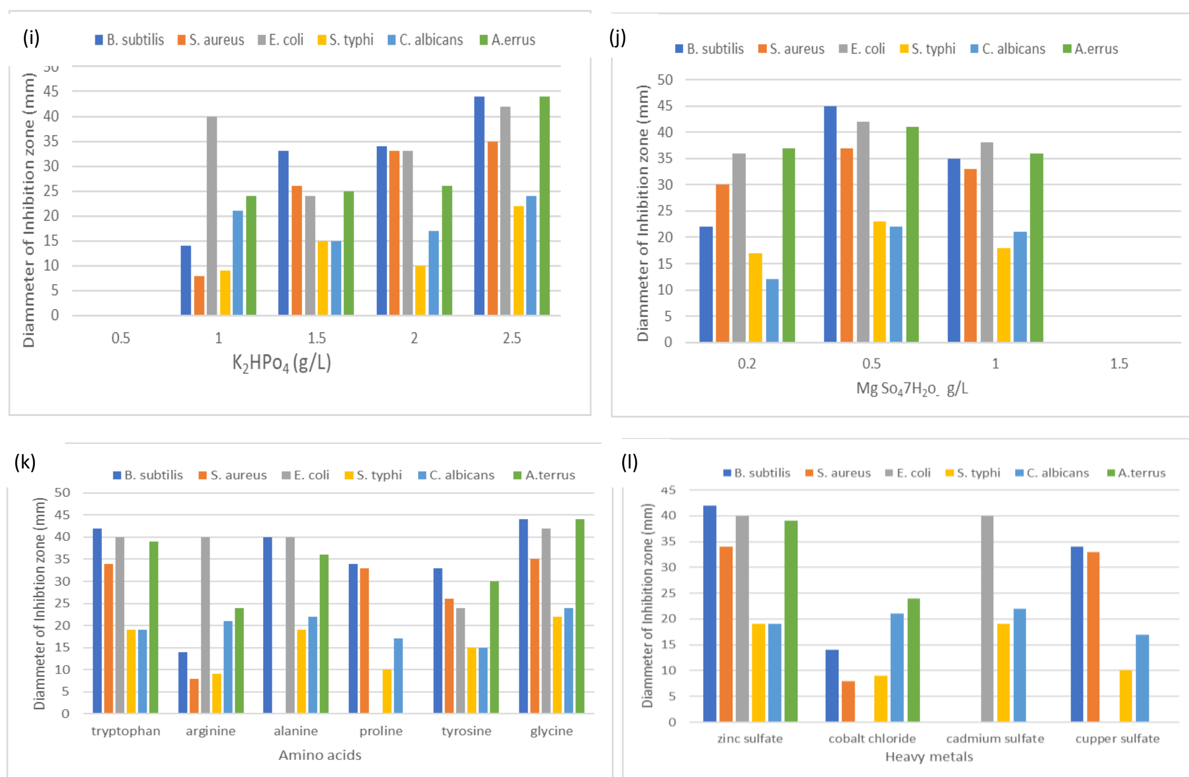


Figure 2: The effect of various physical and biochemical parameters on *Streptomyces thermolilacinus* AZHD22 antimicrobial production, **(a)** pH, **(b)** Temperature, **(c)** Incubation period, **(d)** Na Cl %, **(e)** Carbon source 1%, **(f)** Glucose %, (**g)** Nitrogen source, **(h)** Sodium nitrate %, **(i)** K_2HPO_4 a conc. **(j)** $MgSO_4{\cdot}7H_2O$ conc. **(k)** Amino acid, **(l)** Heavy metals.

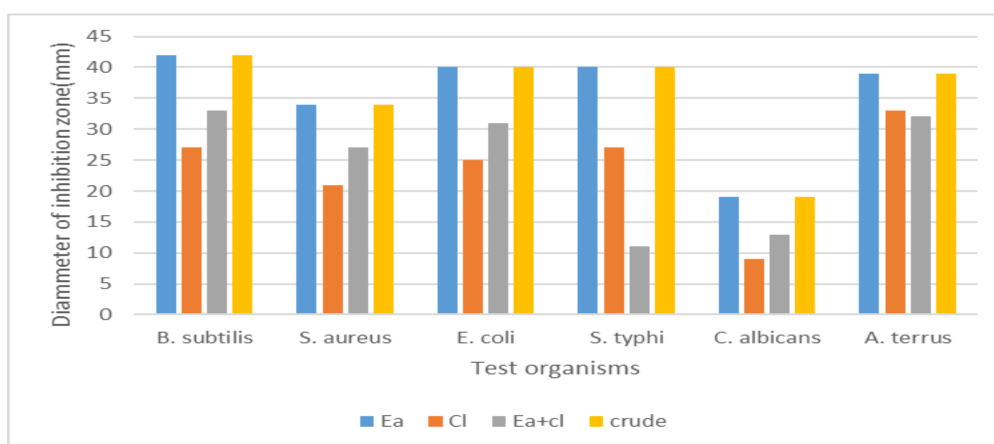
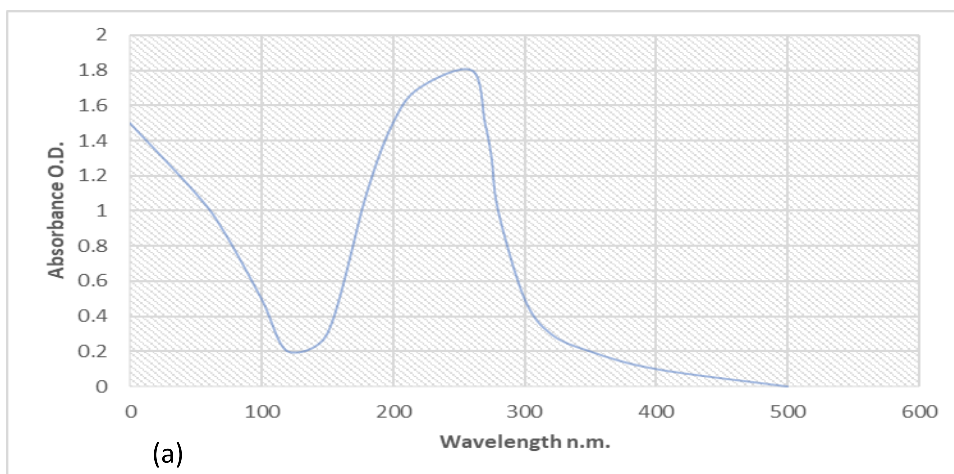
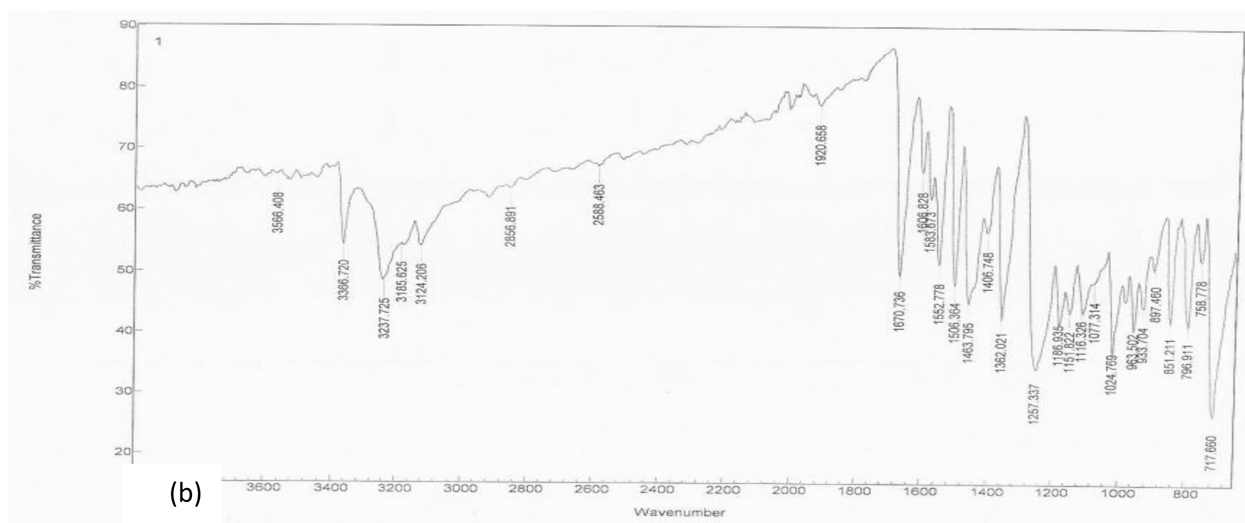
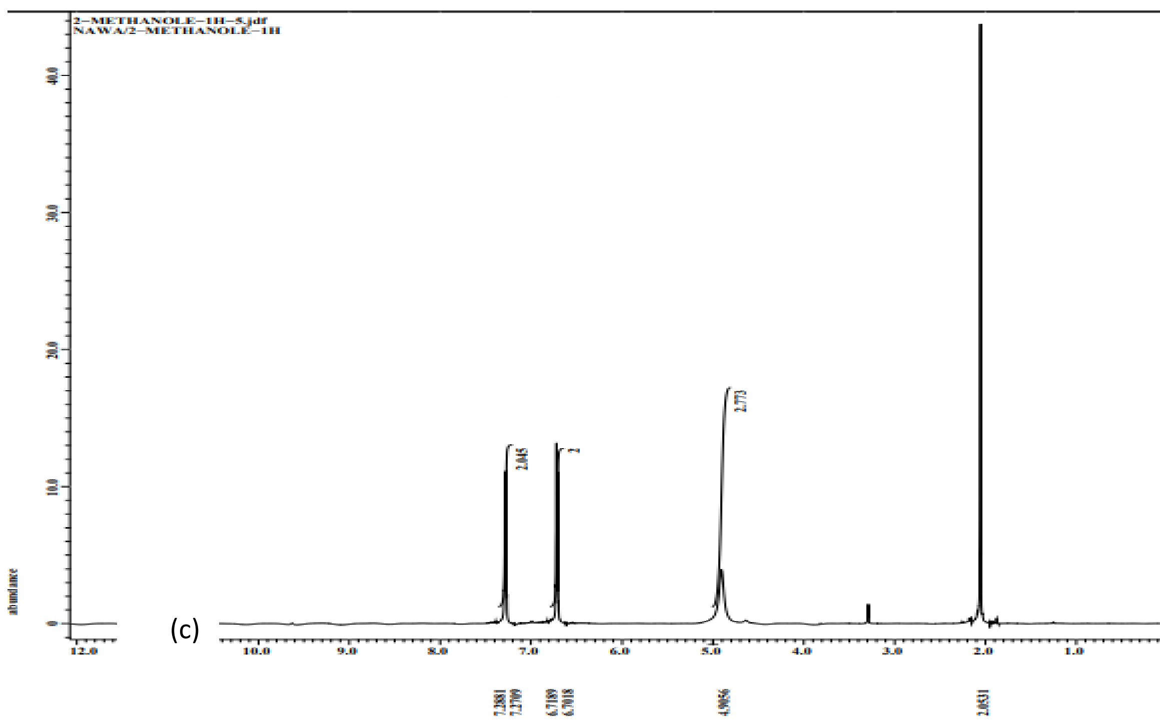


Figure 3: Ethyl acetate extraction the best solvent comparing to chloroform, sum. of ethyl acetate and chloroform.


Absorbance O.D.
Wavelength n.m.
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
0
100
200
300
400
500
600
(a)


%Transmittance
Wavenumber
3566.408
3366.720
3237.725
3165.625
3124.206
2856.891
2588.463
1920.658
1670.736
1606.828
1583.673
1552.778
1506.364
1463.795
1406.748
1362.021
1257.337
1186.935
1151.822
1116.326
1077.314
1024.769
963.502
933.704
897.460
851.211
796.911
758.778
717.660
(b)


2--METHANOLE--1H--5.jdf
NAWA/2--METHANOLE--1H
abundance
2.045
2
2.773
7.2881
7.2709
6.7189
6.7018
4.9056
2.0531
(c)

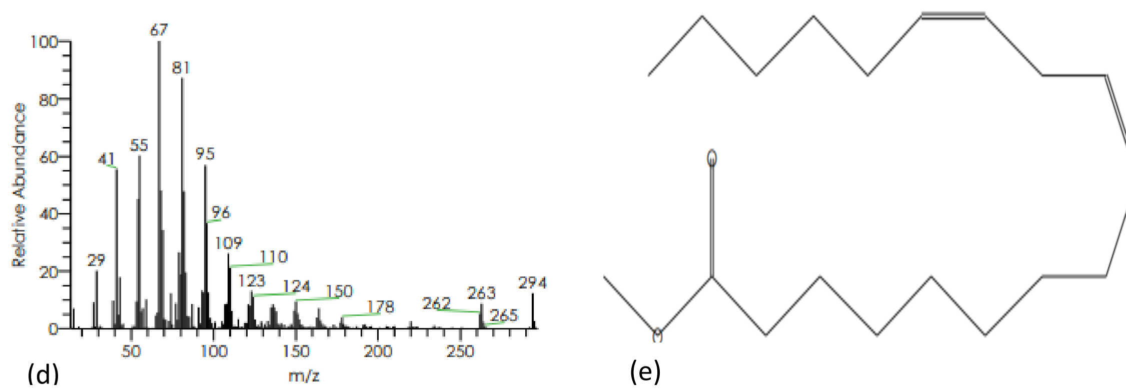


Figure 4: The spectroscopic characteristics of F9**, (a)** UV Spectrum, **(b)** The infrared spectrum (IR), **(c)** [1] H NMR, **(d)** GC-MS analysis, **(e)** Chemical structure of 9,12-Octadecadienoic acid (Z, Z)-, methyl ester with the chemical formula $C_{19}H_{34}O_2$ and a molecular weight of 294.

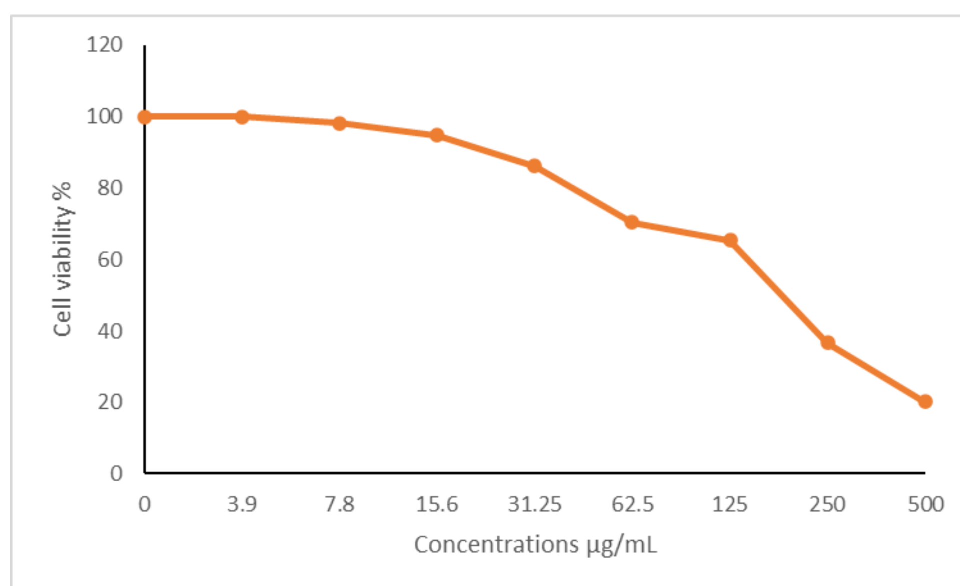


Figure 5: Cytotoxicity assay of 9,12-Octadecadienoic acid (Z, Z)-, methyl ester on the cell line.

استكشاف التنوع الميكروبي لتربة أسوان: عزل وتوصيف مركب قوي مضاد للميكروبات من *streptomyces thermolilacinus* AZHD22

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الملخص العربي

يمكن أن يؤدي اختيار البيئات الطبيعية، مثل التربة ذات المناخ المتنوع، إلى زيادة فرص اكتشاف العزلات الفريدة والمركبات الجديدة المحتملة. تم فحص منطقة أسوان في مصر، المعروفة بتنوعها الجغرافي والمناخي، بحثًا عن عزلات actinomycete. أظهرت إحدى العزلات الخاصة، المعينة باسم Streptomyces thermolilacinus AZHD22، إنتاج مواد نشطة بيولوجيًا ذات خصائص مضادة للميكروبات و كانت أهداف هذا العمل هي اكتشاف عزل أكتينوميسيت ، ووصف الجزيء النشط بيولوجيًا، وتحسين ظروف النمو لتخليق مضادات الميكروبات. لوحظت أفضل الظروف لإنتاج مضادات الميكروبات في مرق النشا والنترات المعقم، الذي تم احتضانه عند 45 درجة مئوية مع درجة حموضة معدلة تبلغ 8 و 1٪ تركيز NaCl. تم تحديد الجلوكوز بتركيز 1٪ كمصدر كربون مثالي، بينما قدم الببتون بتركيز 0.2٪ أفضل مصدر للنيتروجين. تمت إضافة مكونات إضافية، مثل 2.5 جم/لتر $K_2HPO_4 \cdot 3H_2O$ و 0.5 جم/لتر $MgSO_4$، إلى وسائط المرق، تليها وضعها عند حضان هزاز عند 150 دورة في الدقيقة لمدة 9 أيام. تم استخراج المواد ذات التأثير البيولوجي باستخدام أسيتات الإيثيل كمذيب، وكشفت التنقية أن الجزء 9 أظهر نشاطًا قويًا مضادًا للميكروبات ضد الكائنات الدقيقة المختارة. من خلال التوصيفات بما في ذلك تحليل طيف الأشعة فوق البنفسجية والأشعة فوق البنفسجية و ^{1}HNMR و GC-Mass، تم تحديد المركب النشط بيولوجيًا على أنه 9,12-Octadecadienoic acid (Z, Z)-, methyl ester مع الصيغة الكيميائية $C_{19}H_{34}O_2$. أظهر المركب تأثيرات غير مميتة على خلايا فيرو تصل إلى تركيز 250 µ جم/مل.

الكلمات الاسترشادية: *Streptomyces thermolilacinus*، 9,12-Octadecadienoic acid (Z, Z)-, methyl ester ، نشاط مضاد للميكروبات