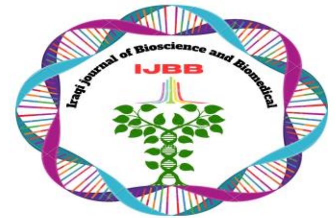

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Streptomyces actinomycinicus CMU-RKDM30 Bacterial Active Compounds Revealed by High-Performance Liquid Chromatography

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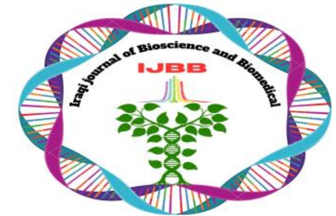
Abstract

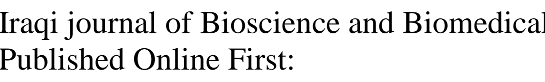
Streptomyces actinomycinicus is a bacterium species from the genus of *Streptomyces* which has been isolated from soil. *Streptomyces actinomycinicus* has the ability to degrade pol(3-hydroxyalkanoate). This species produces Actinomycin, exfoliamycin and many secondary metabolite. The 52 isolated obtained from the isolation processes were sub-cultured on yeast extract-malt extract agar. The results obtained of our isolation *Streptomyces actinomycinicus* CMU-RKDM30 which detected by PCR based on the study of the 16S rRNA gene sequence and phylogenetic relationship. The results of biochemical tests revealed the amylase, urease, catalase, gelatinase, protease, cellulose, phosphatase tests were positive, while the indole production and soluble pigment tests were negative. The HPLC of extracellular crude extract of our isolation *Streptomyces actinomycinicus* showed three different antibiotics (Azithromycin, Amoxicillin, and actinomycin).

Keywords: *Streptomyces,* actinomycin, *actinomycinicus,*16S rRNA, HPLC.

Introduction

Actinomycetes are part of the phylum Actinobacteria, subclass Actinobacteridae, and order Actinomycetes. It is estimated that over 70% of naturally derived antibiotics currently in clinical use are sourced from soil actinomycetes. Some well-known examples of antibiotics produced by actinomycetes include streptomycin, erythromycin, vancomycin and tetracycline. This order is further divided into 14 suborders, 49 families, and over 140 genera. Actinomycetes are widely distributed in various environments, including soil, water, and even in some extreme conditions. They play a crucial role in decomposing organic matter and contributing to the nutrient cycling in ecosystems [1].These antibiotics have been crucial in treating bacterial infections and have played a significant role in advancing medical practices[2]. The ability of





actinomycetes to produce such a wide array of secondary metabolites is thought to be linked to their complex life cycle and their role in competing with other microorganisms in their ecological niche. Research on actinomycetes and their secondary metabolites is focused on discovering therapeutic compounds and addressing antibiotic resistance in pathogenic bacteria[3]. *Streptomyces actinomycinicus* is a bacterium species from the genus of *Streptomyces* which has been isolated from soil. *Streptomyces actinomycinicus* has the ability to degrade poly (3-hydroxyalkanoate). This species produces Actinomycin, exfoliamycin and many secondary metabolite [4]. *Streptomyces* are widely distributed bacteria found on land, known for their strong metabolic powers and adaptability. These bacteria can use new growth and dispersion characteristics as they compete for environmental niches. In addition, they utilize their varied metabolic abilities for a range of purposes, such as optimizing food absorption, impeding phage replication, and suppressing bacterial and fungal proliferation [5]. They are increasingly discovered to exist in symbiosis with plants and insects, frequently providing protective advantages to their host by producing antimicrobial chemicals that inhibit pathogens [6]. One form of liquid chromatography that has proven useful in the separation and quantification of dissolved compounds is high performance liquid chromatography, or HPLC. One way to find out how much of a certain chemical is in a solution is to utilize HPCL. The various solutes in the sample solution interact with the stationary phase in high-performance liquid chromatography (HPLC) and other methods that involve contacting the sample solution with a second solid or liquid phase. As a result of their unique interactions with the column, various components of the sample can be effectively separated into [6]. Various compounds have various retention durations, Rt, because their motilities cause them to escape the column at different times. The objective of this study is to isolate, identify, and analyze the active compounds produced by Streptomyces actinomycinicus CMU-RKDM30, a bacterium isolated from soil. Through a combination of molecular techniques, including PCR-based identification of the 16S rRNA gene, and biochemical testing, the study aims to confirm the strain's identity and its ability to produce bioactive metabolites. Using High-Performance Liquid Chromatography (HPLC), the extracellular crude extract from this strain was analyzed, with the goal of detecting and quantifying the presence of important antibiotics. By comparing the chromatographic data to known standards, the study seeks to reveal the specific antibiotic compounds produced by the isolate, thereby contributing to the growing body of research on the therapeutic potential of Streptomyces species. This investigation has the broader goal of exploring new sources of bioactive compounds, particularly antibiotics, that could address the rising issue of antibiotic resistance.

Materials and Methods

One gram of dried and treated soil samples was used to make suspension by adding it in 99 ml of sterile distilled water (stock suspension) and they were shacked in a shaker at 160 rpm for 30 minutes at room temperature [7]. Serial dilutions from 10^1 to 10^5 were made from the stock suspension and left for 10 minutes. After shaking, 0.1 ml of each dilution were culture on Yeast Extract and Malt Extract (YEME) with Streptomycin 50 ug/ml, then spread by sterile swab for making uniform distribution of the suspension on the surface of the media. The inoculated plates were incubated at 28°C for 7 to 10 days. For the purpose of making a suspension, utilized one gram of treated and dried soil samples [8]. This was mixed with 99 ml of sterile distilled water to create a stock suspension. The mixture was then shaken at 160 rpm for 30 minutes at room temperature. The stock suspension was diluted in a series of steps from 10^1 to 10^5 and then left to sit for 10 minutes. Following shaking, 0.1 ml of each dilution was cultured on Yeast Extract and Malt Extract (YEME) with 50 ug/ml of streptomycin. The suspension was then evenly distributed over the surface of the media using a sterile swab. For 7 to 10 days, the inoculation plates were kept at 37°C for incubation. Based on cultural characteristics, suspected colonies of *Streptomyces* were selected which are characterized as

small, white, pin-point, rough, chalky and a clear zone of inhibition around them, these colonies was confirmed their identification by (types of Gram's stain, aerial and substrate mycelium color, pigment production and pigment color).These colonies were transferred from the mixed culture into separate agar plates and incubated at 28±1°C for 7 days. Streptomyces were re-streaked on the International Streptomyces Project (ISP2) to obtain pure colonies for identification. Cultural characteristics confirmed the identification of small, white, pinpoint, rough, chalky Streptomyces colonies. These colonies were cultured at 28±1°C for 7 days[10].

Standard Preparation

To get a concentration of 200 parts per million (ppm), 10 milligrams of standards were dissolved in 50 milliliters of high-performance liquid chromatography (HPLC) grade methanol. This concentration was then further diluted by dissolving 1 milliliter of the solution in 50 milliliters of methanol [11].

Sample Preparation

Twenty ml of the sample were diluted in 50 ml of HPLC-grade methanol (99%). A 1ml of the solution was added to 50ml of 99% HPLC grade methanol to further dilute it.[12]. Analyzing the extracellular extract of Streptomyces using High-Performance Liquid Chromatography (HPLC). 20 ml of the standard and 20ml of the sample were injected into the HPLC machine to get a chromatogram. The content of the sample was then estimated by comparing it to the standard. The concentrations were determined using the following equation: The formula for concentration is given by multiplying the ratio of the area of the sample to the area of the standard by the sample concentration and the dilution factor.

The equation for determining the concentration of the sample based on the described method can be written as follows:

Concentration of sample = (Area of sample / Area of standard) x Concentration of standard x Dilution factor

Where:

- Area of sample / Area of standard: This is the ratio of the chromatogram peak areas.
- Concentration of standard: This is the known concentration of the standard used in the experiment.
- Dilution factor: The factor accounting for the sample dilution, calculated as:
  Dilution factor = (50/20) x (50/1)

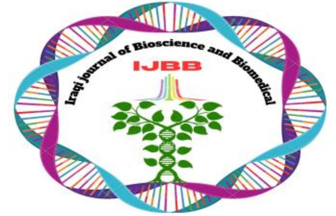
Thus, the final concentration of the sample is:

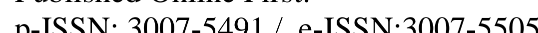
Concentration of sample = (Area of sample / Area of standard) x Concentration of standard x (50/20 x 50/1).

The table 1 shows format to present the circumstances of the deportation and the device used.

Table 1: The circumstances of the deportation and the device used in this study

Item	Description
Device Used	PrimeQ Real-Time PCR System
Deportation Circumstances	Sample preparation and analysis of extracellular extracts from *Streptomyces actinomycinicus* CMU-RKDM30
Purpose of Deportation	Identification and quantification of antibiotic compounds (e.g., Azithromycin, Amoxicillin, Actinomycin) in extracellular extract
Process	1. Sample isolation and preparation using ISP2 medium. 2. Dilution and injection into HPLC machine.
Analysis Method	High-Performance Liquid Chromatography (HPLC)
Results Measurement	Comparison of chromatogram peak areas between the sample and standard
Outcome	Identification of active antibiotic compounds based on retention times and peak areas





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Primers Used in this Study

The following primers were used for detecting *Streptomyces actinomycinicus* 16S ribosomal RNA gene for strain CMU-RKDM30:

- **Forward primer (5'---3')**: AGCGTTGTCCGGAATTATTG
- **Reverse primer (5'---3')**: TCCAGACGTTTCCGGTGTAT

Electrophoresis

Electrophoresis was used to confirm the isolation of genomic DNA and validate PCR results. After PCR amplification, the DNA bands were visualized under UV light and photographed following staining with ethidium bromide. The amplified DNA bands confirmed the presence of the target gene for the detection of *Streptomyces actinomycinicus* gene for 16S ribosomal RNA. in the isolated samples.

Results and Discussion

The results of our isolation of *Streptomyces actinomycinicus* CMU-RKDM30 which detected by PCR based on the study of the 16S rRNA gene sequence and phylogenetic relationship were consistent with other studies findings [13,14], regarding the similar habitats in which *Streptomyces* diversity was observed. The results observed a few different colony types, and the isolation process typically yielded plates with one or a few colony types, ranging from 2-4 colonies as shown in the (figure 1).

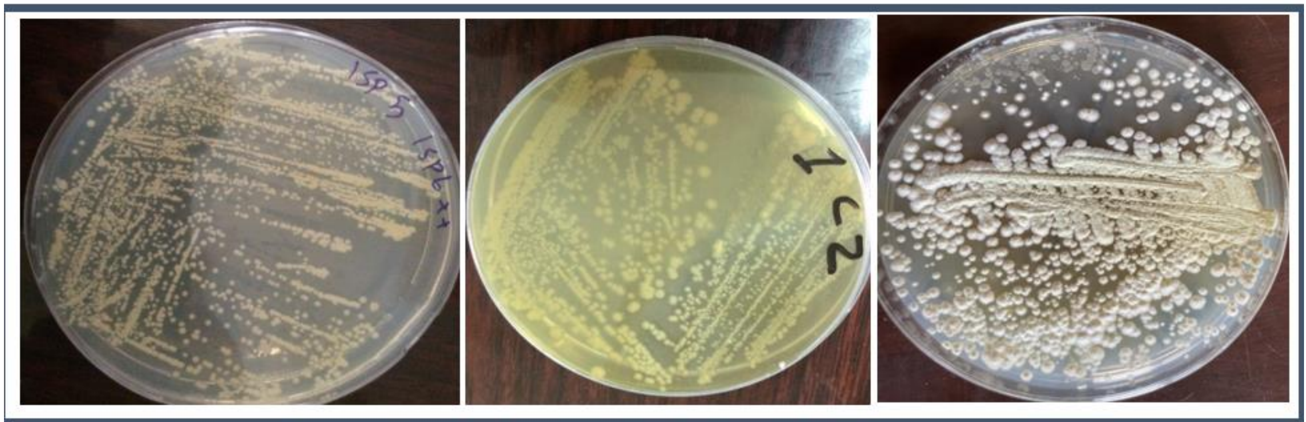
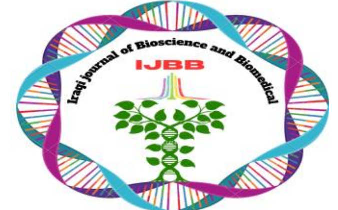


Figure 1. Isolation of *Streptomyces actinomycinicus,* ISP2 (International Streptomyces Project Medium 2) or Starch Casein Agar. The culture was incubated at 28°C for 7-14 days.

Biochemical and physiological characteristics

The *Streptomyces* spp. biochemical and physiological tests are listed in table (2) Amylase, urease, catalase, protease, cellulase, and phosphatase were among the extracellular enzymes produced by the *Streptomyces*. The utilization of citrate was positive, there was no formation of HCN and volatile organic compounds toxicity. Several researchers found consistent results when they studied the *Streptomyces* strains for nutritional uptake, physiological and biochemical characteristics [15]. *Streptomyces actinomycinicus* identification is a laborious procedure. Spore shape, mycelium service, carbon utilization and culture on international *Streptomyces* project (ISP) were the primary criteria for the *Streptomyces* taxonomy. When it comes to classifying members of the Streptomycetaceae family, morphological and biochemical traits are crucial [16].

Table2: Results of biochemical analysis of *Streptomyces* spp.



Reaction	Response	Result
1- Urease	Red to deep pink	Positive
2- Catalase	Bubbles	Positive
3- Amylase	Clear zone	Positive
4- Protease	Clear zone	Positive
5- Gelatinase	Narrow zone	Positive
6- Cellulase	Clear zone	Positive
7- Phosphatase	Clear zone	Positive
8- Indole production	No color zone	Negative
9- Citrate Utilization	Deep blue color	Positive
10- Soluble pigment	No brown	Negative

The results in the table above show that the amylase, urease, catalase, gelatinase, protease, cellulose, phosphatase tests were positive, while the indole production and soluble pigment tests were negative these results are consistent when they studied the *Streptomyces* strains for nutritional uptake, physiological and biochemical characteristics. Isolation and purification of the
extracellular crude extract from the isolated strain the extracellular crude extract was partially purified using High Performance Liquid Chromatography (HPLC) to isolate and identify the bioactive components. This was done by performing bioautographic in the presence of a standard antibiotic[19].

Molecular identification of *Streptomyces*

The study confirmed the isolation of genomic DNAs from Streptomyces isolates through electrophoresis and PCR. 12 isolates were positive, and a single 480 bp band was observed in all tested isolates. The 16S rRNA gene sequence of the T2 strain was identified. The DNA extraction was effective and confirmed through electrophoresis analysis.

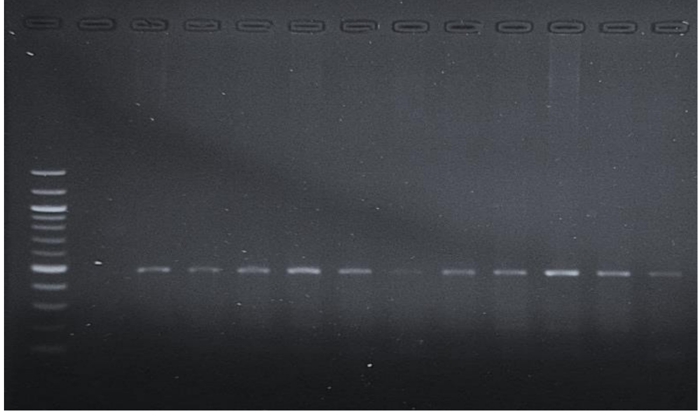
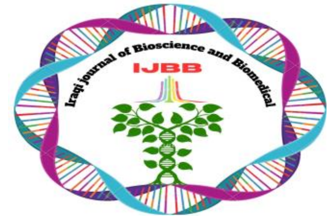


Figure 2: Agarose gel electrophoresis of PCR products detection and isolation *Streptomyces actinomycinicus* gene for 16S ribosomal RNA

Purification by High Performance Liquid Chromatography (HPLC)

The HPLC analysis was done to detect the concentration of important active compounds present in *Streptomyces*. HPLC analysis of extracellular extract of *Streptomyces* indicated the presence of three active



compounds. The figure (2) revealed different peaks of antibiotic present in extracellular extracts of *Streptomyces* in same retention time in compare with a stander but with different area.

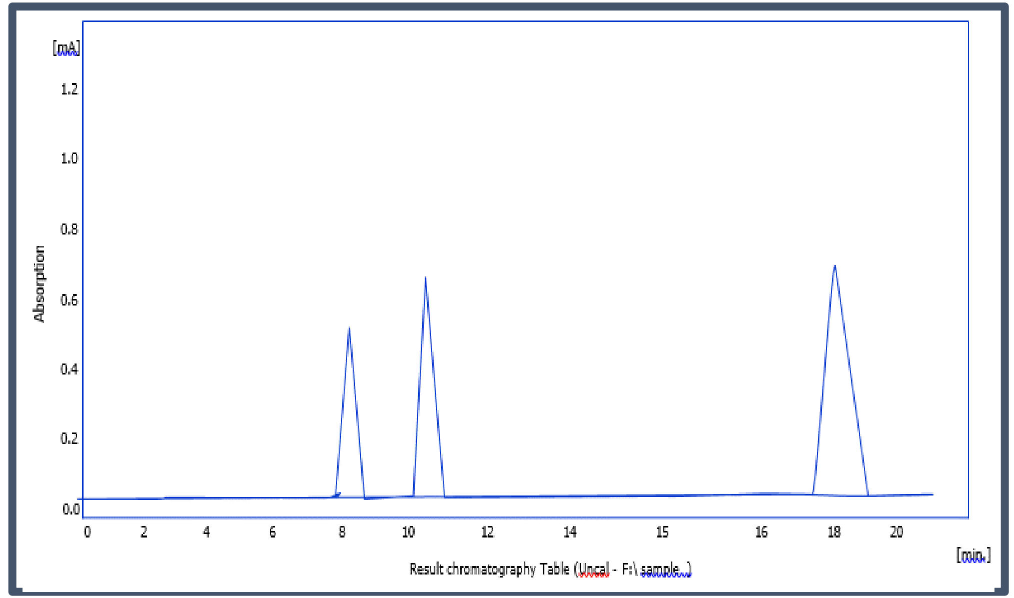


Figure 2: High Performance Liquid Chromatography of standard antibiotic

The HPLC of extracellular crude extract showed three different antibiotics (Azithromycin, Amoxicillin, actinomycin). As presented in figure (3) revealed that the same retention time of the sample in compare with a stander but with different area. The results in table (2) presents the retention times (Rf values), areas, and concentrations of various standard antibiotics identified in *Streptomyces* spp[18]. The table lists three antibiotics: Azithromycin, Amoxicillin, and Actinomycin. Azithromycin, detected at 7.80 minutes, accounts for 30% of the area and height. Amoxicillin, with a longer retention time of 10.75 minutes, contributes to 45% of the total area and height, indicating its significant presence in the sample. Additionally, Actinomycin, detected at 17.21 minutes, shows the highest individual contribution with 65.12% of the total area and 51% of the height, highlighting its dominant concentration within the *Streptomyces* spp. The cumulative area and height percentages across these compounds amount to 100%, reflecting the total analytical output of the chromatographic analysis [17].

Table (2) show the R_f values for each standard antibiotic and area also the concentration of each antibiotic found in *Streptomyces spp*.

N	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	7.80	526.07	600.98	30.00	30.00	0.15	Azithromycin
2	10.75	380.90	800.11	45.00	45.00	0.20	Amoxicillin
3	17.21	509.81	854.12	65.12	51.00	0.26	Actinomycin

The chromatogram in figure (3) shows four distinct peaks at retention times of 7.80, 10.75, and 17.21 minutes. Each peak corresponds to a different compound separated by the chromatographic method. The first peak at 7.80 minutes is slightly larger, corresponding to Azithromycin. The second peak at 10.75 minutes is prominent and sharp, indicating a significant presence of Amoxicillin. Finally, the largest peak occurs at 17.21 minutes, which likely corresponds to Actinomycin, the most abundant compound in this mixture.

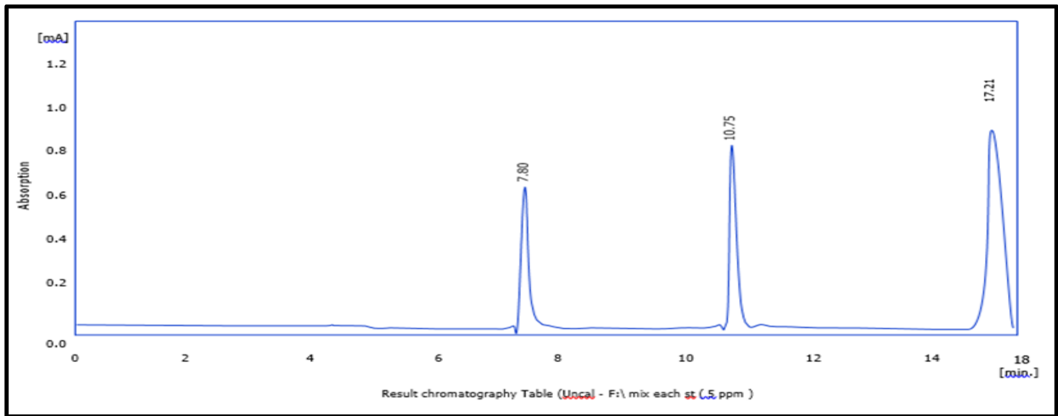


Figure (3) High Performance Liquid Chromatography for extracellular extract of *Streptomyces*.

A study described that thin high performance liquid chromatography are regularly used for analysis and characterization of antimicrobial compounds from producing microorganisms [17].

Conclusions

Actinomycetes were observed in culturing the diluted soil sample (10^{-5}) for 7-10 days on ISP2 agar. The results of our isolation of *Streptomyces actinomycinicus* HPLC analysis of extracellular extract of *Streptomyces* indicated the presence of three active compounds

Author's Declaration

- We hereby confirm that all the Figures and Tables in the manuscript are original and have been created by us.
- We have obtained ethical clearance for our study from the local ethical committee at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.
- Ethical Clearance: The project was approved by the local ethical committee at [Al-Nahrain University/College of Biotechnology], ensuring adherence to ethical standards and the protection of participants' rights and welfare.

Author's Contribution Statement

Bassam Qasim Mohammed: Contributed to the conception and design of the study, conducted some experiments, data rearrangement and drafted the initial manuscript.

Mohsen Hashim Risan: conducted some experiments, collection a part of literature review and conducted some characteristics of the products.

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