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Molecular identification and antimicrobial activity of Streptomyces spp. isolated from soil in Garmian Region, Iraq

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ABSTRACT

Multidrug-resistant pathogens present a significant obstacle in the prevention and treatment of infectious diseases, making the discovery of new antimicrobial agents from diverse sources essential. Actinobacteria, especially *Streptomyces* genus, are well-known for their prolific production of secondary metabolites with antimicrobial capabilities. The geographical niches of Iraq are one of the least explored ones for Actinobacteria strains. Hence, this study aimed to isolate Actinobacteria species from the soil in the Garmian region of Iraq and evaluate their potential antimicrobial activity

A total of forty soil samples were collected and screened using various standard culture media to facilitate isolation, morphological identification, and evaluation of antimicrobial activity. Molecular identification and species confirmation were performed through amplification of the 16S rRNA gene via PCR, followed by sequence comparison with the NCBI database. The obtained two distinct strains of *Streptomyces* were identified, with one demonstrating significant antimicrobial activity against several pathogens, namely, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Candida albicans* ATCC 10231.

Both isolated strains were registered in NCBI under accession numbers OR564031 and OR564032, corresponding to isolates 26 and 29, respectively. Their sequences exhibited high similarity to *Streptomyces rochei* strain SCOS (Accession No. KX575853.1) with 99.04% identity, and *Streptomyces* sp. WAC06273 strain (Accession No. CP042278.1) with 98.81% respectively. These *Streptomyces* strains from Iraqi soils show promise as potential candidates for novel antibiotic discovery.

Further exploration of *Streptomyces* in soils from Garmian region, along with identification and purification of the active metabolites identified in this study, is strongly recommended.

1.Introduction

Multidrug-resistant pathogenic micro-organisms are continually emerging, the need for antimicrobial molecules growing, to fight against these pathogens (Khadayat *et al.,* 2020). This makes different researchers continuously working to find new sources for antimicrobials. Actinobacteria, especially the *Streptomyces* genus is considered one of the favorable sources for antimicrobial production (Bérdy, 2012) which is the most widely distributed microorganisms in the nature, mostly found in the soil (Sayed *et al.,* 2020).

Streptomyces which is the largest genera of Actinobacteria, are aerobic, Gram positive, filamentous bacteria (Chater, 2016; Nikolaidis *et al.,* 2023) with high ratio of GC content in their genome (Ventura *et al.,* 2007). Previously different biochemical and physiological features were employed to identify *Streptomyces*; however, recently molecular characteristics and phylogenetic analysis is used in *Streptomyces* identification and classification (Komaki, 2023). *Streptomyces* demonstrate remarkable adaptability to various environments, with soil being their preferred habitat, where they make up a significant portion of the bacterial population (Sayed *et al.,* 2020).

The *Streptomyces* is well-known for producing a wide range of bioactive compounds, including antibiotics, anticancer agents, antifungals, immunosuppressants, and more (Quinn *et al.,* 2020), which make it a preferred genus for biotechnological applications and a valuable resource for pharmaceutical companies (Bérdy, 2012).

Appearing highly resistant microbial pathogens further underscores the significance of *Streptomyces*, which have the potential to produce novel antimicrobials. previous investigations have led to the discovery of antibiotics like tetracycline, vancomycin, and erythromycin (Quinn *et al.,* 2020). Moreover, genome sequencing of *Streptomyces* species has has provided valuable insights into their genetic diversity and their capacity to produce new active compounds (Harrison and Studholme, 2014). The metabolic versatility and extensive genetic diversity of *Streptomyces* make them a compelling subject for biotechnological research.

Despite its geographical diversity, Iraq's ecosystems remain among the least explored in terms of microbiological diversity, largely due to political and economic instability. There are limited numbers of studies performed on the isolation and identification of *Streptomyces* in different regions of Iraq (Al-Saadi and Mohammad Jaralla 2013; Al-Rubaye, 2016; Qadir *et al.,* 2020; Fawzi *et al.,* 2021; Jassim and Jarallah, 2022)

Additionally, extreme ecosystems screening is one of the interesting approaches for the discovery of new *Streptomyces* strains with novel bioactive compounds (Singh *et al.,* 2014). Accordingly, this study aimed to isolate and identify of *Streptomyces* from soil samples in the Garmian region, Iraqi Kurdistan region, and to screen these isolates for potential antimicrobial compounds.

Garmian region, located at southeast part of the Kurdistan region of Iraq which is a hot and dry area, between the latitudes (34°15–33 = - 35° 11–05 =) above the equator and the longitudes (44° 29–41 = - 45° 54–20 =) of the eastern hemisphere (Al-Bajalan *et al.,* 2018).

2. Materials and methods

2.1. Sample collection and preparation

The soil samples were collected from forty different non-framing areas of the Garmian region from about the depth of 10 to 15 cm. The samples were collected using an open-end soil borer 25 cm wide and 10 cm deep, the collected samples were kept in clean zip bags and transferred to the University of Garmian laboratories. The soil samples were air-dried for 72 hours and stored in clean jars for subsequent analysis. All samples were cultured over a period of two to three weeks, with the work conducted from October 2022 to July 2023.

2.2. Isolation of Streptomyces strains

According to (Risan *et al.,* 2016; Zhao *et al.,* 2019), one gram of each air-dried soil sample was suspended in 9 ml of sterilized distilled water and standard serial dilution till 10^{-5} dilution were prepared. One milliliter from each diluted sample was spread on the International *Streptomyces* Project (Yeast malt extract media; ISP2) and Starch Casein agar medium which previously

supplemented with (streptomycin and Nystatin (30 mg/L). The inoculated plates with soil were incubated at 28°C for 14 days, the characteristics of growing colonies including colony size, shape, surface, edge, elevation, consistency, pigment production were considered, moreover Gram stain was applied. Finally, the suspected colonies were transported and purified to new medium of (ISP2) and stored at 4°C for further study.

2.3. Fermentation and Extraction of Secondary Metabolites

The isolated strains were used for evaluation of antimicrobial activities by carrying out fermentation and extracting their crude bioactive metabolites. The stock solution of bacterial suspension was performed by transferring a loopful of growing purified colonies (4 days age) to 10 ml of ISP2 broth for making stock solution of bacterial inoculum which incubated at 28 °C for 4 days, 1 ml of these stock suspensions were used for inoculating 250 ml (ISP2 broth) in Erlenmeyer flasks (500 ml flask), and incubated in a rotary shaker incubator with (150 rpm, at 28°C for 7 days). Crude extract (cell free supernatant) was obtained by centrifugation of fermented broth at 10000 rpm for 10 minutes, the collected supernatants were used for screening and evaluating antimicrobial effects on pathogenic microorganisms including (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231) which obtained from Medya Diagnostic Center, Erbil, Iraqi Kurdistan Region (Khattab *et al.,* 2016).

2.4. Evaluation of antimicrobial activities

Antimicrobial activities against different microbial pathogens were carried out by agar well diffusion technique. The pathogens were (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231) which grown on nutrient broth for 6 hours at 37°C, the growth and the turbidity was measured and adjusted to the McFarland standard of 0.5.

One hundred Microliters of the microbial pathogen's broth was cultivated on Mueller-Hinton agar (HiMedia, India) and spread over the plates by swab sticks. Six-millimetre diameter wells were created by sterile glass Pasteur pipette, 80 µl of crude extract metabolites (cell free supernatants) were added to the wells. The plates were left at room temperature for 1hr to let the crude extracted metabolites to diffuse. All plates were incubated at 37 °C for 24 hrs, after incubation period the plates were examined, the diameter of the inhibition zone around each well was measured by the means of metric rulers. All antimicrobial activity tests against selected pathogens were done in triplicate (Ibnouf *et al.,* 2022).

2.5. Molecular identification

2.5.1. DNA extraction

A Gram-positive bacterial DNA extraction kit (*Easy Pure* Bacteria Genomic DNA Kit, from Transgenbiotech, China) was used to extract genomic DNA from the isolated Actinomycetes species. Lysis buffer was added to pellet from centrifugation of 2 ml of 96 hrs old bacterial broth culture. The mixture was vortexed thoroughly and manufacturers recommendations were followed, and the extracted DNA was kept at -80°C for later analysis.

2.5.2. PCR reactions

Extracted DNA from *Streptomyces* species was used to amplify the ribosomal RNA gene employing a universal pair of primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) (Chun and Goodfellow, 1995). All amplification reactions were performed in a final volume of 20 µl. The reaction mixture contained 5 µl of genomic DNA mixed with 14 µl of PCR master-mix (Addbio, South Korea) and 0.5 µl (10 µM) of each primer. The PCR reactions were performed in Eppendorf Nexus thermal cycler (Nexus, Eppendorf AG, Germany). The reaction program was as follows: Initial denaturation for 5 minutes at 95°C, then 35 cycles of denaturation at 95°C for 20 seconds, annealing for 30 seconds at 58°C, extension at 72°C for 2 minutes, and final extension for 7 minutes at 72°C. The PCR products were run on 1.5% Agarose gel and gel images were captured using the Gensys gel documentation system (Synoptics Ltd., Surrey, United Kingdom).

2.5.3. DNA sequencings and analysis

The amplified DNA samples were sequenced

by Sanger sequencing (Macrogen Co., Seoul, South Korea) and the sequences were revealed and analyzed using Finchtv software (Geospiza, Inc.; Seattle, WA, USA). The sequences were submitted to NCBI using Bankit tool (Benson *et al.,* 2017). The homology search was achieved by comparing the obtained sequences with the NCBI library employing the standard basic local alignment search tool (BLAST) program. The closest related species and the ratio of similarities were determined.

3. Results

3.1. Isolation of streptomyces strains

After two weeks of incubation, small white colonies among other growing bacteria were appeared on 2 plates from the samples (26 and 29), according to morphological criteria, the isolate no. (26) exhibited the following characteristics; white aerial mycelium, circular, powdery, reverse colony appears as yellow color, while the isolate no. (29) was with gray aerial mycelium, irregular, rough, reverse colony with light brown. Gram's stain was performed for both isolates and both were positive. These results facilitate preliminary identification as Actinomycetes species considering the International *Streptomyces* Project manual (ISP). The suspected colonies were sub-cultured twice on Yeast Malt Extract Agar (ISP2) for acquiring purified culture, which covered the entire plate after seven days incubation (Figure 1).

Figure 1: Growth of *Streptomyces sp.* on yeast malt extract agar. Pure culture colony Panel A: isolate number 26, Panel B: isolate number 29.

3.2. Antimicrobial activity assessment

The purified isolates (26 and 29) were then subjected to evaluating their antimicrobial activities using agar well diffusion against (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231) as presented in (Table 1).

Table 1: Antimicrobial activities of extracellular crude extract from isolated and purified strains of *Streptomyces* sp. against human pathogenic microorganisms

Isolates	Zone of inhibitions (mm)		
	E. coli ATCC 25922 (Mean ∓SD)	*S. aureus* ATCC 25923 (Mean ∓SD)	*C. albicans* ATCC 10231 (Mean ∓SD)
26	23.3∓1.2	32.3∓2.5	17.3∓1.2
29	0.0	0.0	0.0

3.3. Molecular identification

The PCR products were subjected to electrophoresis on a 1.5% agarose gel stained with ethidium bromide. DNA bands approximately 1500 bp in size were visible on the gel, confirming successful amplification of the 16S ribosomal RNA gene (Figure 2). A fraction of the PCR product from each sample was kept for DNA sequencing.

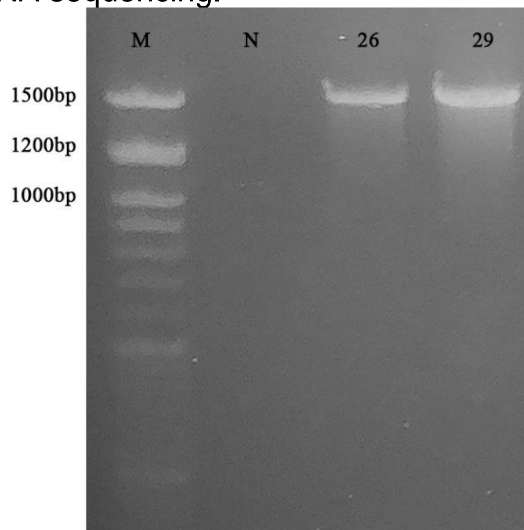

Figure 2: Agarose gel (1.5%) image, loaded with M; Molecular marker, N; Negative control, PCR products of isolates (26) and (29). The molecular weight of PCR products is 1500bp.

3.4. Sequence analysis

The DNA sequences were submitted to NCBI

under accession numbers, OR564031 and OR564032 for sample numbers 26 and 29, respectively. The sequences of two isolates were blasted using NCBI online library and the results of matching of each sequence are demonstrated in (Table 2). Sample number 26 showed (99.04%) similarity to *Streptomyces rochei* strain SCOS, while sample 29 showed (98.81%) similarity to *Streptomyces sp.* WAC06273 strain.

Table 2: Molecular diagnosis of isolated strains including the best matched species comparing to NCBI library and their percentages and accession numbers.

Isolate No.	Our isolates accession No.	Best matched species in NCBI	Similarity %	NCBI Accession No.
26	OR564031	*Streptomyces rochei* strain SCOS	99.04	KX575853.1
29	OR564032	*Streptomyces sp.* WAC06273 strain WAC6273	98.81	CP042278.1

4. Discussion

The emergence of multidrug-resistance microbes indicated the necessity of surveying of unexplored habitats for novel, effective and safe antimicrobials. Each year, thousands of *Actinomycetes* strains are screened by pharmaceutical companies and research laboratories as a source of bio-active compounds (Khandan Dezfully and Gottravalli Ramanayaka, 2015). Secondary metabolites play a crucial role in diverse fields including industrial microbiology and biotechnology. Microorganisms, particularly Actinobacteria, continue to serve as a useful source for picking up secondary metabolites, with the genus *Streptomyces* which is significantly contributed to the discovery of numerous antibiotics. Here, two strains of *Streptomyces* were isolated from Garmian soil samples and have evaluated their antimicrobial activity. One of the isolated strains (26) was exhibited a considerable antimicrobial activity against human microbial pathogen's *Escherichia coli* ATCC 25922, *Staph aureus* ATCC 25923 and *Candida albicans* ATCC 1023, while the second isolated strain (29) failed to manifest any antimicrobial activities against the same human microbial pathogens. Upon molecular identification, the isolate number 26 was closely related to *Streptomyces rochei* strain SCOS, while the isolate number 29 was similar to *Streptomyces sp.* WAC06273 strain comparing to NCBI library.

The rate of success for isolation of Actinobacteria from different sample types could be massively different. The current study has been successful in isolation of two strains from forty soil samples, studies performed in neighboring countries who were successed in isolating 18 isolates from 60 soil samples of which 7 showed antibacterial activity against different ATCC bacterial species, and this is indicative for the relevance of screening soil samples from dry regions for isolation of Actinobacteria with potent antimicrobial compounds (Majidzadeh *et al.,* 2021). Additionally, researchers from Saudi Arabia have also been successed in isolation of 5 *Streptomyces* strains with bioactive compounds from the total 21 isolated strains (Almuhayawi *et al.,* 2021), the current results were agreed with observations recorded by researches in Iraq who tries to isolate *Streptomyces* from soil samples (Al-Ghazali and Omran, 2017; Walaa Isaa and Ahmed Abd, 2020; Mohammad-Amin, 2021; Jassim and Jarallah, 2022)

As microbial pathogens develop resistance to multiple antibiotics, the continuous necessity for effective antimicrobial agents is increasingly critical. Researchers working in this field have achieved diverse results regarding the percentage of isolated *Streptomyces* species with antimicrobial activity, ranging from 4% to 50% (Thakur *et al.,* 2007; Tenebro *et al.,* 2021). In the current study, the antimicrobial activity assessment showed that one of the crude extracts showed potential broad antimicrobial activity, which is agreed with that obtained by other investigators (Sengupta *et al.,* 2015; Abdel-Aziz *et al.,* 2019). However, in Iraq, the number of antimicrobial assessments for isolated *Streptomyces* is limited (Al-Ghazali and Omran, 2017; Walaa Isaa and Ahmed Abd, 2020; Mohammad-Amin, 2021; Jassim and Jarallah, 2022), and comprehensive studies for

determination of the nature of obtained bioactive compounds is rare.

The conventional methods for identification of microorganisms are based on morphological, physiological and biochemical characteristics. However, these methods are classical and necessity for molecular tools is critical and more accurate (Singh *et al.,* 2016). 16S rRNA gene is an effective marker for identification of bacteria and archaea as it is stable, ubiquitous, and conserved subject to horizontal gene transfer (Abdel-Aziz *et al.,* 2019). Molecular identification of the current (26 and 29) isolated strains revealed their similarity to *Streptomyces rochei* strain SCOS and *Streptomyces sp.* WAC06273 strain respectively, with a similarity percent (99.04 and 98.84) respectively, following their alignment with sequences available in NCBI library. Our result for isolate 26 was similar to *Streptomyces rochei* strain CICR7 (Accession No. MW237668.1), which obtained by Habib and Abd Burghal (Habib *et al.,* 2023) in the eastern Hammar marshes in Thi-Qar and Basrah governorates, Iraq. In addition to the limited number of well-documented and scientifically sound research works in this field in Iraq, there is also a lack of research works that used molecular techniques to accurately identify the isolated Actinomycetes. However, the overall outcome of research efforts focused on isolation and molecular identification of Actinomycetes in Iraq, has resulted in publication of relatively small number of strains which were submitted to NCBI (*Streptomyces nigra* strain BA1 accession No. MT239403 and *Streptomyces albogriseolus* strain BA2, accession No. MT239401 by (Jalal and Hasan, 2021), *Streptomyces cellulosae* strain NBRC 13027, accession No. OM978381 by (Mahmood *et al.,* 2022), *Actinoplanes* sp. strain MOSUL, accession No. MN095717.1 and *Amycolatopsis* sp. strain MOSUL, accession No. MN095769.1 by (Salih *et al.,* 2020).

The limitation of this study was the use of only crude extracts to perform the antimicrobial assessments and the inability to purify the crude extract to determine the nature of the bio-active agent which might be a novel antimicrobial compound.

Conclusion

In conclusion, the findings of this study demonstrated that *Streptomyces* are valuable sources of antimicrobial agents. Moreover, the research confirmed the potential of isolating *Streptomyces* strains capable of producing secondary bioactive metabolites from Iraqi soil. We highly recommend further exploration of new actinomycete strains from various habitats in Garmian region, along with screening for their potential to produce novel antimicrobial compounds.

Data Availability

DNA accession numbers are available on GenBank.

Ethical approval

None to be declared.

Conflicts of interest

Authors declare no conflict of interests.

Author contributions

All authors have contributed equally.

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