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RESEARCH ARTICLE OPEN ACCESS

Evaluation of Antimicrobial-producing Actinomycetes Isolated from Regions in Baghdad City

Balqees Yahya Najm*, Sarab Hussein Khallel and Hala Mahmmed Majeed

Basic Science Department, Medicine College, Ibn Sina University of Medical and Pharmaceutical Sciences, Baghdad, Iraq.

Abstract

In this study, we examined 25 actinomycete strains isolated from soil samples collected from different agricultural locations in Baghdad City, Iraq. These isolates were tentatively identified on the basis of their chalky, leathery, waxy, and mucoid colonies. Identifications were confirmed using slide culture techniques and observing the substrate and aerial mycelia and the arrangement of spore chains. Ten of the isolates were established to have antimicrobial-producing activity. To further confirm these isolates as actinomycetes, we performed molecular analyses using 16S rDNA, for which we obtained a characteristic single band of approximately 1500 bp. A selected isolate was studied for its antimicrobial activity against gram-positive and gram-negative bacteria, and we also examined the influence of factors such as carbohydrate sources (glucose, maltose, sucrose, lactose, and starch), and different concentrations of $(NH_4)_2SO_4$ as a nitrogen source, on antimicrobial production.

Keywods: Actinomycetes, Streptomyces, PCR, Extraction, Antibiotic

***Correspondence:** balqees.yahya@ibnsina.edu.iq

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INTRODUCTION

Microbial natural products are at the forefront of the search for bioactive molecules with medicinal potential.[1] Among the diverse range of antimicrobial-producing microorganisms, actinomycetes are the source of more than 80% of all naturally occurring antibiotics currently on the market. Actinomycetes are a group of spore-forming, filamentous, gram-positive, aerobic bacteria with a ubiquitous environmental presence.[2] Their DNA is characterized by a high GC content (>55 mol%), and they are noted for their atypical spore-producing ability and mycelial structure, with the aerial hyphae developing into spore chains.[3] Many microorganisms, particularly actinomycetes, are dependent upon soil to produce a range of bioactive natural resources, including compounds with relevant pharmacological properties.[4] Actinomycetes are noted for the large numbers genes encoding enzymes that contribute to the production of bioactive secondary metabolites,[5] and in this regard, it has been established that strain growth conditions have a considerable influence on secondary metabolite production. Consequently, numerous studies have examined the effects of manipulating nutritional and physiochemical conditions throughout the fermentation process and performed genome sequencing to identify the genes involved in secondary metabolite production.[6] It has been found that the majority of bioactive compounds are synthesized via metabolic pathways comprising molecules that are encoded by genes on neighboring chromosomes (biosynthetic gene clusters). [7]

Multi-drug resistance is currently of considerable concern, the severity of which is predicted to increase in the future, owing to a lack of new antibiotics. This accordingly highlights the necessity to identify novel antimicrobial-producing bacterial and fungal strains. Consequently, extensive research is now being focused on identifying next-generation medicinal compounds, particularly among the actinomycetes. Significant antibiotics produced by actinomycetes include actinomycetin, mycetin, and micromonosporin lysozyme, actinomycin, streptothricin, proactinomycin, and streptomycin, the structural, antibacterial, and cytotoxic properties of which vary considerably.[8] However, in the past two decades, there has been a worrying reduction in the number of new antibiotic compounds being discovered.[9, 10] In this regard, it is anticipated that surveying alternative sites and environments for microbe isolation, could potentially yield novel sources of antibiotics,[11] and thus research is currently focused on identifying next-generation pharmaceutical compounds from previously untapped sources. Some of the most important antibiotic compounds, including actinomycetin, mycetin, and micromonosporin, of actinomycete origin are characterized by an extensive variation in structure and antibacterial and toxic properties.[8] The largest actinomycete genus found most frequently in terrestrial habitats is *Streptomyces*, which includes the species Streptomyces kanamyceticus, Streptomyces fradiae, *Streptomyces griseus, Streptomyces antibioticus, Streptomyces venezuelae, Streptomyces lincolnensis, Streptomyces roseosporus,* and *Actinoplanes teichomyceticus*, which are well known for producing a range of extracellular enzymes and bioactive secondary metabolites with a broad range of structural and functional diversity that are used as pesticides and herbicides and also as antibacterial, antiprotozoal, antifungal, antiviral, antihelminthic, anticholesterol, anticancer, and immunosuppressant agents.[12] Given these beneficial properties, the bioactive secondary metabolites produced by *Streptomyces* have attracted considerable attention, particularly from the perspective human health-related applications.[13] Thus, given the importance of discovering novel secondary metabolites as a source for the development of new drugs,[14] we sought in the present study to isolate actinomyces from a previously unsurveyed area and characterize their secondary metabolites profiles with the objective of identifying novel compounds with antibacterial activity against pathogenic bacteria.

MATERIALS AND METHODS

Sample collection

Over a 1-month period during the spring of 2022, we collected 200 soil samples from different area in agricultural regions in Baghdad City, Iraq, from depths of between 6 and 12 cm.

These sample were dried, placed in sterile plastic zipper bags, and maintained in a refrigerator at 5°C until used for analysis.

Isolation of actinomycetes

Soil samples were resuspended in distilled water to give a dilation series, to which 1% sodium dodecyl sulfate (SDS) was added to disrupt the mycelia. Actinomycetes were cultured on nutrient medium containing nistatin as an antifungal agent, and L50 of each dilution was spread on enrichment media plates and incubated at 31°C for 7-13 days.[15]

Morphological identification of actinomycetes

Actinomycete strains were identified morphologically using traditional microscopy procedures at ×10 to ×100 magnification and a range of techniques, with the morphology of the isolated actinomycetes being compared with that of known species.

Molecular characterization of actinomycetes

Actinomycetes were identified molecularly using polymerase chain reactions (PCR) to amplify the 16S rRNA gene. DNA was isolated from cells using a standard protocol. To amplify a fragment of the 16S rDNA gene, we used the primers pair St-F): 5'-AAGCCCTGGAAACGGGGT-3' and St-R: 5'-CGTGTGCAGCCCAAGACA-3'). PCR amplification was performed using reaction mixtures comprising a Master Mix (Operon Technologies), 0.4 µM of each primer, and 40 ng chromosomal DNA in a final volume of 25 µL. PCR amplification was performed using the following thermal cycler program: 94ºC for 5 min as a primary denaturation step, followed by 35 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 105 s, and a final extension at 72°C for 10 min. The PCR products were visualized using electrophoresis on 1% agarose gels and compared with a 10 kb DNA ladder.[16]

Antibiotic production in liquid cultures

For the culture of isolates, we used an antibiotic production medium containing (per liter) 0.8 g of NaCl, 1 g NH4Cl, 0.1 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.04 g $CaCl_2$, 10 g glucose, and 3 g yeast extract dissolved in distilled water at pH 7.2. Prepared medium was placed in 500-mL flasks and autoclaved for 20 min at 121°C and 15 atm pressure. The sterilized medium was inoculated with actinomycete spores under sterile conditions. In order to induce the production of secondary metabolites, the cultures were cultivated in a shaking incubator at 120 rpm and 30°C for 8 days. All processes we conducted in duplicate.[17]

Extraction of antimicrobial compounds and examination of their activity

Secondary metabolites were extracted using previously described methods.[18]

Tests for antimicrobials

Following fermentation, 1.5 mL aliquots of supernatant from culture flasks were transferred to Eppendorf tubes for antimicrobial assays. To assess the antibacterial activity of the actinomycete isolates, we used nutrient agar plates containing colonies of *Escherichia coli, Staphylococcus aureus, Bacillus subtilis,* and *Pseudomonas aeruginosa,* which were clinical isolates obtained from Al-Yarmook Hospital.[19]

Antibiotic bioassay

As a test medium, we used Mueller–Hinton agar in 9-cm-diameter Petri dishes, and performed assays using the paper-disc diffusion technique. Agar surfaces were inoculated with 0.1 mL of each test bacteria (3×10^6 cells). Sterile paper discs (6.0 mm diameter, Whatman antibiotic test discs) were placed on the surface of the medium and inoculated with 20 µL aliquots of actinomycete culture filtrate. Having been inoculated, the Petri dishes were placed in a fridge for 2h to enable the antibiotic to spread. The diameter of the inhibitory zone was measured in millimeters.[20]

Analyses of factors influencing antibiotic synthesis

In this study, using a selected isolate, we also assessed the effects of certain factors on antimicrobial production, and sought to establish the optimal conditions for production. The basal medium used contained (per liter) 1.0 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.04 g $CaCl_2.2H_2O$, 0.005 g $FeSO_4.7H_2O$, and 0.0005 g $ZnSO_4.7H_2O$, with the pH adjusted to 7.5. The carbon sources examined were glucose, maltose, sucrose, lactose, and starch at a concentration of 1%, and $(NH_4)2SO_4$ at concentrations of 0.05%, 0.10%, 0.15%, and 0.20%

was assessed as a nitrogen source. These nutrients were sterilized separately and added immediately prior to inoculation. Erlenmeyer flasks containing 250 mL of medium and inoculated with 10 mL of *Streptomyces* inoculum. For each condition, we used triplicate flasks. Flasks were incubated for 7 days in a rotary shaker operating at 220 rpm, following which, a disc diffusion assay was used to assess the respective antibacterial activities.[21]

RESULTS AND DISCUSSION

From 200 rhizosphere soil samples collected from agricultural regions in the vicinity of Baghdad City, we obtained 25 (12%) isolates of the genus *Streptomyces*, identified based on the chalky appearance of colonies and development of a damp, earthy odor. This initial identification was subsequently verified using a slide culture technique, based on the color of spores, development of aerial and substratum mycelia (which upon maturation divided into spiral spore chains), and the generation of diffusible pigments. Morphological analysis confirmed these isolates to be members of the genus *Streptomyces*.[22]

Light microscopy observations of the isolates revealed mycelial growth and spiral spore chain formation, and cultural features were characterized by cultivating on the following media: peptone-glycerol-yeast extract agar, glycerol asparagine agar, tyrosine agar, and starch mineral salt agar (Table 1). Heat treatment and the addition of $CaCO_3$ increased the value of hydrogen power, which inhibits the development of most fungi and promotes actinomycete growth.

Observations also revealed differences in the aerial mycelia when isolates were cultured in different media, with white-colored mycelia tending to be more prevalent among the isolates. Such differences in the morphological characteristic of isolates are assumed to be associated with nuclear material, which reached up to 10.5 × 10. The DNA of these isolates was characterized by a high GC content reaching up to 78,69%, which makes it difficult taxonomically classify isolates to the species level.[23] Our findings are consistent with the morphological and biochemical characteristics of the isolates reported by Saadoun et al.[24], Al- Obaidi[21], and Antonieta et al.[25] the latter of whom isolated 71 *Streptomyces* from soil samples collected at different locations in Venezuela.

Biochemical tests revealed that 13 out of the 25 isolates in the gray series were able to utilize all of the assessed carbon sources, whereas the remainder of the series were characterized by variable utilization. Isolates grouped in the white series were found to be less efficient in their utilization of the same carbohydrates. Comparatively few isolates (7 of 25) were able to use cellulose as a carbon source, whereas 12 of the isolates could degrade urea. With regards to the utilization of casein, tyrosine, and xanthine, we obtained variable results. Only a few isolates were unable to degrade tyrosine (2) and xanthine (5), whereas 20 of the 25 isolates could degrade casein.

To further confirm the preliminary identification of the 25 isolates as *Streptomyces*, we performed PCR reactions to amplify a fragment of the bacterial 16S rDNA gene, the results of

Figure 1. PCR amplification of the 16S rDNA gene of 25 local *Streptomyces* isolates[15]

Table 1. morphological characteristics of 25 *Streptomyces* spp. Isolated from agricultural soil region in Baghdad city

Isolate No.	Medium	Growth	Areal mycelium	Substrate mycelium	Diffusible pigment
Strp1	GAA/SMSA/ PGYEA/TA	Good/moderate/ good/good	Gray/white/ gray/brown	White/brown/ red/brown	None/gray/ red/white
Strp2	GAA/SMSA/ PGYEA/TA	Good/good/ good/moderate	White/brown/ none/gray	Green/red/ red/gray	Brown/gray/ white/red
Strp3	GAA/SMSA/ PGYEA/TA	Moderate/good/ good/moderate	Red/white/ white/red	None/gray/ none/red	Gray/red/ red/white
Strp4	GAA/SMSA/ PGYEA/TA	Good/good/ moderate/moderate	Brown/gray/ white/none	Gray/white/ white/white	Brown/white/ white/none
Strp5	GAA/SMSA/ PGYEA/TA	Moderate/good/ moderate/good	Brown/gray/ white/red	Brown/gray/ white/red	Brown/gray/ white/red
Strp6	GAA/SMSA/ PGYEA/TA	Good/moderate/ good/good	Gray/red/ red/white	None/gray/ none/red	Gray/red/ red/white
Strp7	GAA/SMSA/ PGYEA/TA	Moderate/good/ good/good	Gray/gray/ white/white	None/gray/ none/red	None/brown/ red/brown
Strp8	GAA/SMSA/ PGYEA/TA	Moderate/good/ good/moderate	Gray/none/ none/red	White/brown/ none/white	Gray/red/ red/white
Strp9	GAA/SMSA/ PGYEA/TA	Moderate /good/ moderate /good	None/gray/ none/red	Gray/red/ red/white	Brown/brown/ none/brown
Strp10	GAA/SMSA/ PGYEA/TA	Good/good/ moderate/good	None/gray/ none/red	Gray/red/ red/white	Brown/gray/ white/red
Strp11	GAA/SMSA/ PGYEA/TA	Moderate /good/ good/moderate	White/brown/ none/white	Brown/brown/ none/none	Gray/red/ red/white
Strp12	GAA/SMSA/ PGYEA/TA	Good/good/ moderate /moderate	None/gray/ none/red	Gray/white/ white/white	Gray/red/ red/white
Strp13	GAA/SMSA/ PGYEA/TA	Good/moderate/ good/good	Gray/white/ white/white	None/gray/ none/red	Brown/gray/ white/red
Strp14	GAA/SMSA/ PGYEA/TA	Good/good/ moderate/good	Brown/gray/ white/red	Brown/brown/ none/none	Brown/brown/ none/none
Strp15	GAA/SMSA/ PGYEA/TA	Good/moderate/ good/good	Gray/white/ white/white	None/gray/ none/red	Brown/gray/ white/red
Strp16	GAA/SMSA/ PGYEA/TA	Moderate /good/ good/moderate	Brown/brown/ none/none	None/brown/ none/brown	White/brown/ none/white
Strp17	GAA/SMSA/ PGYEA/TA	Good/moderate/ good/good	Gray/white/ white/white	Brown/white/ white/none	Brown/white/ white/none
Strp18	GAA/SMSA/ PGYEA/TA	Good/moderate/ good/good	White/brown/ none/white	Brown/brown/ none/none	Brown/gray/ white/red
Strp19	GAA/SMSA/ PGYEA/TA	Moderate/good/ good/good	Gray/white/ white/white	Brown/gray/ white/red	White/brown/ none/white
Strp20	GAA/SMSA/ PGYEA/TA	Good/moderate/ good/good	Gray/red/ red/white	Brown/brown/ none/none	Gray/red/ red/white
Strp21	GAA/SMSA/ PGYEA/TA	Good/good/ moderate/good	Brown/brown/ none/none	Gray/red/ red/white	Brown/gray/ white/red
Strp22	GAA/SMSA/ PGYEA/TA	Good/moderate/ good/good	Gray/white/ white/white	White/brown/ none/white	None/none/ red/none
Strp23	GAA/SMSA/ PGYEA/TA	Moderate/good/ good/good	Gray/red/ red/white	Gray/red/ red/white	Brown/gray/ white/red
Strp24	GAA/SMSA/ PGYEA/TA	Moderate/good/ good/good	Gray/white/ white/white	Brown/brown/ none/none	Gray/red/ red/white
Strp25	GAA/SMSA/ PGYEA/TA	Good/good/ moderate/good	Brown/white/ white/none	Gray/red/ red/white	Brown/white/ white/none

GAA: Glycerol asparagine agar, SMSA: Glycerol asparagine agar medium,PGYEA: Peptone-Glycerol-Yeast extract agar, TA:throsine agar

which revealed a single band of approximately 1500 bp in all assessed isolates are shown in Figure 1 and 2. These findings are consistent with those reported by Hadi et al.,[26] who isolated 140 strains from the northwest of Iran, among which, 12 selected *Streptomyces* isolates characterized by high antibacterial activity against pathogenic bacteria were subjected to 16S rDNA gene PCR, which similarly yielded a single specific band of approximately 1500 bp.

Microbial susceptibility

Among the 25 *Streptomyces* isolates obtained in this study, we found that only 10 were characterized by antimicrobial activity against colonies of *E. coli, S. aureus, B. subtilis,* and *P. aeruginosa,* thereby indicating that the secondary metabolites produced by these isolates differed in their antimicrobial activity. Our findings in this regard are consistent with those reported by Latif et al.,[27] who isolated five strains of *Streptomyces* (designated S, N, W, E, and C) from the rhizosphere soil of an area cultivated with palm in Al Madina, Saudi Arabia.

It is clear from Table 2 that in the disc assay conducted in this study, *S. aureus* and B. subtilis were inhibited to a greater extent by *Streptomyces* culture substrates than were *E. coli* and *P. aeruginosa,* which is consistent with the findings of Scherrer and Gerhardt[28] who found that gram-positive bacteria are characterized by a greater susceptibility to metabolites produced by *Streptomyces* than are gram-negative species.

Table 2. Comparison of antimicrobial activity of local *Streptomyces* spp. against standard clinical bacteria

Isolates	*E.coli,*	*S.aureus*	*B.subtilis*	*P.aeruginosa*
Strep1	5	11	10	2
Strep10	5	12	15	8
Strep11	8	30	10	10
Strep15	6	13	30	9
Strep16	3	22	20	2
Strep18	4	10	10	5
Strep19	8	12	10	4
Strep21	5	25	12	3
Strep23	2	5	11	4
Strep24	5	11	10	5

The most important bioactive secondary metabolites for treating infectious illnesses are antibiotics. However, owing to the lack of novel antibiotics produced during the past two decades, extensive multi-drug resistance has developed, which represents a fundamental obstacle to the efficient treatment of infectious diseases.[29] As the burden of multi-drug resistance has intensified in recent years, there has been a corresponding growth in interest among researchers with respect to the discovery new bioactive secondary metabolites that have efficacy in combating infections attributable to multi-drug-resistant strains.[30] In this regard, actinomycetes are considered a rich source of such secondary metabolites that could be developed for the treatment of infectious diseases. Accordingly, with the objective of isolating actinomycetes, we collected soil samples from a range of different

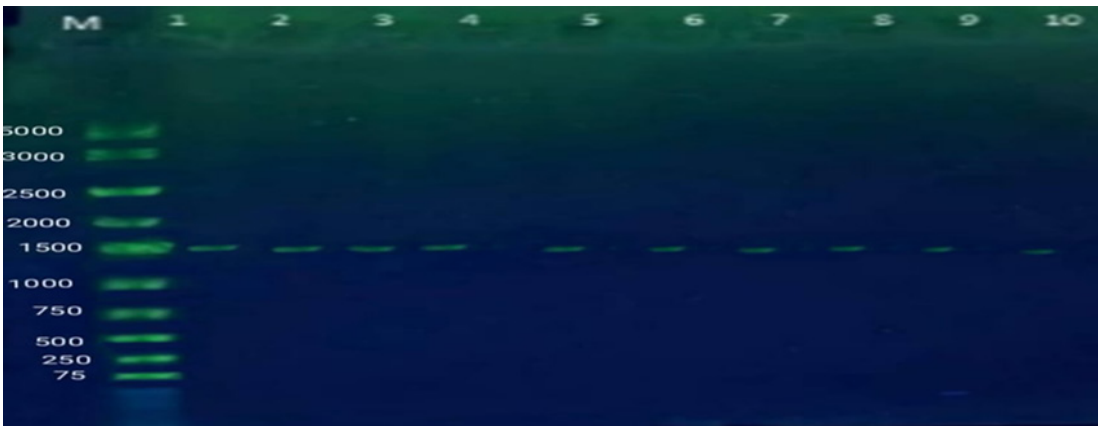

Figure 2. PCR amplification of the 16S rDNA gene of 10 local Streptomyces isolates

Table 3. Concentration of Ammonium sulfate on growth and antimicrobial production of clinical bacteria

Con. of $(NH_4)2SO_4$	Biomass	*E. coli*	*S. aureus*	*B. subtilis*	*P. aurgenosa*
0.05	14.20	10	12	10	9
0.10	20.60	12	14	13	10
0.15	19.60	10	12	12	12
0.20	16.80	10	12	12	10

Table 4. Influence of diffident carbohydrate on production of antibiotic

Carbo-hydrate	Biomass g/L	*E. coli*	*S. aureus*	*B. subtilis*	*P. aeruginosa*
Glucose	16.36	-	15	9	2
maltose	15.26	4	14	10	5
Sucrose	6.20	1.5	-	-	-
Lactose	8.26	-	2	-	-
Starch	5.04	-		3	2

localities, as the findings of previous studies have established that novel actinomycetes are most frequently found in soil.[13]

Clear zones surrounding the wells in actinomycete-inoculated plates containing bacterial lawns are taken to be evidence of the antibacterial efficacy of secondary metabolites against test organisms. In this regard, Gurung et al.[31] recorded inhibitory zone ranging in diameter of up to 18 mm against test organisms, which compares with the inhibitory zone ranging from 2 to 30 mm observed in the present study.

Influence of selected growth factors on antibiotic production

In this study, using a selected isolate (strep15), we examined the effects of different carbon and nitrogen sources on growth and the production of antimicrobials.

Effects of carbohydrates on antibiotic production

Among the selected carbohydrates used to examine their influence on the growth and antibiotic production of isolate strep15, we identified glucose as a particularly good source of carbon for the synthesis of antibiotics, of which enhanced production was observed. The inhibition zone shows that S. regions produced 15 mm diameter and the inhibition zone. All results are shown in Table 4. These finding are consistent with those reported by Al-Obaidi,[21] who isolated *Streptomyces* from rhizosphere soil in an agricultural region of Mosul, Iraq. The author used different concentrations of glucose to assess its influence on growth and antimicrobial production, and accordingly observed higher antibiotic productivity in cultures cultivated in the presence of 15 g/L glucose, with a corresponding inhibition zone of 20 mm against *S. aureus*, which compares with the inhibition zones of up to 14 mm against *S. aureus* in the present study using *Streptomyces* cultured in maltose-supplemented medium. Comparatively supplementing media with sucrose and starch as carbon sources was found to result in relatively poor antibiotic production. We suspect that these differential efficacies can be attributed to the fact that the latter carbon sources are rapidly utilized for the synthesis of cellular components, thereby leaving little residual carbon and energy for the production of secondary metabolites. Our findings in this regard tend to be consistent with those of Pandey et al.,[32] who assessed the effects of a range of carbon and nitrogen compounds on the synthesis of antimicrobials by *Streptomyces kananmyceticus* M27. However, whereas cultures grown in the presence of maltose, sucrose, and soluble starch produced only moderate antimicrobial yields, dextrose was identified as the most suitable carbon source.

Effects of nitrogen sources on antibiotic production

In term of nitrogen sources, (NH4)H2PO4 and yeast extract have been identified as being suitable for the synthesis of antibiotics. In the present study, we assessed the effects of different supplementary concentrations of $(NH_4)_2SO_4$, with a view to optimizing the concentration for antimicrobial production. As shown in Table 3, the highest levels of antimicrobial production were obtained from cultures cultivated in medium supplemented with $(NH_4)_2SO_4$ at concentration of 0.10%, with culture supernatants producing clear inhibition zones reaching up to 14 and 13 mm against *S. aureus* and *B. subtilis,* respectively. Nitrogen sources play an important role in

determining antimicrobial production, and in this regard, Fraid et al.[33] observed that compared with a concentration of 2.5 g/L, a higher production of the antimicrobial natamycin by *Streptomyces natalensis* was obtained in response to the addition of ammonium nitrite as nitrogen source at concentrations of 8 and 2 g/L.[34]

In addition to carbon and nitrogen, it has been established that phosphate also contributes to the synthesis of a large number of antibiotics.[35] Conversely, however, an excess of inorganic phosphate has been demonstrated to have inhibitory effects on the synthesis of certain antibiotics, including tetracycline, actinomycin, and candicidin.[36] These findings are consistent with those reported by other researchers.[37]

CONCLUSION

On the basis of the findings of this study, we identified microorganisms isolated from the rhizosphere soil of an agricultural region in Baghdad City as members of the genus *Streptomyces*. Moreover, we established that these isolates are characterized by antimicrobial production, thereby indicating that the soil in this region may represent an important source for the isolation of novel secondary metabolites. In future research we intend to conduct a more extensive survey of this area to identify further beneficial compounds with antimicrobial properties. The *Streptomyces* isolates obtained in this study produced antibiotics with growth inhibitory effects that were clearly visible on solid media, and we believe that these isolates may represent potentially novel sources for the synthesis of bioactive substances in the biotechnology industry.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All data sets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors

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