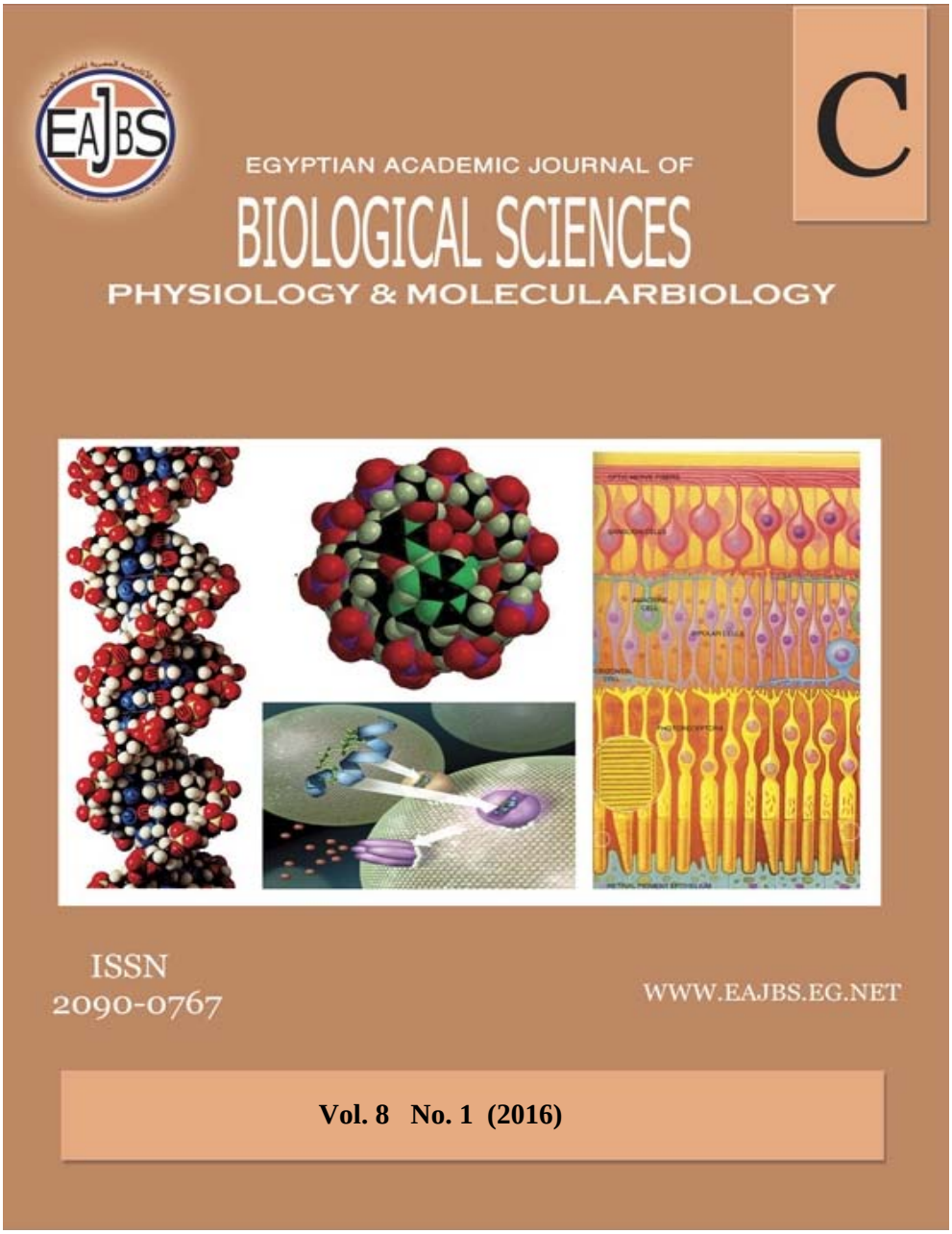

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Isolation and Molecular Identification of Streptomyces spp. with Antibacterial Activity from KSA

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ABSTRACT

Introduction: The genus *Streptomyces* represents a group of microorganisms that are widely distributed in nature. The genus *Streptomyces*, filamentous soil bacteria, has been described as the greatest source of the commercially available antibiotics. *Streptomyces* are also reported to produce other valuable bioactive secondary metabolites acting as antitumor agents, immune-osuppressive agents, and enzymes. In this study, a wide survey was conducted across Saudi Arabia. Soil samples were collected from different governorates representing different climatic conditions.

Methods: The soil sample was collected randomly from the agricultural lands in Riyadh and Qassim in Saudi Arabia. All the samples were pre-treated with calcium carbonate to reduce the number of vegetative bacterial cells, while allowing many Actinomycetes spores to survive. The collected samples were isolated by serial dilution method and identified based on cultural, morphological, microscopic, biochemical, and sequence analysis of 16S RNA gene parameters. The collected samples were analyzed for antimicrobial activities by perpendicular streak and disc diffusion methods, against the Gram-positive bacteria *Staphylococcus aureus, Bacillus subtilis*), Gram-negative *Escherichia. coli*, *Enterococcus aerogenes*, and filamentous fungi *Candida albicans* .

Results: Analysis of morphological and biochemical characteristics and the 16S rDNA gene sequence indicated that all selected isolates belonged to the genus Streptomyces. Moreover, screening of the isolates with regard to their antimicrobial activity against indicator bacteria, the optimum growth and antimicrobial compound production was found to be a maximal pH 8, in the shaker incubator at 28ºC, for a period of 10 days.

Conclusions: The *S. flavogriseus* showed a broad spectrum of antimicrobial activities against the test organisms and this opened further research investigations on purification and structural characterization of the active compounds from the crude extract.

INTRODUCTION

In this study, a wide survey was conducted along Saudi Arabia. Soil samples were collected from different governorates representing different climatic conditions (Abdulaziz *et al*., 2014). The search for isolating novel antibiotics, effective against resistant pathogenic microorganisms from unexplored habitats around the world, continues to be an important sector of research. The search for novel antibiotics continues to be of immense

importance in research programs around the world for pharmaceutical, industrial, and agricultural applications.

Filamentous soil bacteria, belonging to the *Streptomyces* genus, are widely used as an important biological tool for their ability to produce a wide range of novel secondary metabolites, such as "antibiotics." (Dezfully *et al*., 2015).

The genus *Streptomyces*, filamentous soil bacteria, has been described as the greatest source of the commercially available antibiotics. *Streptomyces* are also reported to produce other valuable bioactive secondary metabolites acting as antitumor agents, immunosuppressive agents and enzymes. (Mahmoud *et al*., 2014).

The genus *Streptomyces* represents a group of microorganisms that are widely distributed in nature. (Salih *et al*., 2014).

Streptomyces is a genus of Gram-positive bacteria that grows in various environments, and its shape resembles filamentous fungi. The morphological differentiation of *Streptomyces* involves the formation of a layer of hyphae that can differentiate into a chain of spores. The most interesting property of *Streptomyces* is the ability to produce bioactive secondary metabolites, such as antifungals, antivirals, antitumorals, anti-hypertensives, immunos-uppressants, and especially antibiotics. (Rudi *et al*., 2012).

The *Streptomyces* exhibited potent antimicrobial activity against various plant pathogenic fungi as well as against bacteria. (Minh *et al*., 2015).

Streptomyces avermitilis, belonging to Actinomycetes, is specialized for production of avermectin, used as an anthelmintic and insecticidal agent. It is mostly found in soil and its isolation is very crucial for medically important avermectin production.(Qureshi *et al*., 2014).

Streptomyces genus of the order Actinomycetales constitutes a distributed group of bacteria. They have many properties that favour their predominance among other saprophytic microorganisms. They are best known for their economic importance as producers of antibiotics, vitamins, and enzymes, and are certain to have a significant role in future biotechnology.

Moreover, the majority of antibiotics in use today were discovered in the 1950's. 1000 antibiotics are known today and most of them (58%) are produced by Actinomycetales especially the genus *Streptomyces*.

Some species of *Strepmyces* are causative agents of important human and animal diseases, plant pathogens, and the rest are involved in the turnover of organic matter revealing that *Streptomyces sp*. had been investigated and their antagonistic properties were known and some species produced famous antibiotics.

This research was aiming at isolating bacterial strains from soil and review if they are able to produce antibiotic and then to study the different conditions affecting its productivity. In recent years, the search for novel pharmaceutical agents against drug resistant microbes from common species of the genus *Streptomyces* has become a more difficult and costly task. Hence, more attention has been focused on isolating streptomycetes from unusual and unexplored environments, such as the oceans (Veyisoglu & Sahin, 2014) and desert soil (Santhanam *et al*., 2013).

To date, tens of thousands of actinomycete-derived compounds have been isolated and characterized, many of which have been developed into drugs to treat various diseases (Aislabie *et al*., 2008). Actinomycetes like streptomyces isolated from different environments

have produced the same compound due to frequent genetic exchange between species (Bredholt *et al*., 2008). Hence, this results in reduced chances of finding genuinely new biologically active molecules from streptomyces and actinomycetes. (Baltz, 2006; Busto *et al*., 2006).

Therefore, the exploration of new habitats with unusual environment and poorly explored areas of the world has become important and useful to the discovery of novel compounds and actinomycetes (Learn-Han *et al*., 2012).

Consequently, Saudi Arabia, one of the harshest and poorly explored areas on Earth, has now emerged as a potential area for the discovery of novel bioactive compounds form actinomycetes. In our study, we have focalized the Riyadh and Qassim regions, situated in the center of the Kingdom, which is famous by its geographical biodiversity. We have isolated soil actinomycetse from different locations in order to determine their antagonist effect against selective gram+ and gram- bacteria, fungi, and cancerous cells. This study will be useful for the extraction of eventual new antibiotics. In a second step, we have characterized the species of actinomycetes that has the most effect on bacteria, using molecular technique (PCR) and subsequently the sequencing of the amplified region of the 16s rDNA in order to do phylogenetic analysis.

Objectives:

The objective of this study was to isolate, identify, and evaluate the antimicrobial activity for optimization of the strain *Streptomyces* obtained from a soil sample of the Kingdom Saudi Arabia.

MATERIALS AND METHODS

Sample Collection

Soil samples were collected randomly from agricultural lands of the Riyadh region and Qassim region, situated in the Center of the Kingdom Saudi Arabia during 2016.

Using an open-end soil borer (20 cm in depth and 25 cm in diameter) from a depth of 10 - 20 cm, which were then air dried for 24 to 48 hours. The soil sample was then treated with 1% $CaCO_3$ and kept at ambient temperature for 2 - 3 days for the next step of analysis.

Isolation of Actinomycetes

Soil samples were serially diluted and plated on Actinomycetes Isolation Agar medium and incubated at 28˚C for 6 days. The suspected colonies were picked up and purified on yeast extract-malt extract agar medium (ISP- 2).

Taxonomical Characterization of the Isolates

The Isolates were identified based on cultural, morphological, physiological, biochemical, and sequence analysis of 16S rRNA gene parameters.

Cultural and Morphological Characteristics

The cultural characteristic of pure isolate was studied after incubation of Isolates for 7-14 days at 28˚C.

Antimicrobial Activity

The test organisms employed included the following: Gram-positive *Staphylococcus aureus, Bacillus subtilis*, and Gram-negative E. coli, *Enterococcus aerogenes*. All the test organisms used in this study were obtained from the Microbial Type Culture Collection, Institute of Microbial Technology. The filamentous fungi *Candida albicans* were obtained from the Department of Studies in Microbiology and Department of Studies in Botany.

Actinomycetes were inoculated in Mueller Hinton Medium in the form of a horizontal line at the top of the environment.

Dishes were incubated for 48 hours to give them a chance to produce antibiotics as a by-product at a temperature of 37 m, then inoculated microorganisms tested in the same dish

is perpendicular to the horizontal vaccine, and incubated for 24 hours.

Read the result based on the emergence zone appealing zones of inhibition guide the production of antibiotics.

The inhibition zone around the disk was measured with a millimeter scale and the results were tabulated and analyzed.

Genomic DNA Isolation

For DNA isolation the organism was grown with shaking for 24h at 37°C in baffled flasks containing 50 ml Nutrient broth medium.

PCR Primers: In order to identify *Streptomyces* isolates and classify them according to their phylogenetic group, the advantage of the sequence diversity of the 16s -23s intergnic spacer regions (SRs) of *Streptomyces* was considered. The SR primer was paired with a specific primer from within the 16s rDNA gene. The primer set 24 f and 1492r, illustrated in Table (1) was used in the amplification 1.5 kbp product of 16s rDNA gene. To increase the sensitivity of PCR, the primer sets ACT235f and ACT878r designed to amplify a portion of 16s rDNA gene was used in the nested-PCR.

Table 1: Sequence specificity of PCR Primers used for amplification.

	Primers	Sequence
Set 1	24 f 1492 r	(5'-AGAGTTTGATCCTGGCTCAG-3') (5'-TACGGTTACCTTGTTACGACTT-3')
Set 2	ACT235f ACT878r	(5'-CGCGGCCTATCAGCTTGTTG-3') (5'-CCGTACTCCCCAGGCGGGG-3')

PCR Amplifications: All amplification reactions were performed in a final volume of 50 ul, containing 50 mM KCl, 10 mM Tris HCl, pH 8.3, each primer at 500 nM, each dNTP at 0.2 mM, 1.5^3.5 mM MgC l2, 1.5^2.5 U of Ampli Taq Gold DNA Polymerase (Applied Biosystems) and 3 ul of 10UDenhardt s reagent (per liter: 2 g Ficoll 400, 2 g polyvinylpyrrolidone, 2 g bovine serum albumin) (Volossioule *et al*., 1995). Optimized cycling parameters, enzyme, and MgCl2 concentration for each primer pair are shown in Table (1).

Each amplification program was initiated by denaturation for 5 min at 95° C (10 min when using AmpliTaq Gold) and finished with an extension step of 10 min at 72 °C. Reactions were performed either in a PE 9600 in a PCRExpress thermal cycler (HybaidLtd.) equipped with a gradient block for optimization of the annealing temperature. As a positive control, all DNAs yielded the expected amplification fragment of about 1.5 kb when tested with the degenerated primers, specific for all bacteria (Heuer *et al*., 1997).

Electrophoretic Analysis: Aliquots of 10 µl each of the amplification products were loaded onto 1.2% agarose slabs and run in TBA (40 mM Tris-acetate, 1 mM EDTA) buffer at 80 V for two hours. Slabs were stained with 0.4 µg of ethidium bromide/ml. After electrophoresis, the PCR patterns were visualized with UV transilluminator and documented with a Gel Doc 2000 gel system (Bio-Rad). In addition, gels were photographed using a Polaroid camera. Molecular weight analysis of the resulted patterns was performed with the Quantity One version 4.2.1 software (Bio-Rad), with the 1-kb DNA ladder (Invitrogen) as a molecular weight marker.

RESULTS AND DISCUSSION

The results in Table 2 showed that the Cultural Characteristics of Streptomyces Isolates on Different Media after 14 Days of Incubation at 28˚C and they are growth in Yeast Malt Agar

media and poor growth in Nutrient agar media.

The results of the optimal nutritional media showed that antibiotic production was higher in the medium containing glucose. The importance of glucose in the nutritional medium for the synthesis of a wide range of antibiotics by different *Streptomyces* species has been reported by many investigators.

Although the Isolates ability to grow in five different media tested, the highest growth (0.8 mg/50 mL) and antimicrobial activity were obtained in MISP-2 Broth medium supplemented with starch (as a carbon source) and CaCO3 (as growth promoters).

This result is quite comparable with the *S. fulvissimus* and *Pseudonocardi* species, for which MISP-2 was found to be a suitable medium for the antibiotic production. Optimal cultural conditions at different pH levels and incubation temperatures and time for antimicrobial production by *S. flavogriseus* improved in the medium containing MISP-2 Broth.

Table 2: Cultural Characteristics of *Streptomyces* Isolates on Different Media After 14 Days of Incubation at 28˚C

Sl. No.	Medium	Growth	Aerial Mycelium	Substrate Mycelium	Spores	Soluble Pigment
1	Yeast Malt Agar	Moderate good	Moderate Grayesh White	Whitish gray	gray	Non
2	Yeast Malt Agar	Good	Abundant Gray	Whitish gray	gray	Non
3	Yeast Malt Agar	Poor	Moderate Ashes	Yellowish brown	Whitish gray	Red White
4	Yeast Malt Agar	V. Good	Moderate Ashes	Purple	Non	White pink
5	Yeast Malt Agar	Moderate	Moderate green	green	green	Whitish green
6	Yeast Malt Agar	Good	white	white	Non	Non
7	Nutrient agar	Poor	Poor green	green	greenish	Non

The results revealed that the maximum growth (0.8 mg/50 mL) and in-vitro antimicrobial compound production by *S. flavogriseus* could be obtained in MISP-2 medium having a pH 8, for 10 days of incubation at 28˚C.

The growth profile, closely coupled with the metabolic capacities of the produced organism *S. flavogriseus*, which greatly influenced the biosynthesis of antibiotics.

The findings confirm that the nature of the medium composition and cultural conditions strongly affect and enhance the antimicrobial compound of Isolates, which is comparable to the surveys conducted on different Streptomyces spp by other investigators.

In this study, the *S. flavogriseus* designated as Isolates, isolated from agricultural soil sample of Riyadh and Qassim in Saudi Arabia showed broad spectrum antimicrobial activity against Gram-positive, Gram-negative bacteria, and the fungus.

Further studies on the purification and chemical characterization of the antimicrobial compound from the strain of *S. flavogriseus* will be useful for pharmaceutical applications.

The emerging crisis of antibiotic resistant pathogens indicates an increasing necessity for the survey of unexplored and underexplored niche habitats.

The isolation of actinomycetes can contribute to the discovery of novel, safe, effective, and broad-spectrum antimicrobial compounds, as part of the

strategy to control the drugresistant pathogens.

During the course of screening of bioactive compounds for the isolation of new antibiotics each year, thousands of actinomycetes, particularly *Streptomyces* strains, are screened by pharmaceutical research laboratories as sources for novel antimicrobial compounds.

The results in Table 3 and Fig.1 and Fig. 2 showed The Antimicrobial Activity of *Streptomyces* Isolates, inhibiting the growth of tested bacteria by perpendicular streak method.

In the present study, a broad spectrum of antimicrobial producing actinomycetes, isolated from agricultural soil sample lands of Riyadh and Qassim in Saudi Arabia were selected for optimization of their antimicrobial activities. The Isolates were identified through conventional and molecular methods.

Table 3: The Antimicrobial Activity of *Streptomyces* Isolates inhibiting the growth of tested bacteria by perpendicular streak method.

	The test organisms			
No. *Streptomyces*	**1 Staphylococcus**	**1 Bacillus**	**2 E.coli**	**3 Candida**
1	0	.5	.5	0
2	0	0	0	0
3	0	0	0	0
4	2	4	1.5	0
5	1	.5	1	0
6	0	4	.5	0
7	0	0	0	0

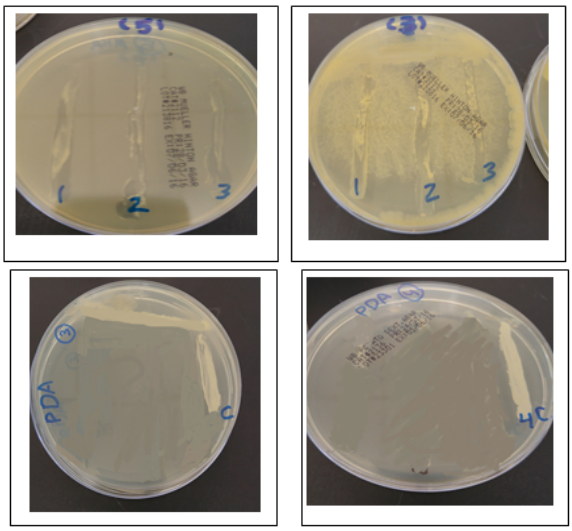

Fig. 1: The Antimicrobial Activity of *Streptomyces* Isolates inhibiting the growth of the test organisms by perpendicular streak method

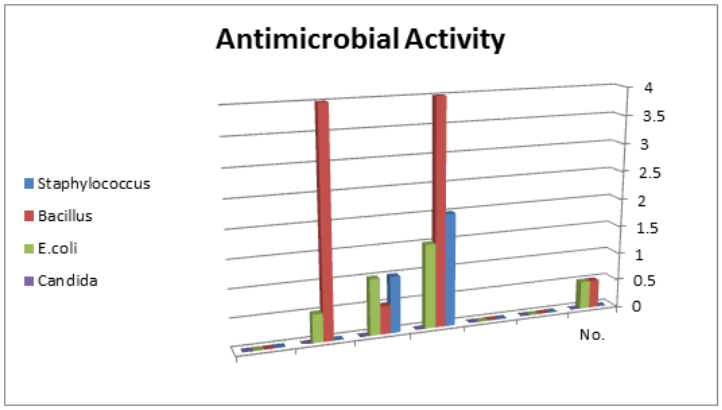

Fig. 2: The Antimicrobial Activity of *Streptomyces* Isolates. inhibiting the growth of the test organisms.

The isolate exhibited broad antimicrobial activities against Gram-positive, Gram-negative bacteria, and in the primary and secondary screening process.

In a study conducted in Iran with different strains of *Streptomyces*, there was no significant correlation between the activity of intact bacteria in primary screening and their extract in secondary screening, which was similar to the present study on Isolates.

After studying the morphology Identification of *Streptomyces using universal primers in* Table (1) and in Figure 3 *using* Gel electrophoresis image for PCR of *Streptomyces* Isolates.

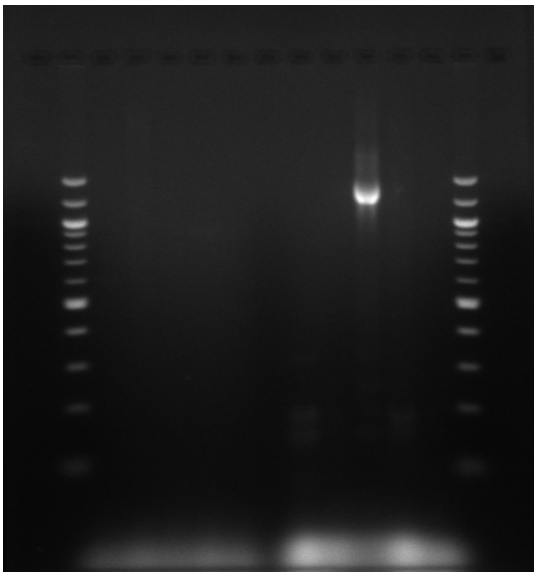

Fig. 3: Gel electrophoresis image for PCR of *Streptomyces* Isolates.

CONCLUSION

This study shows that the test actinomycetes isolates have the potential to act as sources of new antibacterial compounds against pathogenic micro-rganisms to humans. Here, we found that Riyadh is a good region of biodiversity and has been adequately acceptable due to its vast floral diversity and also microbial diversity. The results showed that all the isolates were able to inhibit the extracellular growth of filaments in the test organism.

So, further intensive studies are required on the actino-bacterial diversity of exclusive biotopes in Riyadh, which could form an important input into pharmaceutical industries.

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