Molecular and Biological Studies of *Streptomyces* sp. Producing Antibacterial Agents against Some Pathogenic Bacteria

Afra M. Baghdadi*, Naylah M. Alblady

Department of Biology Sciences, Faculty of Science, University of Jeddah, Jeddah, Saudi Arabia.

ABSTRACT

The genus *Streptomyces*, known as prolific antibiotic products and many other bioactive secondary metabolites, is a unique subgroup of actinomycetes bacteria. This study aims to isolate *Streptomyces* strains from plant rhizosphere that has antibacterial activity. Soil samples were collected from Saudi Arabia. About 39 isolates of *Streptomyces* sp. were obtained from 12 soil samples, using serial dilution and plating techniques on the solid starch nitrate medium. The *Streptomyces* strains were cross streaked against some human pathogen bacteria. Against various pathogenic bacteria, only 16 out of 39 isolates demonstrated strong antimicrobial activity. The most active strain *Streptomyces* NM38 was chosen for secondary screening and identification. The molecular identification was confirmed using 16S rRNA as *Streptomyces globosus,* the phylogenetic tree was built; the sequence was presented to the GenBank under the accession number: MN538259.1.

Key Words: *Antibacterial activity, Streptomyces, Pathogenic bacteria, Identification*.

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INTRODUCTION

Microbial diseases are growing year after year and have become a major threat to public health [1, 2]. The greatest challenge is the emergence of drugs and multidrug-resistant pathogens. Therefore, new antimicrobial agents from natural sources with new mechanisms of action in the medical and pharmaceutical sectors are urgently needed. Soil is the most significant *Streptomycetes* territory; it makes 40% of soil microbes. Under dry and soluble conditions, *Streptomyces* sp. are the various microbial populace in soil. On account of their filamentous structure, they cause soil surface quality and shield it from wind and rained annihilation [3]. *Streptomyces* is the biggest sort of actinobacteria and the sort class of the family Streptomycetaceae [4].

Actinobacteria are a category of Gram-positive bacteria in their DNA that have a rich guanine and cytosine source. Actinobacteria generate a range of highly pharmacological and commercially attractive secondary metabolites. A variety of antibiotics were identified with the detection of actinomycin from actinobacteria, particularly from the genus Streptomyces [5].

Streptomyces' morphological divergence includes arranging a layer of hyphae that can separate into a chain of spores. This procedure is remarkable among Gram-positive, requiring specific and facilitated digestion [6].

Streptomyces sp. are Gram-positive filamentous bacteria living in the soil with a complex morphological differentiation cycle [7]. Members of the *Streptomyces* genus used for human and animal therapy are potential sources of secondary metabolites possessing a broad range of biological activities, including antibacterial activity [8, 9]. It is estimated that more than 7,000 metabolites are synthesized by these bacteria [10].

This study aimed at the isolation of *Streptomyces* strains from plant rhizosphere produces antibacterial activity.

Corresponding author: Afra M. Baghdadi
Address: Department of Biology Sciences, Faculty of Science, University of Jeddah, Jeddah, Saudi Arabia.
E-mail: ✉ amboghdadi @ uj.edu.sa

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MATERIALS AND METHODS

Soil samples

This investigation was carried out in March 2018. The soil samples have been gathered from plants rhizosphere from 12 localities in Saudi Arabia, Al-Baha region (*Olea* sp., *Rosa*, *Cymbopogon schoenanthus*, *Juniperus*, *Dodonaea,* and *Olea oleaster*) and Jeddah province (*Ocimum basilicum*, *Spathiphyllum wallisii*, *Plumeria alba*, *Calotropis gigantean*, *Conocarpus,* and *Phoenix sylvestris*). Soil samples obtained from different sites were carefully collected with a spatula down a 5-15 cm depth from the plant rhizosphere. The samples were stored in plastic bags. The soil samples were incubated in Petri dishes in an oven at 50 C^{o} to kill the vegetative bacteria.

Test indicator bacteria

For test (indicator), the pathogenic bacteria employed included Gram-negative bacteria: *Salmonella* sp., *Pseudomonas earuginosa* ATCC27853*, Escherichia coli* ATCC35218, and *Escherichia coli* ATCC25922 and Gram-positive bacteria: *Streptococcus pneumonia* ATCC49619 and *Staphylococcus aureus* ATCCBAA977. American Type Culture Collection (ATCC) was used for this experiment.

Isolation of *Streptomyces* sp.

Starch-nitrate agar medium was used for isolation of *Streptomyces* sp. from the soil samples. It is consists of starch 10 g, potassium nitrate 2g, di-potassium hydrogen phosphate1g, magnesium sulphate 0.5g, sodium chloride 0.5g, calcium carbonate 3g, and ferrous sulfate 0.01g at pH 7.5. Soil samples were serially diluted (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) using sterile distilled water. About 0.1 ml of each dilution was transferred to the starch-nitrate agar medium and distributed on the medium surface using a sterile L-shape glass rod. The dishes were placed upside down after being incubated at 28 ºC for seven days. The *Streptomyces* sp. were picked up.

Screening of *Streptomyces* antimicrobial activity

The selected test pathogens bacteria were gotten from Microbial Type Culture Collection (MTCC). The seven indicator bacteria have been grown for 18 hours in the broth of MH. A sterile cotton swab was immersed into a correctly calibrated cell density solution and swabbed on the nutrient agar surface. *Streptomyces* isolates were examined for their antibacterial activity against selected nosocomial infection bacteria species using the disc diffusion method. *Streptomyces* were grown on starch-nitrate agar. After incubation for 7 days at 28 ºC, agar discs (6 diameters) were cut off by a sterilized cork borer. Petri plates were prepared of sterile nutrient agar and inoculated by the test bacteria and left to dry for 5 minutes at room temperature. Two agar discs (6 diameters) of each *Streptomyces* isolate were placed over the plate's surface previously swabbed with indicator bacteria. Growth inhibition of the indicator bacterium was detected by measuring inhibition zone diameters (including the cork borer diameter) and recorded in mm at 37 ºC, after overnight incubation.

Identification of *Streptomyces* sp. isolate that possesses the most potent antibacterial activity

In addition to 16SrRNA, the most active isolate was initially chosen and defined at the genus level by morphological and physiological tests. To identify an unknown isolate (obtained in pure culture), preliminary observations and tests, including reaction to certain stains, morphology, physiological and biochemical tests, were carried out. The molecular identification was also done.

Cultural characteristics

Cultural characteristics were determined and detect growth, the color of aerial mycelium, substrate mycelium color, and soluble pigments, after 7 days of incubation at 30 ºC on different media (ISP2, ISP5, ISP7, ISP9, nutrient agar, and starch-nitrate agar).

Morphological characteristics

Morphological studies have been performed using a light microscope (Olympus Ch20bimf200sa-Olympus Optical. Co. Ltd., Japan). Examination of sporophore and spore morphology surface features were observed by scanning electron microscope (JSM 7600F Field Emission Gun Ultra-High Resolution) at the Electron Microscope Unit, Faculty of Science, King Abdul-Aziz University.

For the selected *Streptomyces* isolate different physiological and biochemical characteristics including the production of melanin pigments, cellulose decomposition, gelatin liquefaction (production of proteolysis enzymes), starch-hydrolysis, and the use of various sources of carbon and nitrogen have been investigated.

Molecular characterization of bacterial isolate

At the Princess Al-Jawhara Center for Molecular Medicine and Inherited Disorders, the sample was sequenced, and 10 sequencing reactions were graded as forward and reverse reactions, using bioinformatics software, the sequence was trimmed to eliminate the low quality ends Codon Code aligner ver. 6.0.2 by 100 to 250 bases from ends.

Briefly, genomic DNA isolation was performed using GeneJET, Genomic DNA Purification Kit (Thermo Fisher Scientific). An amplicon of 1500 bp fragments representing the full length of the 16S rRNA gene was

amplified using highly conserved universal primers pA 5'AGA GTT TGA TCC TGG CTC AG 3' and pH 5'AAG GAG GTG ATC CAG CCG CA 3' [11]. Fifty ng of DNA, 1µL of each 10 µM primer, 12.5µL GoTaq® Green Master Mix (Promega, USA) and sterile dH_2O up to 25 µl were used.

PCR amplification conditions

Initial: at 94 °C for 5 minutes, denaturation: at 94°C for 1 min, annealing: at 55°C for 1 min and extension: at 72 °C for 2 min. The number of cycles: 35. The final extension: at 72°C for 10 min. The sequences have been analyzed and compared to those in GenBank to check for close evolutionary relatives using the BLAST algorithm and RDP database. The 16S rRNA nucleotide sequence was determined to characterize the strain and the phylogenetic tree-structured by the Neighbour- Joining (N-J) method using Clustal-W in MegaAlign tool of DNASTAR version 12.3.1. DNA Star inc. The final phylogenetic tree was gartered using the iTOL tree of life tool [12].

Bioinformatics analysis

Using BLAST, the obtained nucleotide sequences of 16S rRNA genes were aligned with NCBI. In the Gene Bank database, the resulting DNA sequences were analyzed phylogenetically and compared to the available analogous type strain sequences.

RESULTS AND DISCUSSION

Isolation of *Streptomyces* sp.

About 39 isolates of *Streptomyces* were obtained from 12 soil samples. Data in Table 1 showed *Streptomyces* ratios that isolates from samples collected and described. There were 24 isolates (61.5%) gathered from the Al-Baha region and 15 isolates (38.5%) gathered from Jeddah province. *Streptomyces* were gray color (14 isolates about 35.89%), white color (12 isolates about 30.77%), pink color (5 isolates about 12.82%), brown color (4 isolates about 10.24%), yellow color (2 isolates about 5.14%), black color (one isolate about 2.57%), and orang color (one isolate about 2.57%). About 39 colonies of *Streptomyces* were purified on starch-nitrate agar medium and used for further studies.

Soil is an ecological niche where several organisms live together, some of which produce useful natural products, including therapeutically effective antibiotics. Microorganisms are abundant in the soil, especially those belonging to the genus *Streptomyces*. *Streptomyces* is relatively slower in growth compared to other bacteria. A large number of isolates are needed to search for the producer of novel metabolites. The diversity of actinomycetes can be affected by the variety of plants grown on a particular soil, referring to Oskay et al. [13]. It was also noticed that various plants produce various chemical metabolites that may be useful for the microbes around them and vice versa. The microbes (actinomycetes in this case) must adapt to the environment to survive in a threatened environment [13].

Table 1. Distribution of *Streptomyces* isolates in soil samples collected from different localities at Al-Baha region and Jeddah province.

Location	Soil from plants rhizosphere	Ratio of isolates	The color of mycelium					
			White	Gray	Yellow	Pink	Brown	Black
Soil samples from Al-Baha	*Olea* sp.	7.71 %	2.57 %	2.57 %	-	-	2.57 %	-
	Rosa	10.25 %	5.11 %	2.57 %	-	-	2.57 %	-
	Cymbopogon schoenanthus	7.68 %	2.57 %	5.11 %	-	-	-	-
	Juniperus	12.81 %	5.11 %	7.70 %	-	-	-	-
	Dodonaea	10.26 %	-	10.26 %	-	-	-	-
	Olea oleaster	12.82 %	2.57 %	-	2.57 %	5.11 %	-	2.57 %
Soil samples from Jeddah	*Ocimum basilicum*	5.14 %	-	-	-	2.57 %	-	-
	Spathiphyllum wallisii	7.7 %	7.7 %	-	-	-	-	-
	Plumeria alba	7.71 %	2.57 %	-	-	2.57 %	2.57 %	-
	Calotropis gigantea	2.57 %	-	-	-	-	2.57 %	-
	Conocarpus	10.25 %	2.57 %	5.11 %	2.57 %	-	-	-
	Phoenix sylvestris	5.14 %	-	2.57 %	-	2.57 %	-	-
Total		100 %	30.77 %	35.89 %	5.14 %	12.82 %	10.24 %	2.57 %

Antibacterial activity of *Streptomyces* isolates

Primary screening of antibacterial activity of seven days old experimental *Streptomyces* isolates were studied on starch-nitrate medium. The cultures have been checked for their antagonistic activities against Gram-negative and Gram-positive bacteria using diffusion in the agar by disc method. Data in Figure 1 show that 16 from 39 isolates showed antibacterial activity. These isolates had an inhibitory effect on Gram-positive bacteria more than Gram-negative bacteria. *Streptomyces* isolates inhibited Gram-positive bacteria more than inhibited Gram-negative bacteria. About 14 *Streptomyces* isolates hindered the growth of *Streptococcus pneumonia* ATCC49619, and 9 *Streptomyces* isolates hindered the growth of *Staphylococcus aureus* ATCCBAA977. There were 4 *Streptomyces* isolates that hindered the growth of *Escherichia coli* ATCC35218. Also, 4 *Streptomyces* isolates hindered the growth of *Escherichia coli* ATCC25922, one *Streptomyces* isolate hindered the growth of *Salmonella* sp., while no *Streptomyces* isolate hindered the growth of *Pseudomonas earuginosa* ATCC27853. These findings align with prior research that indicated that the activity of most isolated *Streptomyces* sp. was against both the majority of Gram-positive bacteria [14] and some Gram-negative bacteria. There are two possible reasons for this effect, first, solid media and liquid media cultivation may lead to the production of various secondary metabolites [15]; second, during the method of organic solvent extraction, some compounds may be lost.

In our screening for new *Streptomyces'* possible antibacterial activity, soil samples were gathered from different plant rhizosphere from Saudi Arabia regions. During our ongoing search, 39 isolates from soil samples were recovered. It has been shown in previous studies that the isolation rate of actinomycetes with antimicrobial activity is greater than 40 % [16] and less than 10 % in others [17]. These findings confirm that antifungal activity can be produced by a various collection of antibiotics from actinomycetes. Besides, novel antibiotic molecules are directed at isolating strains that have not been exploited before from specific ecosystems and hostile environments [18].

Based on the results obtained in this experiment, *Streptomyces* isolates NM38 had the most potent antibacterial activity against both Gram-negative and Gram-positive bacteria. This isolate was chosen for the subsequent experiments (Figures 1 and 2).

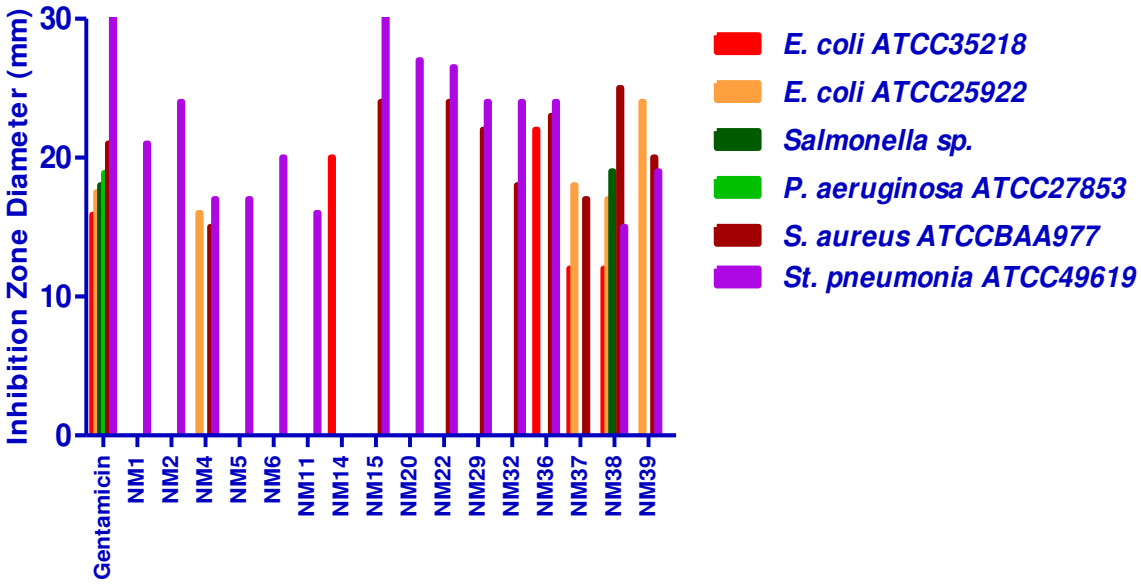

Figure 1. Antibacterial activity of *Streptomyces* isolates against some pathogenic bacteria

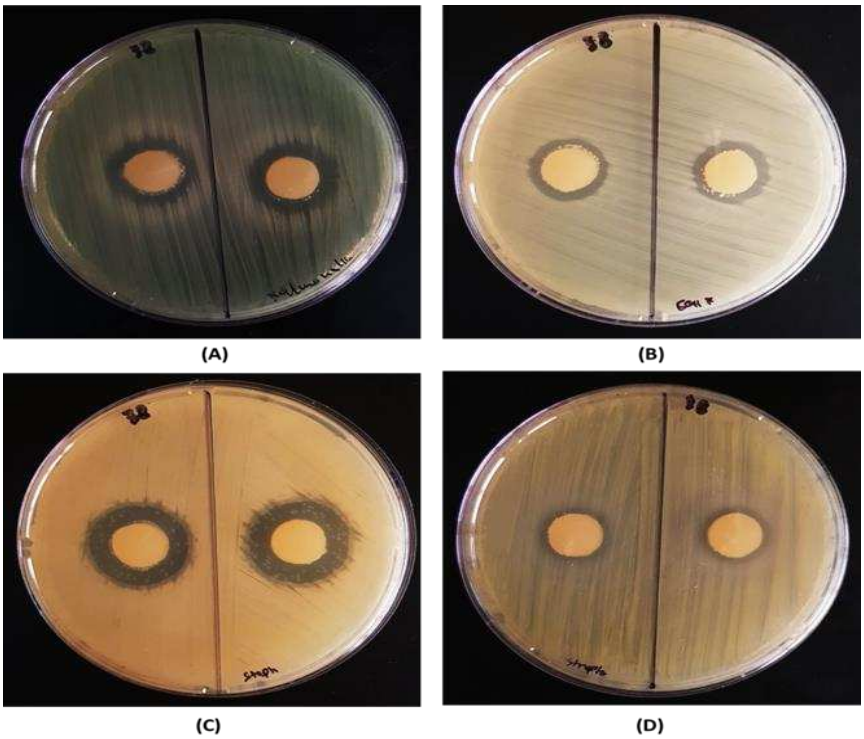

Figure 2. Antibacterial activity of *Streptomyces* NM38 against (A) *Salmonella* sp., (B) *E. coli* ATCC25922, (C) *S. aureus* ATCCBAA977 and (D) *St. pneumonia* ATCC49619.

Identification of the most potent *Streptomyces* isolate (NM38)

Cultural characteristics

There was good growth on some media, moderate growth or trace growth occurred on other media. On starch-nitrate agar, the isolate gave moderate growth, yellowish-white color aerial mycelium, but the substrate mycelium was yellow and produced yellow soluble pigment. On yeast malt agar (ISP-2), the isolate gave heavy growth, pale gray aerial mycelium, but the substrate mycelium was brown and produced brown soluble pigment. On glycerol asparagine agar (ISP-5), the isolate gave feeble growth, yellow aerial and substrate mycelium, and produced no soluble pigments. On tyrosine agar (ISP-7), isolate gave moderate growth, yellowish-white color aerial mycelium, but the substrate mycelium was dark yellow and produced no soluble pigments. On E-medium (ISP-9), the isolate gave moderate growth, pale gray aerial mycelium, but the substrate mycelium was yellowish-white color, and produce no soluble pigment (Table 2 and Figure 3).

Table 2. Cultural characteristics of *Streptomyces* NM38 on different media

Media	Growth	Color of aerial mycelium	Color of substrate mycelium	Presence of soluble pigments
Starch-nitrate agar	Moderate	Yellowish-white	Yellow	Yellow
Yeast Malt agar (ISP-2)	Heavy	Pale gray	Brown	Brown
Glycerol asparagine agar (ISP-5)	Feeble	Yellow	Yellow	No pigment
Tyrosine agar (ISP-7)	Moderate	Yellowish-white	Dark yellow	No pigment
E-medium (ISP-9)	Moderate	Pale gray	Yellowish-white	No pigment

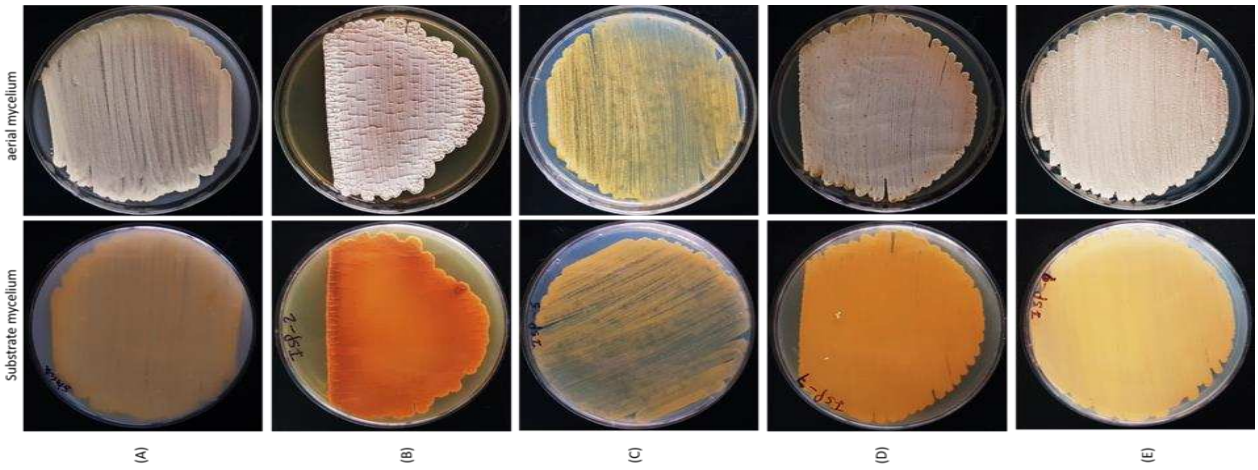

Figure 3. Cultural characteristics of *Streptomyces* NM38 on: (A) Starch-nitrate agar, (B) ISP-2, (C) ISP-5, (D) ISP-7 and (E) ISP-9 medium.

Morphological characteristics

The direct microscopic study of spore chains of *Streptomyces* NM38 on starch-nitrate agar after 7 days revealed that the isolate produces long filamentous mycelium with few branches and long spore chains.

The spores' electron microscopic study shows that the isolate had aerial hyphae that differentiated into smooth-surface spores on straight chains (Figure 4). As described in Bergey's Manual of Determinative Bacteriology, most actinomycetes isolates have the typical morphology of *Streptomycetes* [19]. They were slow-growing, aerobic, and chalky, with a variety of colors in both aerial and substrate mycelia. The isolate was selected identified using 16S rDNA [20].

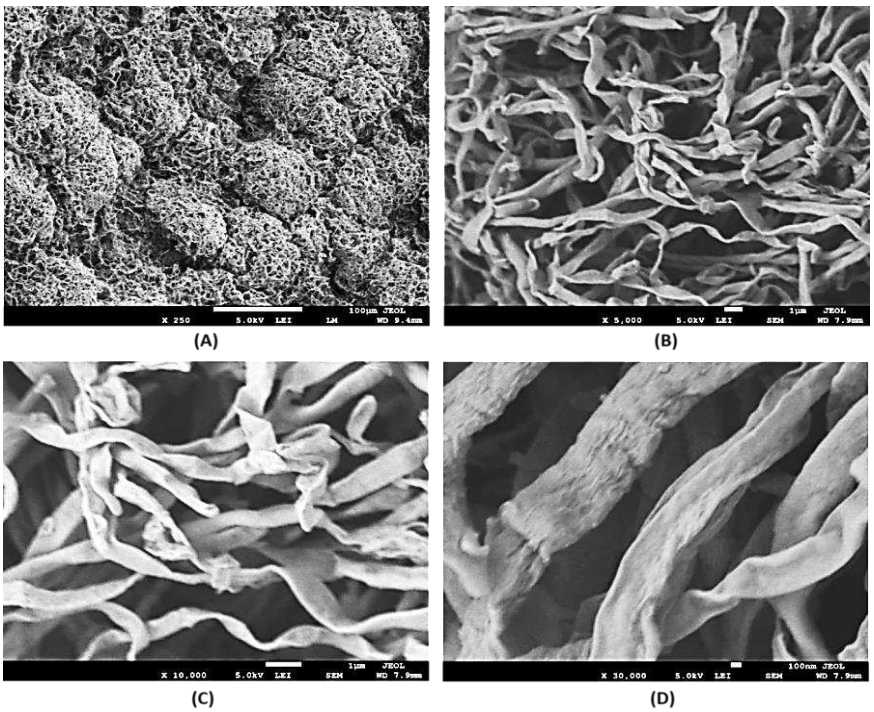

Figure 4. Scanning electron microscope of *Streptomyces* NM38. (A) x 250, (B) x 5,000, (C) x 10,000 and (D) x 30,000.

Physiological and biochemical characteristic

Melanoid pigments (brown soluble pigments) were produced after 7 days of the isolate incubation on tyrosine-glycerol agar at 30 °C (Figure 5).

Cellulose decomposition

After 7, 15, and 21 days of incubation at 30 °C, the filter paper strips were examined. There was no growth of isolate, indicating that Streptomyces NM38 can't decompose cellulose.

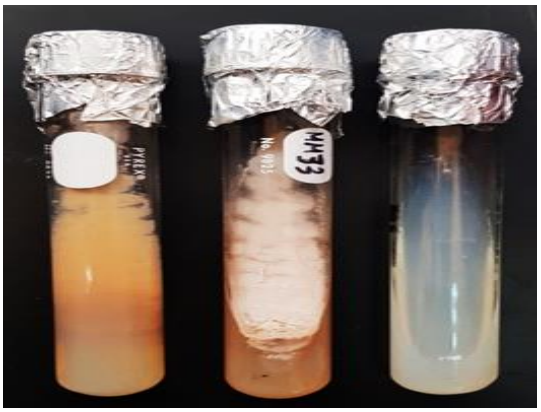

Figure 5. Melanin pigment of *Streptomyces* NM38 on Tyrosine-Glycerol Agar.

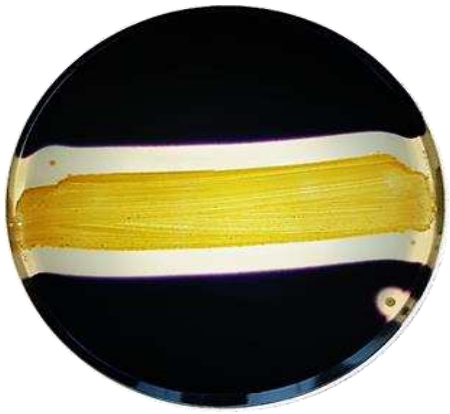

Figure 6. *Streptomyces* NM38 analysis of starch.

Gelatin liquefaction (production of proteolysis enzymes)

After 7, 15, and 21 days of the isolate incubation at 30 °C, the intensity of gelatin liquefaction was determined by comparison with uninoculated gelatin medium. The isolate has a very high degree of gelatin liquefaction.

Starch hydrolysis

Figure (6) showed a clear zone around the growth area of *Streptomyces* NM38, indicating that this isolate produces a high degree of starch analysis.

Utilization of different carbon and nitrogen Sources

Data in Figure (7) showed that the isolate could grow in the presence and absence of a carbon source. Like starch, glucose, or galactose. Feeble growth to very feeble growth was observed on lactose, sucrose, maltose, fructose, and mannitol. However, glycerol and xylose arrested the growth of the isolate. Figure (7) also showed that the isolate could assimilate potassium nitrate, sodium nitrate, calcium nitrate, ammonium sulphate, and ammonium chloride. Very feeble growth was observed on organic sources as urea and casein, whereas no Peptone, tryptone, or yeast extract's growth presence.

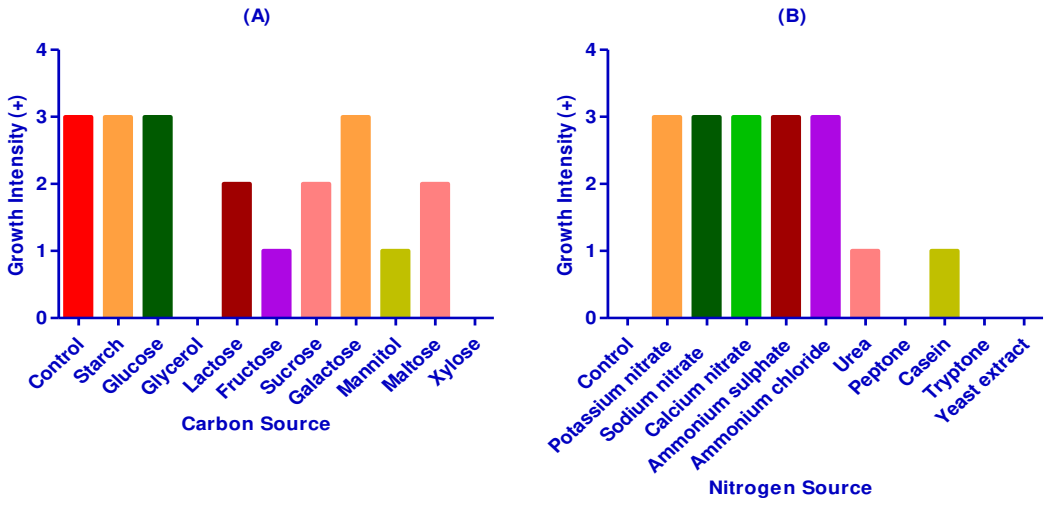

Figure 7. Usage of various carbon and nitrogen sources by *Streptomyces* NM38. 4 (+): good growth, 3 (+): moderate growth, 2 (+): feeble growth, 1 (+): very feeble growth, 0 (+): no growth.

Genetic identification

Molecular identification of the selected *Streptomyces* isolate (NM38) based on 16S rDNA gene

This strain displayed a taxonomic correlation with the isolated strain based on the comparative analysis of *Streptomyces* NM38 with the sequencing of the closest type species obtained by the NCBI BLAST method based on the trimmed and merged 16s rDNA sequencing analyzed using gene bank nucleotide blast alignment tools. *Streptomyces* NM38 was known as *Streptomyces globosus* with 100 % similarity percentages, under the accession number (MN538259.1) as shown in Table (3) and Figure (8). The 1500 bp 16S rDNA amplicon agarose gel electrophoresis after the purification compared with DNA Ladder 1kb on a 1 % agarose gel. Lane: 1kb DNA leader Figure (9).

16S rRNA sequencing is used to identify *Streptomyces* sp. [21, 22]. Even though 16S rRNA gene sequences are extremely beneficial for the classification of bacteria, it has a little amount of phylogenetic power and low discriminatory power for specific genera at the species level [23, 24].

Table 3. Molecular identification of the 16S rDNA gene-based isolate

Strain	Strains	Gene bank accession numbers	Identity %	Coverage %
Streptomyces NM38	*Streptomyces globosus*	MN538259.1	100%	100%

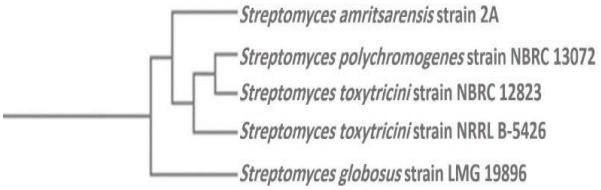

Figure 8. Based on 16S rRNA sequence analysis and the relation among *Streptomyces* and the most closely associated bacterial species, the Neighbor-joining phylogenetic tree.

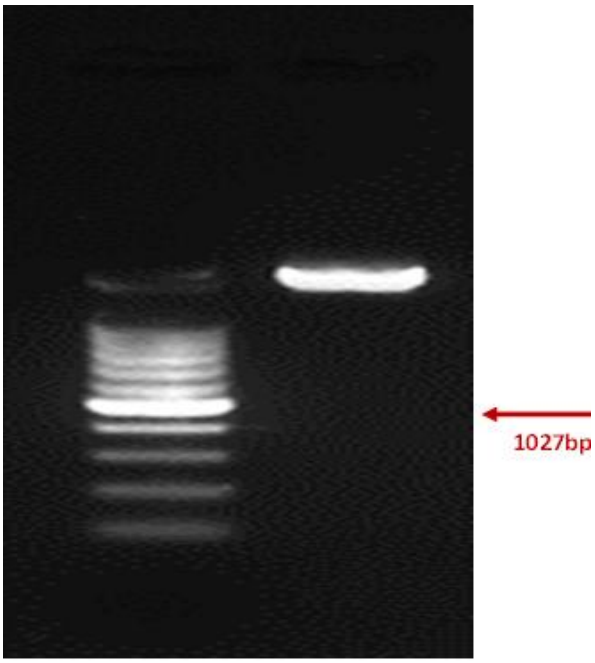

Figure 9. Compared to DNA Ladder 1 kb, the 1500 bp 16S rDNA amplicon agarose gel electrophoresis after purification on a 1 % agarose gel. Lane 1: 1kb DNA leader. Lane 2: Bacterial isolate.

CONCLUSION

In conclusion, there was a rich source of streptomycetes in plant rhizospheric soils. There was no previous report stating *S. globosus* antimicrobial activity. This study has demonstrated the ability of this isolate to produce Gram-positive and antibacterial agents. However, to discover the antibacterial compound, antibiotic production by this strain needs to be further analyzed in the future and other compounds as anticancer agents and to understand the genetic regulation of antibiotic production.

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