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Taxonomic Studies and Phylogenetic Characterization of *Streptomyces gancidicus* isolated from Al-Khurmah Governorate, KSA

Sami A. Al-Dhumri[1] and Houssam M. Atta[2]

[1]*Biology Department. Faculty of Al Khurmah University College, Taif University - KSA.*

[2]*Botany & Microbiology Department., Faculty of Science (Boys)-Al-Azhar University, Cairo, Egypt. & Biology Department. Faculty of Al Khurmah University College, Taif University - KSA.*

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Corresponding Author:
Houssam M. Atta

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ABSTRACT

This research aims to isolate , purify and identify some actinomycetes, that having antimicrobial substances against some pathogenic bacteria (Gram positive and Gram negative) and unicellular & filamentous fungi, from some soil samples collected from different localities in Al Khurmah governorate, KSA. Only one actinomycete culture KH-2019-38 exhibited to produce wide spectrum of antibacterial activities. Morphological characterization by scanning electron microscopic analysis followed by chemotaxonomic, physiological and biochemical characterizations were performed. The potent strain was identified by the 16S rDNA gene sequence (950 base pairs) and the Phylogenetic tree revealed the local isolate KH-2019-38 is closely related to Streptomyces gancidicus (99% similarity).Thus, it was given the suggested name Streptomyces gancidicus, KH-2019-38.

Introduction

Soil considered as a good source of potent microorganisms and is an excellent resource for identification and characterization of novel antibiotic producing microorganisms among which Actinomycetes are known for their potential features in the production of various antibiotics (Sreenivasa *et al.,* 2018). Actinomycetales are Gram-positive bacteria and capable of producing antibiotics, secondary metabolites and bioactive compounds. (Manteca *et al.,* 2005). Among them actinomycetes are the most economically and biotechnologically important prokaryotes; hold a prominent position due to their diversity and proven ability to produce novel bioactive compounds (Ghosh *et al.,* 2017). Actinomycetes produce approximately two-thirds of all know antibiotics in the market, most of these are from members of the genus *Streptomyces* (Barka *et al.,* 2016). The genus *Streptomyces* is a prominent source of pharmaceutically bioactive compounds (antibiotics, antitumor agents, anti-inflammatory compounds, and enzyme inhibitors) (Labeda *et al*. 2012 and Mohammed *et al.,* 2018). (Zhang *et al.;* 2020) reported that, Streptomycetes are prolific producers of antibiotics, and are responsible for producing more than 50% of our clinically relevant antibiotics (Van der Heul *et al.,* 2018). Identification of Actinomycetes filamentous structure using microscopic and scanning electron microscope is not enough further, the biochemical methods help in identification of isolates for genus level (Prakasham *et al.,* 2014). Presently with advanced technology, many researchers performing 16S rDNA sequence analysis of the Actinomycetes isolates for the species level identification (Tamura *et al.,* 2007).

In the present study, we have focused on isolation, morphological, physiological, biochemical and molecular characterization of Actinomycetes from soil samples.

Materials and Methods

Actinomycete isolate

The actinomycete isolate KH-2019-38 was isolated from soil sample collected from Al-Khurmah governorate, Saudi Arabia kingdom. It was purified using the soil dilution plate technique described by (Williams and Davies, 1965)

Test organisms

The references strains of Gram positive & Gram negative bacteria and unicellular & filamentous fungi used for screening of antimicrobial activity were collection from National Research Centre, Dokki-Giza, Egypt.

Culture media

The seed medium had the following composition (in g/L distilled water): glycerol, 15; bacto-peptone, 10; malt extract, 10; yeast extract, 1.0; K_2HPO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 0.75; $MnCl_2 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; and $ZnSO_4 \cdot 7H_2O$, 0.001. The pH of the medium was adjusted to 6.8 with NaOH 5.0 M before autoclaving at 121 °C for 15 min. The inoculum medium used in the cultivations, based on that proposed by (Maranesi *et al*., 2005), had the following composition (in g/L distilled water): soluble starch, 20; KNO_3, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 and $CaCO_3$, 0.1. pH 7.2 before sterilization. After 5 days of incubation at 30°C, the filtration was conducted followed by centrifugation at 4000 rpm for 15 min for investigating its potency to produce antimicrobial agents.

Screening for antimicrobial activity

The antimicrobial activity was determined by agar-well diffusion assay according to **(**Zamanian *et al*., 2005**).**

Conventional Taxonomy

The cultural, morphological, physiological and biochemical characteristics of strain KH-2019-38 were assessed following the guidelines adopted by the International *Streptomyces* Project (ISP) **(**Shrilling and Gottlieb, 1966**).** The diaminopimelic acid (LL-DAP) isomers (chemotaxonomy character) in the cell wall were analysed as described by **(**Lechevalier and Lechevalier, 1980**).** The media composition and the cultivation conditions were implemented as described by **(**Shrilling and Gottlieb, 1966**).** Colors characteristics were assessed on the scale developed by (Kenneth and Deane, 1955).

DNA Isolation and Manipulation

The locally isolated actinomycete strain was grown for seven days on a starch agar slant at 30°C. Two ml of a spore suspension were inoculated into the starch- nitrate broth and incubated for five days on a shaker incubator at 200 rpm and 30°C to form a pellet of vegetative cells (pre-sporulation). The preparation of total genomic DNA was conducted in accordance with the methods described by **(**Sambrook *et al*., 1989**).**

Amplification and Sequencing of the 16_S rDNA Gene

PCR amplification of the 16S rDNA gene of the local actinomycete strain was conducted using two primers, StrepF; 5.-ACGTGTGCAGCCCAAGACA-3. and Strep R; 5.ACAAGCCCTGGAAACGGGGT-3., in accordance with the method described by (Edwards *et al.,* 1989). The PCR mixture consisted of 30 pmol of each primer, 100 ng of chromosomal DNA, 200 µM dNTPs, and 2.5 units of Taq polymerase, in 50 µl of polymerase buffer. Amplification was conducted for 30 cycles of 1 min at 94°C, 1 min of annealing at 53°C, and 2 min of extension at 72°C. The PCR reaction mixture was then analyzed via agarose gel electrophoresis, and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The 16_S rDNA gene was sequenced on both strands via the dideoxy chain termination method, as described by **(**Sanger *et al*., 1977**).**

Sequence Similarities and Phylogenetic Analysis

The BLAST program (www.ncbi.nlm.nih. gov/blst) was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluating using BioEdit software (Hall, 1999). The phylogenetic tree was displayed using the TREE VIEW program.

Results

Screening for the antimicrobial activities

The metabolites of the *Streptomyces* sp. exhibited various degrees of activities against Gram positive and Gram negative bacteria viz: *Staphylococcus aureus*, NCTC 7447; *Bacillus subtilis*, NCTC 1040 ; *Bacillus pumilus*, NCTC 8214 ; *Micrococcus luteus*, ATCC 9341.*; Bacillus sphaericus ; Escherichia coli*, NCTC 10416; *Salmonella typhi; Klebsiella pneumonia*, NCIMB 9111; *Pseudomonas aeruginosa*, ATCC 10145 (Table 1).

Table 1. Mean diameters of inhibition zones (mm) caused by 100µl of the antimicrobial activities produced by KH-2019-38 in the agar-well diffusion assay (The diameter of the used cup assay was 10 mm).

Test organism	Mean diameters of inhibition zone (mm)
A-Bacteria	
1- Gram Positive	
Staphylococcus aureus, NCTC 7447	**29.0**
Bacillus pumilus, NCTC 8214	**27.0**
Micrococcus luteus, ATCC 9341	**28.0**
Bacillus subtilis, NCTC 1040	**29.0**
Bacillus sphaericus	**29.0**
- Gram Negative	
Escherichia coli, NCTC 10416	**25.0**
Salmonella typhi	**24.0**
Klebsiella pneumonia, NCIMB 9111	**23.0**
Pseudomonas aeruginosa, ATCC 10145	**21.0**
B- Fungi	
1-Unicellular fungi	
Candida albicans, IMRU 3669	**0.0**
Saccharomyces cervisiae ATCC 9763	**0.0**
2-Filamentous fungi	
Aspergillus niger IMI 31276	**0.0**
Aspergillusfumigatous, ATCC 16424	**0.0**
Aspergillus flavus, IMI 111023	**0.0**
Fusarium oxysporum	**0.0**
Penicillium chrysogenum	**0.0**

Identification of the Most Potent Actinomycete Isolate

Morphological Characteristics

The vegetative mycelia grew abundantly on both synthetic and complex media. The aerial mycelia grew abundantly on Starch-nitrate agar medium Oat-meal agar medium (ISP-3) and Inorganic salts-starch agar medium (ISP-4). The Spore chains were spirals, and had a spiny surface (Plate 1). Neither both sclerotic granules and sporangia nor flagellated spores were observed.

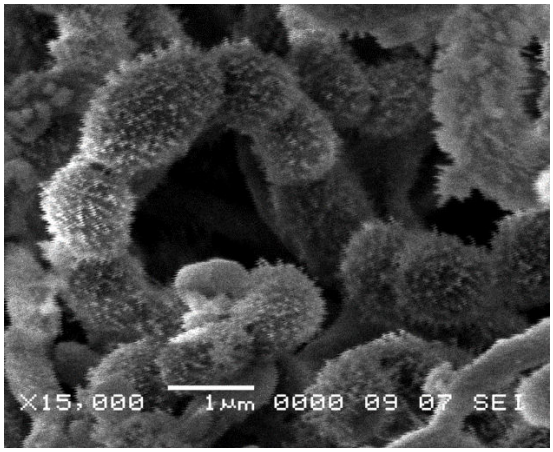

Plate 1. Scanning electron micrograph of the actinomycete isolate KH-2019-38 growing on starch nitrate agar medium showing spore chain Spiral shape and spore surfaces spiny (X15,000).

Cell Wall Hydrolysate

The cell wall hydrolysate contains LL-diaminopimelic acid (LL-DAP) and sugar pattern not detected.

Physiological and Biochemical Characteristics

The actinomycete isolate KH-2019-38 could hydrolyzes starch, protein, and lecithin, lipid, pectin hydrolysis and catalase test are negative, melanin pigment is negative, degradation of esculin & xanthin was positive, H_2S production, nitrate reduction, urea and KCN utilization were positive, whereas citrate utilization is negative (Table 2).The isolate KH-2019-38 utilizes D-mannose, D-mannitol, D-glucose, D-xylose, D-galactose, L-arabinose, D-fructose, L-rhamnose, maltose, lactose, *meso*-inositol, starch, L-arginine, L-tyrosine and L-Valine, but do not utilize sucrose, raffinose, L-histidine, L-cycteine and L-phenylalanine. Growth was detected in presence of up to (5%) NaCl. The isolate KH-2019-38 utilizes thallous acetate (0.001) but do not utilize in sodium azid (0.01%) and phenol (0.01%). Good growth could be detected within a temperature range of 25 to 40 ºC. Good growth could be detected within a pH value range of 5 to 8. Moreover, the actinomycete isolate KH-2019-38 are active against *Staphylococcus aureus*, NCTC 7447; *Bacillus subtilis*, NCTC 1040 ; *Bacillus pumilus*, NCTC 8214 ; *Micrococcus luteus*, ATCC 9341.*; Bacillus sphaericus ; Escherichia coli*, NCTC 10416; *Salmonella typhi; Klebsiella pneumonia*, NCIMB 9111; *Pseudomonas aeruginosa*, ATCC 10145(Table 2).

Colour and Culture Characteristics

The actinomycete isolate KH-2019.38 shows the aerial mycelium is light gray; substrate mycelium is light yellowish brown, and the diffusible pigment moderate brown to grayish yellowish brown for ISP-3, 4 , 6 & 7 (Table 3).

Taxonomy of Actinomycete Isolate

This was performed basically according to the recommended international Key's viz. (Williams, 1989; Hensyl, 1994 and Holt *et al*., 2000). On the basis of the previously collected data and in view of the comparative study of the recorded properties of actinomycete isolate in relation to the closest reference strain, viz. *Streptomyces gancidicus*, it could be stated that the actinomycetes isolate KH-2019-38 is suggestive of being likely belonging to *Streptomyces gancidicus*, KH-2019-38.

Molecular phylogeny of the selected isolate

The 16_S rDNA sequence of the local isolate was compared to the sequences of *Streptomyces* spp. In order to determine the relatedness of the local isolate to these *Streptomyces* strains. The phylogenetic analysis using the 16S rDNA gene sequence (950 bp) confirmed that KH-2019-38 strain belonged to the genus *Streptomyces.* The highest similarity Level was 99% with *Streptomyces gancidicus.* The phylogenetic tree illustrated in Fig. 1 showed that the KH-2019-38 strain forms a distinct phylogenetic position with closely related *Streptomyces gancidicus*.

Table 2. The morphological, physiological and biochemical characteristics of the actinomycete isolate KH-2019-38

Characteristic	Result	Characteristic	Result
Morphological characteristics:		Mannitol	++
Spore chains	Spirals	L- Arabinose	+
Spore mass	Gray	*meso*-Inositol	+
Spore surface	Spiny	Lactose	+
Colour of substrate mycelium	Grayish yellow brown	Maltose	+
Motility	Non-motile	D-fructose	+
Cell wall hydrolysate		**Utilization of amino acids:**	
Diaminopimelic acid (DAP)	LL-DAP	L-Cycteine	-
Sugar Pattern	Not-detected	L-Valine	+
Physiological and biochemical properties:		L-Histidine	-
Hydrolysis of:-		L-Phenylalanine	-

Starch	+	L-Arginine	+
Protein	+	L-Tyrosine	+
Lipid	+	**Growth with (% w/v)**	
Pectin	+	Sodium azide (0.01)	-
Lecithin	+	Phenol (0.1)	-
Catalase test	-	Thallous acetate (0.001)	+
Production of melanin pigment on:		**Growth at different temperatures (˚C):**	
Peptone yeast- extract iron agar	-	20	±
Tyrosine agar medium	-	25 - 40	++
Tryptone – yeast extract broth	-	45	±
Degradation of**:**		50	-
Xanthin	+	**Growth at different pH values:**	
Esculin	+	4	-
H_2S Production	+	5-8	+
Nitrate reduction	+	9	-
Citrate utilization	-	**Growth at different concentration of NaCl (%)**	
Urea test	+	1-7	+
KCN test	+	8	-
Utilization ofcarbon sources		**Antagonistic Effect:**	
D-Xylose	+	*Staphylococcus aureus*, NCTC 7447; *Bacillus subtilis*, NCTC 1040 ; *Bacillus pumilus*, NCTC 8214 ; *Micrococcus luteus*, ATCC 9341.*; Bacillus sphaericus ; Escherichia coli*, NCTC 10416; *Salmonella typhi; Klebsiella pneumonia*, NCIMB 9111; *Pseudomonas aeruginosa*, ATCC 10145	+
D- Mannose	+		
D- Glucose	+		
D- Galactose	+		
Sucrose	-		
L-Rhamnose	++		
Raffinose	-		
Starch	+++		

+=Positive, - = Negative and± = doubtful results, ++ = good growth.

Table 3. Cultural characteristics of the actinomycete isolate KH-2019-38

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
1- Starch-nitrate agar medium	Good	264-1. gray light gray	76-1-y-br Light yellowish Brown	-
2- Yeast extract - Malt extract agar medium (ISP-2)	Good	264-1. gray light gray	60-I.gy.Br Light grayish brown	57-I.Br Light brown
3- Oat-meal agar medium (ISP-3)	Good	264-1. gray light gray	95-m.OI.Br Medium orange brown	80-gy.yBr Grayish yellowish brown
4- Inorganic salts-starch agar medium (ISP-4)	Good	264-1. gray light gray	95-m.OI.Br Medium orange brown	80-gy.yBr Grayish yellowish brown
5- Glycerol-Asparagine agar medium (ISP-5)	No growth	-	-	-
6- Melanin test: Tryptone-yeast extract broth (ISP-1)	No growth	-	-	-
b- Peptone yeast extract-iron agar medium (ISP-6)	Good	264-1. gray light gray	76-1-y-br Light yellowish Brown	58-m. Br Moderate brown
c- Tyrosine agar (ISP-7)	Good	264-1. gray light gray	57-I. Br Light brown	77—m.ybr moderate yellowish brown

The colour of the organism under investigation was consulted using the ISCC-NBS colour - Name charts II illustrated with centroid colour.

Discussion

The aerobic actinomycetes are soil-inhabiting microorganisms which are the major source of interest worldwide for the commercial drug industry and have been considered as an extremely useful microorganism for producing novel antimicrobial agents (Baltz 2007; Kampfer *et al.*, 2009 and Prakasham *et al.,* 2014). The *Streptomyces gancidicus* was isolated from Al-Khurmah governorate, KSA. Many similar observations were reported such as, 31 Actinomycetes strains were isolated from soil samples from sheopur (Hotam *et al.,* 2013), and Ninety-seven Actinomycetes strains were isolated from fifty soil samples collected from the Taif City, Saudi Arabia (Atta, 2011). The isolate was growing on production medium had the following composition (in g/L distilled water): soluble starch, 20; KNO_3, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 and $CaCO_3$, 0.1. pH 7.2 for investigating its potency to produce antimicrobial agents. The actinomycete isolate, exhibited a wide spectrum antibacterial agents against Gram positive and Gram negative bacteria. Previous studies showed that many species of *Streptomyces* had antimicrobial

activity (Reddy *et al.,* 2011; Atta *et al.,* 2014, Saravanakumar *et al.,* 2014 and Hosny *et al.,* 2015). Due to the selective isolation of soil actinomycetes for finding novel strains which can produce useful bioactive compounds, thus various culture media and techniques have been developed (Hozzein *et al*., 2008 and Dhananjeyan *et al*., 2010). Identification process had been performed (Williams, 1989; Hensyl, 1994 and Holt *et al*., 2000). The morphological characteristics and microscopic examination emphasized that the spore chain is spiral. Spore mass is gray, while spore surface is spiny, substrate mycelium is light yellowish-brown and diffusible pigment moderate brown to grayish yellowish brown. The results of physiological, biochemical characteristics (Table 2) and cell wall hydrolysate of actinomycete isolate, exhibited that the cell wall containing LL-diaminopimelic acid (DAP). These results emphasized that the actinomycetes isolate related to a group of *Streptomyces* as previously studied (Raja and Prabakarana, 2011; Muharram *et al*., 2013 and Ghosh *et al.,* 2017). The phylogenetic tree (diagram) revealed that the local isolate is closely related *Streptomyces gancidicus*, similarity matrix is 99% as identified strain of *Streptomyces gancidicus*. In view of all the previously recorded data, the identification of actinomycete isolate KH-2019-38 was suggestive of being belonging to *Streptomyces gancidicus*, KH-2019-38. Similar results were reported earlier in 16S rDNA gene sequencing as a powerful method for identification of prokaryotic organisms (Sreenivasa *et al.,* 2018). The number of Actinomycetes are identified by sequencing the 16S rDNA gene as *Streptomyces flavogriseus* (Dezfully and Ramanayaka, 2015).

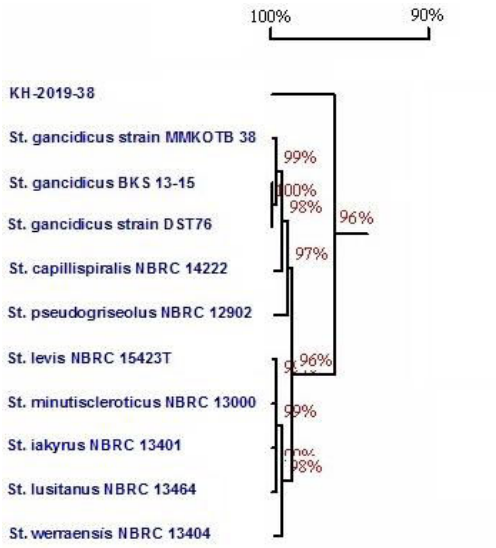

Fig. 1. The phylogenetic position of the local *Streptomyces* sp. strain among neighboring species. The phylogenetic tree was based on the multiple alignments options of 16_S rDNA sequences.

Conclusion

The present investigation was carried out on isolation and functional screening of potential soil Actinomycetes for potent antimicrobial properties. Only one actinomycete culture KH-2019-38 exhibited to produce wide spectrum of antibacterial activities. Actinomycete culture KH-2019-38 are identified by sequencing the 16S rDNA gene as *Streptomyces gancidicus*. Hence the *Streptomyces gancidicus,* KH-2019-38 has to be subject for further analysis for its ability in quality production of antibiotics and antitumor also be screened for synthesis of nanoparticles for its potential applications in nano-agriculture as such application are greatly encouraged nowadays.

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