Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 9 (1): 1-15, 2014
ISSN 1816-4935 / DOI: 10.3923/jm.2014.1.15

Microbiological Studies on the Production of Antimicrobial Agent by Actinomycete Isolated from Saudi Arabia

[1,2]Magdy M. Afifi, [3,4]Houssam M. Atta and [3,4]Reda A. Bayoumi
[1]Department of Botany and Microbiology, Faculty of Science, Al-Azhar University, Assuit, 71524, Egypt
[2]Department of Applied Medical Science, Faculty of Applied Medical Science, King Khalid University, Bisha, 551, KSA
[3]Department of Botany and Microbiology, Faculty of Science (Boys), Al-Azhar University, Cairo, Egypt
[4]Department of Biotechnology, Faculty of Science and Education, Al-Khurmah Branch, Taif University, KSA

Corresponding Author: Magdy M. Afifi, Department of Botany and Microbiology, Faculty of Science, Al-Azhar University, Assuit, 71524, Egypt

ABSTRACT

This research aims to isolate and identify some actinomycetes, that having antimicrobial substances against some pathogenic bacteria and fungi, from soil samples in Al-Khurmah governorate. Only one actinomycete culture KSA-20 exhibited to produce wide spectrum of antibacterial activities. The nucleotide sequence of the 16S rRNA gene of this strain evidenced an 95% similarity with *Streptomyces griseoviridis*. From the taxonomic features, the actinomycete isolate KSA-20 matches with *Streptomyces griseoviridis* in the morphological, physiological and biochemical characters. Thus, it given the suggested name *Streptomyces griseoviridis* KSA-20. The parameters controlling the biosynthetic process of antibacterial agent were fully investigated. The separation of the active ingredient and its purification were performed using both Thin Layer Chromatography (TLC) and Column Chromatography (CC) techniques. The minimum inhibition concentrations "MICs" of the purified antibiotic were also determined. The purified antibiotic suggestive of being belonging to griseoviridin antibiotic produced by *Streptomyces griseoviridis* KSA-20.

Key words: *Streptomyces griseoviridis*, griseoviridin, 16S rRNA, spectroscopic analysis, MIC

INTRODUCTION

Actinomycetes play an important ecological role in recycling substances in the nature and able to inhibit both Gram-positive and Gram-negative pathogen (Khucharoenphaisan *et al*., 2012). In addition, *Streptomyces griseoviridis* is known to produce antimicrobial metabolites and be value in a biological control program (Cuppels *et al*., 2013). Griseoviridin/viridogrisein from *Streptomyces griseoviridis* follow streptogramin family (Ahmed and Donaldson, 2007; Barriere *et al*., 1998). Several pharmaceutical companies described various similar compounds in streptogramin antibiotic but did not develop them further (Greenwood, 2008). Their regulation is highly complex and influenced by general physiological conditions (Zhang *et al*., 2012). Many investigations have neen carried out for synthesizes of antibiotics which possess biological activities from *Streptomyces* from soil samples (Afifi *et al*., 2012a, b; Atta *et al*., 2011).

In the present study, the production of the bioactive substances that exhibited inhibitory effects on some pathogenic bacteria and fungi from *Streptomyces griseoviridis* were reported, along with some physico-chemical properties of secondary metabolites with high biological effects.

MATERIALS AND METHODS

Actinomycete strain: Strain KSA-20 was isolated from a suspension of a soil sample (Williams and Davies, 1965) collected from Al-Khurmah governorate, Saudi Arabia and inoculated onto a Starch-nitrate agar medium (SNA). Plates were incubated at 35°C for five days. The isolates were individually maintained on Starch-nitrate agar at 4°C and stored as a mixture of hyphae and spores in 20% glycerol at 80°C. The selected isolate was allowed to grow on (SNB) in a purpose to get a clear supernatant for antimicrobial activity.

Test organisms

Bacteria

Gram-positive bacteria: *Staphylococcus aureus*, NCTC 7447; *Bacillus subtilis*, NCTC 1040; *Bacillus pumilus*, NCTC 8214 and *Micrococcus luteus*, ATCC 9341.

Gram-negative bacteria: *Escherichia coli*, NCTC 10416; *Klebsiella pneumonia*, NCIMB 9111 and *Pseudomonas aeruginosa*, ATCC 10145.

Fungi

Unicellular fungi: *Candida albicans*, IMRU 3669 and *Saccharomyces cerevisiae,* ATCC 9763.

Filamentous fungi: *Aspergillus niger*, IMI 31276; *A. flavus*, IMI 111023, *A. fumigatous*, ATCC 16424; *Fusarium oxysporum and Penicillium chrysogenium*.

Screening for antimicrobial activity: The antimicrobial activity was determined by cup method (Kavanagh, 1972).

Taxonomic studies of actinomycete isolate: Morphological characteristics of the most potent produces strain KSA-20 grown on SNA medium at 35°C for 5 days was examined under scanning electron microscopy (JEOL Technics Ltd.) (Fig. 1).

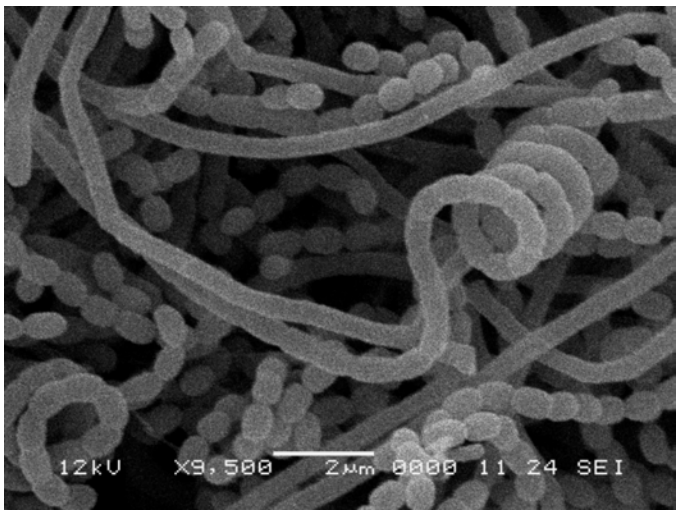

Fig. 1: Scanning electron micrograph of the actinomycete isolate KSA 20 growing on SNA showing spiral shape spore chain and smooth spore surface (X 9.500)

Physiological and biochemical characteristics: The ability of the strain to produce different enzymes was examined by using standard methods. Lecithinase was conducted on egg-yolk medium (Nitsch and Kutzner, 1969), Lipase (Elwan *et al.*, 1977), Protease (Chapman, 1952), Pectinase (Hankin *et al.*, 1971); α-amylase (Cowan, 1974) and Catalase (Jones, 1949). Melanin pigment (Pridham *et al.*, 1957). Degradation of Esculin and Xanthine (Gordon *et al.*, 1974). Nitrate reduction (Gordon, 1966). Hydrogen sulphide production and oxidase test (Cowan, 1974). The utilization of different carbon and nitrogen sources (Pridham and Gottlieb, 1948). Cell wall was performed (Becker *et al.*, 1964; Lechevalier and Lechevalier, 1970). Cultural characteristics such as color of aerial mycelium, color of substrate mycelium and pigmentation of the selected actinomycete were recorded (Shirling and Gottlieb, 1966). Color characteristics were assessed (Kenneth and Deane, 1955).

DNA isolation and manipulation: The locally isolated actinomycete strain was grown for 5 days on a starch agar slant at 35°C. Two milliliter of a spore suspension were inoculated into the starch-nitrate broth and incubated for 3 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 (Sambrook *et al.*, 1989).

Amplification and sequencing of the 16S rRNA gene: PCR amplification of the 16S rRNA gene of the local actinomycete strain was conducted using two primers, StrepF; 5-ACGTGTGCAGCCCAAGACA-3 and StrepR; 5-ACAAGCCCTGGAAACGGGGT-3 (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 16S rRNA gene was sequenced on both strands via the dideoxy chain termination method (Sanger *et al.*, 1977). The 16S rRNA gene (1.5 kb) sequence of the PCR product was acquired using a Terminator Cycle Sequencing kit (ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA).

Sequence similarities and phylogenetic analysis: The BLAST program (www.ncbi.nlm.nih.gov/blast) 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.

Fermentation: The *Streptomyces griseoviridis*, KSA-20 inoculum was introduced aseptically into each sterile flask containing the following ingredients (g L^{-1}): glucose, 20; KNO_3, 2.0; K_2HPO_4, 0.8; $MgSO_4.7H_2O$, 0.7 and KCl, 0.5. The pH was adjusted at 7.0 before sterilization. After 5 days of incubation at 35°C. Filtration was carried out through cotton wool and followed by centrifugation at 5000 rpm for 15 min.

Extraction: The culture filtrates was extracted twice with n-Butanol and the pooled solvent extracts were evaporated to dryness under vacuum to yield a crude residue.

Precipitation: The precipitation process of the crude compound was carried out using petroleum ether (bp 60-80°C) followed by centrifugation at 5000 rpm for 15 min.

Purification by TLC: Separation of the antimicrobial compound into its individual components was conducted by thin layer chromatography using chloroform and methanol (24: 1, v/v) as a solvent system.

Purification by column chromatography: The purification of the antimicrobial compound was carried out using silica gel column (2.5×50) chromatography. Chloroform and methanol 10:2 (v/v), was used as an eluting solvent. The column was left overnight until the silica gel (Prolabo) was completely settled. One milliliter crude extract to be fractionated was added on the silica gel column surface and the extract was adsorbed on top of silica gel. Fifty fractions were collected (each of 5 mL) and tested for their antimicrobial activities.

Physicochemical properties

Elemental analysis: The elemental analysis including C, H, O, N and S was carried out at the microanalytical center, Cairo University, Egypt.

Spectroscopic analysis: The IR, UV and Mass spectrum were determined at the micro analytical center of Cairo University, Egypt.

Biological activity: The Minimum Inhibitory Concentration (MIC) has been determined by the cup method (Kavanagh, 1972).

Characterization of the antimicrobial agent: The antibiotic produced by *Streptomyces griseoviridis*, KSA-20 was identified according to the recommended international references of Berdy (1974) and Umezawa (1977).

RESULTS

Screening for the antimicrobial activities: The active metabolites produced by *Streptomyces griseoviridis*, KSA-20 exhibited various degrees of activities against Gram positive and Gram negative bacteria as reported in Table 1.

Identification of the actinomycete isolate

Morphological characteristics: Spore chain is spiral, spore mass is red; spore surface is smooth and the reverse is light grayish yellow; diffusible pigment production is grayish red orange.

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

Physiological and biochemical characteristics: The actinomycete isolate KSA-20 could hydrolyze protein, starch, cellulose, lipid, lecithin and pectin, while catalase and melanin pigment are negative. In addition, degradation of xanthine, esculine production of H_2S, decomposition of

Table 1: Mean diameters of inhibition zones (mm) caused by 100 µL of the antimicrobial activities produced by *Streptomyces griseoviridis*, KSA-20 in the agar plate diffusion assay (The diameter of the used cup assay was 10 mm)

Test organism	Mean diameters of inhibition zone (mm)
Bacteria	
Gram positive	
Staphylococcus aureus NCTC 7447	33.0
Bacillus subtilis NCTC 1040	33.0
Bacillus pumilus NCTC 8214	32.0
Micrococcus luteus ATCC 9341	33.5
Gram negative	
Escherichia coli NCTC 10416	29.0
Klebsiella pneumonia NCIMB 9111	28.0
Pseudomonas aeruginosa ATCC 10145	27.0
Fungi	
Unicellular fungi	
Candida albicans IMRU 3669	0.0
Saccharomyces cerevicea ATCC 9763	0.0
Filamentous fungi	
Aspergillus niger IMI 31276	0.0
Aspergillus fumigatus ATCC 16424	0.0
Aspergillus flavus IMI 111023	0.0
Fusarium oxysporum	0.0
Penicillium chrysogenum	0.0

urea, nitrate reduction and utilization of citrate are positive but coagulation of milk are negative. The isolate under study utilizes raffinose, mannitol, L-arabinose, meso-inositol, lactose, maltose, trehalose, D-meliztose, D-mannose, D-glucose and rhamnose but do not utilize D- galactose, D- fructose and sucrose, whereas, D-xylose and sodium citrate are doubtful. Good growth on L-cycteine, L-histidine and L-arginine. No growth on L-valine, L-phenyl alanine, L-lysine, L-Hydroxyproline and L-glutamic acid. Moreover, good growth in the presence of 4% NaCl. The growth is not inhibited in the presence of sodium azide and phenol. The actinomycete isolate showed non-resistant to Pencillin G, Amoxicillin, Gentamycin and Cephalosporin "Keflex". Moreover, it resistant to Augmentin, Carpenicillin, Cefadroxil, Cephardine Ceptrine, Chloramphinicol, Cloxacillin, Doxycycline, Neomycin, Erythromycin, Rifampicin and Noroxin. The isolate KSA-20 is active against Gram positive and Gram negative bacteria (*S. aureus*, NCTC 7447; *B. subtilis*, NCTC 1040; *M. luteus*, NCTC 1089; *E. coli*, NCTC 10416 and *K. pneumonia* NCIMB 9111) (Table 2).

Color and culture characteristics: The isolate KSA-20 exhibited good growth on SNA, the aerial mycelium is medium red, substrate mycelium is light yellowish brown and the diffusible pigment grayish red orange. No growth on tryptone-yeast extract broth medium (ISP-1), yeast extract-malt extract agar medium (ISP-2) and glycerol-asparagine agar medium (ISP-4). Moderate growth was detected on Oat-meal agar medium (ISP-3), aerial mycelium is medium is red, substrate mycelium light garish orange and failed to produce diffusible pigment. Moderate growth was detected on inorganic salts-starch agar medium (ISP-4), aerial mycelium reddish gray, substrate mycelium light yellowish brown and diffusible pigment grayish red orange. Moderate growth was detected on peptone yeast extract-iron agar medium (ISP-6), aerial mycelium medium

Table 2: Morphological, physiological and biochemical characteristics of the actinomycete isolate

Characteristic	Result*	Characteristic	Result
Morphological characteristic		Raffinose	+
Spore chain	Spiral	Mannitol	+
Spore mass	Red	L-arabinose	+
Spore surface	Smooth	Meso-Inositol	+
Color of substrate mycelium	Yellow brown	Lactose	+
Diffusible pigment	Grayish red orange Non-	Maltose	+
Motility	motile	Trehalose	+
Cell wall hydrolysate		D-melizitose	+
		D-fructose	-
Diaminopimelic acid (DAP)	LL-DAP	Sodium citrate	±
Sugar pattern	Not-detected	**Utilization of different amino acids**	
Physiological and biochemical properties		L-Cycteine	+
Hydrolysis		L-Valine	-
Protein, starch, cellulose, lipid	+	L-histidine	+
Egg-yolk (lecithin) and Pectin	+	L-Phenylalanine	-
Catalase test	-	L-Arginine	+
Resistance of different antibiotics		L-Lysine	-
Pencillin G, Amoxicillin,	-	L-Hydroxproline	-
Gentamycin and		L-Glutamic acid	-
Cephalosporin (Keflex)		**Growth inhibitors**	
Augmentin Carbenicillin,	+	Thallous acetate (0.001)	+
Cefadroxil, Cephardine		Sodium azide (0.01)	+
Ceptrine, Chloramphinicol,		Phenol (0.1)	+
Cloxacillin, Doxycycline,		**Growth at different temperatures (°C)**	
Neomycin Erythromycin,		10	-
Rifampicin and Noroxin		20-45	+
Production of melanin pigment		50	-
Peptone yeast-extract iron	-	**Growth at different pH values**	
Tyrosine agar medium (ISP-7)	-	4	-
Tryptone-yeast extract broth	-	10	-
Degradation		**Growth at different concentrations of NaCl (%)**	
Xanthin	+	1-7	+
Esculin	+	10	-
H_2S production	+	**Active against**	
Nitrate reduction	+	*Staph. aureus*, NCTC 7447 *B. subtilis*, NCTC 1040; *M. luteus*, NCTC 1089, *E. coli*, NCTC 10416. and *K. pneumonia* NCIMB 9111	+
Citrate utilization	+		
Urea test	+		
Coagulation of milk	-		
Utilization of different carbon sources			
D-Xylose	±		
D-Mannose	+	*C. albicans* IMRU 3669, *S. cervicea*, *Asp. niger*, IMI 31276; *Asp. Fumigatus*, *Asp. flavus* IMI 111023, *Fusarium oxysporum* and *Penicillium chrysogenum*	-
D-Glucose	+		
D-galactose and sucrose	-		
Rhamnose	+		

*+: Positive, -: Negative and ±: Doubtful results

Table 3: Cultural characteristics of the actinomycete isolate

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Starch nitrate agar medium	Good	15- med. Red	76-1-y-br	39-gy.ro
		Medium red	Light yellowish brown	Grayish red orange
Tryptone yeast extract broth (ISP-1)	No growth	-	-	-
Yeast extract malt extract agar medium (ISP-2)	No growth	-	-	-
Oat-meal agar medium (ISP-3)	moderate	15- med. Red	109. I. gy.or	-
		Medium red	Light grayish orange	
Inorganic salts starch agar medium (ISP-4)	Good	22-r. Gray	76-1-y-br	39-gy.ro
		Reddish gray	Light yellowish brown	Grayish red orange
Glycerol-Asparagine agar medium (ISP-5)	No growth	-	-	-
Peptone yeast extract iron agar medium (ISP-6)	moderate	15- med. Red	76-1-y-br	39-gy.ro
		Medium red	Light yellowish brown	Grayish red orange
Tyrosine agar medium (ISP-7)	Poor	22-r. Gray	90-gy. y	58 m-br
		Reddish gray	Grayish yellow	moderate brown

*The color of the organism under investigation was consulted with the ISCC-NBS color-name charts illustrated with centroid color

red, substrate mycelium light yellowish brown and diffusible pigment grayish red orange. Poor growth was detected on tyrosine agar medium (ISP-7), aerial mycelium reddish gray, substrate mycelium grayish yellow and diffusible pigment moderate brown (Table 3).

Taxonomy of actinomycete isolate, KSA-20: This was performed basically according to the recommended international Key's (Buchanan and Gibbons, 1974; Hensyl, 1994; NTP, 1989). On the basis of the previously collected data and in view of the comparative study of the recorded properties of KSA-20 in relation to the most closest reference strain, viz., *Streptomyces griseoviridis*, it could be stated that actinomycetes isolate, KSA-20 is suggestive of being likely belonging to *Streptomyces griseoviridis*, KSA-20 (Table 4).

Amplification of the 16S rRNA gene: The 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) using the universal primers. The primers that was used to 16S rRNA sequencing were of the sequence strepF; 5'-ACGTGTGCAGCCCAAGACA-3' and strepR; 5'-ACAAGCCCTGGAAACGGGGT-3', the product of the PCR was analyzed on 1.5% ethidium bromide gel.

Molecular phylogeny of the selected isolate: The 16S 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 *Streptomyces* strains, the phylogenetic tree (as displayed by the Tree View program) revealed that the locally isolated strain is closely related to *Streptomyces* sp., rather related to *Streptomyces* sp. rather than to *Streptomyces griseoviridis* (Fig. 2). Multiple sequence alignment was conducted the sequences of the 16S rRNA gene of *Streptomyces griseoviridis*. Computer assisted DNA searches against bacterial database similarly revealed that the 16S rRNA sequence was 95% identical *Streptomyces griseoviridis* (Fig. 2).

Fermentation, extraction and purification: The fermentation process was carried out for five days at 35°C. After incubation period, the filtration was conducted followed by centrifugation at 4000 rpm for 15 min. The entire culture broth (20 L) was centrifuged (4000 rpm, 15 min) to

Table 4: Numerical taxonomy of *Streptomyces* species program (PIB WIN)

Characteristic	KSA-20*	*Streptomyces griseoviridis*
Dianinopimelic acid (DAP)	LL-diaminopimelic acid	LL-diaminopimelic acid
Sugar pattern	Not detected	Not detected
Spore chain rectiflexibles	-	-
Spore mass Spiral	+	+
Spore mass red	+	+
Spore mass gray	-	-
Diffusible pigment red/orange	+	+
Diffusible pigment yellow/brown	-	-
Melanin pigment		
Peptone yeast extract-iron agar medium	-	-
Tyrosine agar medium	-	-
Active against		
B. subtilis and *M. luteus*	+	+
C. albicans and *A. niger*	-	-
Hydrolysis of: Lecithin-lipid-Pectin	+	+
Nitrate reduction	-	-
H_2S production	+	+
Degradation of Xanthin	+	+
Resistance of		
Neomycin	-	-
Rifampicin and Pencillin G.	+	+
Growth at 45°C	+	+
Growth at NaCl 7% (w/v)	+	+
Growth inhibitors		
Phenol (0.1% w/v) and Thallus acetate (0.001% w/v)	+	+
Utilization of		
L-Cysteine, L-Histadine	+	+
L-Valine, L-phenylalanine and L-Hydroxproline	-	-
Sucrose	-	-
Rhamnose; Raffinose and meso-Inositol	+	+

No.	Key	Source	Identification	ID Score
1	KSA-20	KSA	*Streptomyces griseoviridis*	0.99963

*+: Positive, -: Negative, ±: Doubtful results

separate the mycelium and the supernatant. The supernatant was extracted with n-butanol (1:1, v/v) and the organic layer was evaporated to give an oily material. The oily material was then dissolved in 15% aqueous methanol and defatted by partitioning with petroleum ether (bp 60-80°C) to give a solid extract. Its color is yellowish. Separation of antimicrobial agent into individual components was carried out by TLC using a solvent system composed of chloroform and methanol (24: 1, v/v). Only one band at R_f = 0.7 showed antimicrobial activity. The purification process through column chromatography packed with silica gel, revealed that the most active fractions against the tested organisms ranged between 20 to 30.

Physicochemical characteristics: The purified antibacterial agent produced by *Streptomyces griseoviridis*, KSA-20 has a characteristic odour and a melting point of 155°C. The compound is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10% isopropyl alcohol but insoluble in petroleum ether, hexan and benzene.

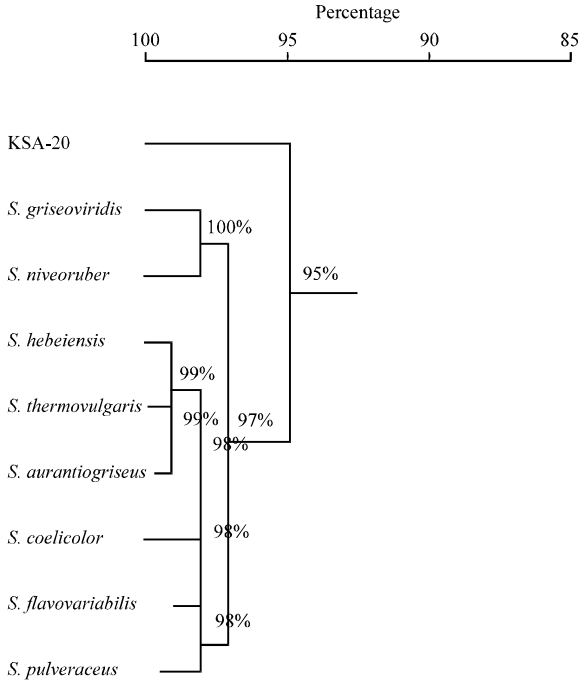

Fig. 2: The phylogenetic position of the local *Streptomyces* sp. strain among neighboring species. The phylogenetic tree was based on the pairwise comparisons of 16S rRNA sequences

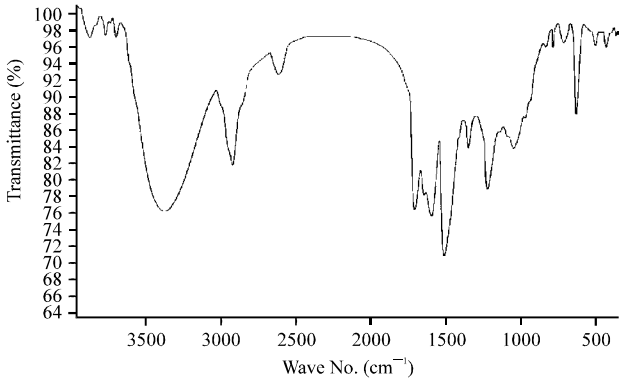

Fig. 3: IR spectrum of antibacterial agent produced by *Streptomyces griseoviridis*, KSA-20

Elemental analysis: The elemental analytical data of the antibacterial agent produced by *Streptomyces griseoviridis*, KSA-20, given the following percent composition: C = 55.33%, H = 5.70%, N = 8.80%, O = 23.45% and S = 6.71%. This analysis indicates a suggested empirical formula of $C_{22}H_{27}N_3O_7S$.

Spectroscopic characteristics: The spectroscopic analysis of the purified of antibacterial compound produced by *Streptomyces griseoviridis*, KSA-20; showed the following data: The infrared (IR) sepectrum showed characteristic band corresponding to 26 peaks (Fig. 3), the ultraviolet (UV) absorption spectrum recorded a maximum absorption peak at 221 nm (Fig. 4) and the mass spectrum showed molecular weight at 477.4 (Fig. 5).

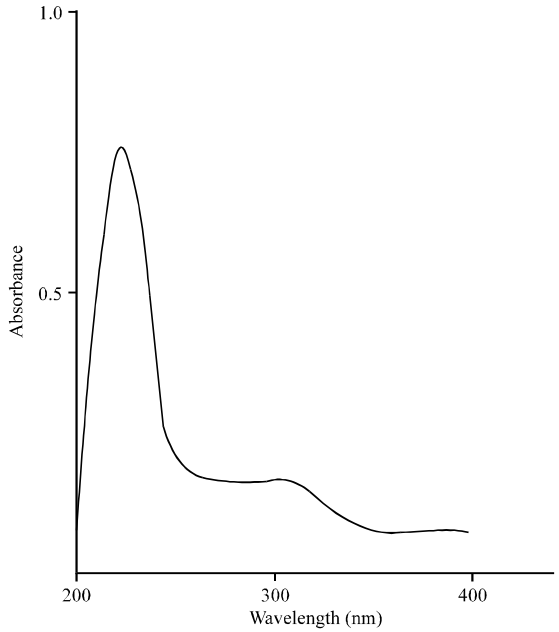

Fig. 4: Ultraviolet absorbance of antibacterial agent produced by *Streptomyces griseoviridis*, KSA-20

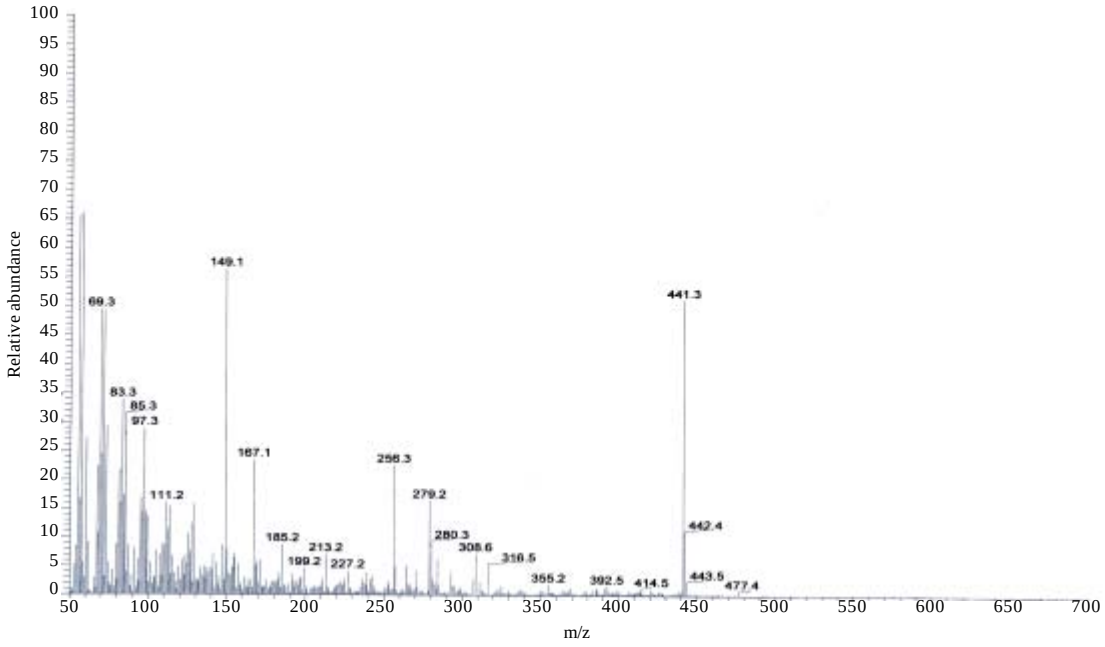

Fig. 5: Mass spectrum of antibacterial agent produced by *Streptomyces griseoviridis*, KSA-20

Biological activities of the antimicrobial agent: Data recorded in Table 5 indicated that the antimicrobial agent is fairly active against both Gram positive and Gram negative bacteria.

Table 5: Biological activities (MIC) of the antibacterial agent by cup method assay

Test organisms	MIC (μg mL^{-1}) concentrations
Bacteria	
Gram positive cocci	
Staph. aureus NCTC 7447	7.8
Micrococcus luteus ATCC 9341	7.8
Gram positive bacilli	
Bacillus pumilus NCTC 8214	15.6
Bacillus subtilis NCTC 10400	11.7
Gram negative bacteria	
Escherichia coli NCTC 10416	23.43
Klebsiella pneumonia NCTC 9111	31.25
Pseudomonas aeruginosa ATCC 10415	62.50

Identification of the antimicrobial agent: On the basis of the recommended keys for the identification of antibiotics and in view of the comparative study of the recorded properties of the antimicrobial agent, it could be stated that the antibacterial compound is suggestive of being belonging to griseoviridin antibiotic (Berdy, 1974; Umezawa, 1977).

DISCUSSION

Actinomycetes had been recognized as the potential producers of metabolites such as antibiotics, growth promoting substances for plants and animals, immunmodifiers, enzyme inhibitors and many other compounds of use to man. The actinomycetes have the ability to produce secondary metabolites with biological activities such as antibiotic, antifungal compounds (Afifi *et al*., 2012a, b; Atta *et al*., 2011). The *Streptomyces griseoviridis*, KSA-20 was isolated from Al-Khurmah governorate and produce a wide spectrum antimicrobial agent against Gram positive and Gram negative bacteria and unicellular and filamentous Fungi. *Streptomyces griseoviridis*, KSA-20 isolate was growing on SNA to investigate its potency to produce antimicrobial agents.

The *Streptomyces* species under investigation exhibited a wide spectrum antibacterial agent as previously reported (Dhananjeyan *et al*., 2010; Kavanagh, 1972; Zamanian *et al*., 2005). 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 deveoped (Dhananjeyan *et al*., 2010; Hozzein *et al*., 2008). Identification process had been performed (Hensyl, 1994; NTP, 1989; Williams, 1989). The morphological characteristics and microscopic examination emphasized that the spore chain is spiral. Spore mass is red, while spore surface is smooth, substrate mycelium is light yellow-brown and diffusible pigment grayish red orange. The results of physiological, biochemical characteristics (Table 2) and cell wall hydrolysate of actinomycetes 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 (Afifi *et al*., 2012a, b; Muharram *et al*., 2013; Raja *et al*., 2010; Zamanian *et al*., 2005).

The phylogenetic tree (diagram) revealed that the local isolate is closely related *Streptomyces griseoviridis*, similarity matrix is 95% as identified strain of *Streptomyces plicatus* (strain 101) by Zamanian *et al*. (2005) and *Streptoverticillium* sp. and two *Streptomyces* sp. by Raja *et al*. (2010). In view of all the previously recorded data, the identification of actinomycete isolate KSA-20 was suggestive of being belonging to *Streptomyces griseoviridis*, KSA-20, which can

produce a broad-spectrum antibacterial agents as previously reported (Ghadin *et al*., 2008; Ubukata *et al*., 2007).

The species under investigation belonging to the genus *Streptomyces* constitute 50% of the total population of soil actinomycetes and 75-80% of the commercially and medicinally useful antibiotics have been derived from this genus (Mellouli *et al*., 2003). The organic phase was collected and evaporated under reduced pressure using a rotary evaporator. The extract was concentrated and treated with petroleum ether (b.p. 40-60°C) for precipitation process where only one fraction was obtained in the form of yellowish ppt. and then tested for their antimicrobial activity. Separation of antibiotic into individual components has been tried by TLC using a solvent system composed n-butanol-acetic acid-water (3:1:1, v/v); as developing solvent (Atta *et al*., 2009; Zhang *et al*., 2007).

For the purpose of purification process, the antibiotic were allowed to pass through a column chromatography packed with silica gel and eluting solvent was composed of chloroform and methanol (10:2 v/v), fifty fractions were collected and tested for their activities. The most active fractions against the tested organisms ranged between 24 to 30. Similarly, many workers used a column chromatography packed with silica gel and an eluting solvent composed of various ratios of chloroform and methanol (Sekiguchi *et al*., 2007). The compound under investigation is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10% isopropyl alcohol but insoluble in petroleum ether, hexan and benzene; similar results were recorded (Atta, 2010; El-Tayeb *et al*., 2004; Mellouli *et al*., 2003).

Isolation of antimicrobial agents from Streptomyces griseoviridis isolated from Al-Khurmah governorate showed remarkable antimicrobial activity against tested organisms. This isolate was identified according to the recommended keys as Streptomyces griseoviridis KSA-20 as antimicrobial agents isolated from actinomycetes from EL-Taif area, Kingdom of Saudi Arabia (Al-Humiany, 2011).

In this investigation, a study of the elemental analysis of the antibiotic showed C = 55.33%, H = 5.70%, N = 8.80%, O = 23.45% and S = 6.71%. This analysis indicates a suggested empirical formula of $C_{22}H_{27}N_3O_7S$. The spectroscopic characteristics of antibiotic revealed the presence of the maximum absorption peak in UV at 221 nm, infra-red absorption spectrum showed characteristic band corresponding to 26 peaks. Mass-spectrum showed that the molecular weight is 477.4. The MIC of antibiotic under study exhibited fairly active against both Gram positive and Gram negative bacteria. Similar investigations and results were attained (Atta *et al*., 2009; Inagaki *et al*., 2006; Sekiguchi *et al*., 2007). Identification of antibiotic according to recommended international keys indicated that the antibiotic is suggestive of being belonging to griseoviridin antibiotic (Berdy, 1974; Umezawa, 1977).

CONCLUSION

The present study mainly involved in the isolation of actinomycetes based on the cultural, morphological, physiologcial and biochemical characteristics, as well as 16S rRNA methodology. Further work should be focused in most potent *Streptomyces* isolate for production the antibacterial activities against Gram positive and Gram negative bacteria and studies parameters controlling the biosynthetic process of antibacterial agent formation. The bioactive substance was suggestive of being belonging to griseoviridin antibiotic.

ACKNOWLEDGMENT

This research was supported by a program to support research and researchers at King Khalid University, Saudi Arabia No. (KKU_S133_33).

REFERENCES

Afifi, M.M., H.M. Atta, A.A. Elshanawany, U.M. Abdoul-Raouf and A.M. El-Adly, 2012a. Biosynthesis of hygromycin-B antibiotic by *Streptomyces crystallinus* AZ151 isolated from Assuit, Egypt. Bacteriol. J., 2: 46-65.

Afifi, M.M., H.M. Atta, A.A. Elshanawany, U.M. Abdoul-Raouf and A.M. El-Adly, 2012b. Identification of luteomycin like antibiotic produced by *Streptomyces tanashiensis* AZ-C442 isolated from luxor governorate at Upper Egypt. Microbiol. J., 2: 1-22.

Ahmed, F. and W.A. Donaldson, 2007. Chemistry and biology of the streptogramin a antibiotics. Mini-Rev. Org. Chem., 4: 159-181.

Al-Humiany, A.A.A., 2011. Taificidin1 and taificidin2, two anti-microbial agents isolated from the fermentation broth of *Streptomyces roseodistaticus* TA15 and *Streptomyces lavendofoliae* TA17. Res. J. Microbiol., 6: 328-342.

Atta, H.M., S.M. Dabour and S.G. Desoukey, 2009. Sparsomycin antibiotic production by *Streptomyces* sp. AZ-NIOFD1: Taxonomy, fermentation, purification and biological activities. Am.-Eur. J. Agric. Environ. Sci., 5: 368-377.

Atta, H.M., 2010. Production, purification, Physico-chemical characteristics and biological activities of antifungal antibiotic produced by *Streptomyces antibioticus*, AZ-Z710. Am.-Eur. J. Sci. Res., 5: 39-49.

Atta, H.M., M.H. El-Sehrawi, N.M. Awny and N.I. El-Mesady, 2011. Cirramycin-B antibiotic production by *Streptomyces cyaneus*-AZ-13Zc: Fermentation, purification and biological activities. N. Y. Sci. J., 4: 35-42.

Barriere, J.C., N. Berthaud, D. Beyer, S. Dutka-Malen, J.M. Paris and J.F. Desnottes, 1998. Recent development in *Streptogramin* research. Curr. Pharm. Dis., 4: 155-180.

Becker, B., M.P. Lechevalier, R.E. Gordon and H.A. Lechevalier, 1964. Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Applied Microbiol., 12: 421-423.

Berdy, J., 1974. Recent developments of antibiotic research and classi?cation of antibiotics according to chemical structure. Adv. Applied Microbiol., 18: 309-406.

Buchanan, R.E. and N.E. Gibbons, 1974. Bergey's Manual of Determinative Bacteriology. 8th Edn., Williams and Wilkins Co., Baltimore.

Chapman, G.H., 1952. A simple method for making multiple tests of a micro-organism. J. Bacteriol., 63: 147-149.

Cowan, S.T., 1974. Cowan and Steels Manual for the Identification of Medical Bacteria. 2nd Edn., Cambridge University Press, Cambridge, London.

Cuppels, D.A., J. Higham and J.A. Traquair, 2013. Efficacy of selected streptomycetes and a streptomycete + pseudomonad combination in the management of selected bacterial and fungal diseases of field tomatoes. Biol. Control, 67: 361-372.

Dhananjeyan, V., N. Selvan and K. Dhanapal, 2010. Isolation, characterization, screening and antibiotic sensitivity of actinomycetes from locally (Near MCAS) collected soil samples. J. Biol. Sci., 10: 514-519.

Edwards, U., T. Rogall, H. Blocker, M. Emde and E.C. Bottger, 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res., 17: 7843-7853.

El-Tayeb, O.M., A.A. Salama, M.M.M. Hussein and H.F. El-Sedawy, 2004. Optimization of industrial production of rifamycin B by *Amycolatopsis mediterranei* I. The role of colony morphology and nitrogen sources in productivity. Afr. J. Biotechnol., 3: 266-272.

Elwan, S.H., M.R. El-Nagar and M.S. Ammar, 1977. Characteristics of Lipase(s) in the growth filtrate dialystate of *Bacillus stearothermophilus* grown at 55°C using a tributryin- cup plate assay. Bull. Fac. Sci. Riyadh Univ., 8: 105-119.

Ghadin, N., N.M. Zin, V. Sabaratnam, N. Badya, D.F. Basri, H.H. Lian and N.M. Sidik, 2008. Isolation and characterization of a novel endophytic streptomyces SUK 06 with antimicrobial activity from malaysian plant. Asian J. Plant Sci., 7: 189-194.

Gordon, R.E., 1966. Some criteria for the recognition of *Nocardia madurae* (vincent) blanchard. J.Gen. Microbiol., 45: 355-364.

Gordon, R.E., D.A. Barnett, J.E. Handerhan and C.H.N. Pang, 1974. *Nocardia coeliaca*, *Nocardia autotrophica* and the Nocardin strain. Int. J. Syst. Evolut. Microbol., 24: 54-63.

Greenwood, D., 2008. Antimicrobial Drugs: Chronicle of a Twentieth Century Medical Triumph. 1st Edn., Oxford University Press, Oxford, UK., ISBN-13: 978-0199534845, Pages: 368.

Hall, T.A., 1999. BioEdit: A user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acid Symp. Ser., 41: 95-98.

Hankin, L., M. Zucker and D.C. Sands, 1971. Improved solid medium for the detection and enumeration of pectolytic bacteria. Applied Microbiol., 22: 205-209.

Hensyl, W.R., 1994. Bergey's Manual of Systematic Bacteriology. 9th Edn., Williams and Wilkins, Baltimore, Philadeiphia, Hong Kong, London, Munich.

Hozzein, W.N., M.I.A. Ali and W. Rabie, 2008. A new preferential medium for enumeration and isolation of desert actinomycetes. World J. Microbiol. Biotechnol., 24: 1547-1552.

Inagaki, T., K. Kaneda, Y. Suzuki, H. Hirai and E. Nomura *et al.*, 2006. CJ-12,373, a novel topoisomerase II inhibitor: Fermentation, isolation, structure elucidation and biological activities. J. Antibiotics, 51 : 112-116.

Jones, K.L., 1949. Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristic. J. Bacteriol., 57: 141-145.

Kavanagh, F., 1972. Analytical Microbiology. Vol. 2, Academic Press, New York, London.

Kenneth, L.K. and B.J. Deane, 1955. Color universal language and dictionary of names. United States Department of Commerce. National Bureau of standards. Washington, D.C., 20234

Khucharoenphaisan, K., N. Sripairoj and K. Sinma, 2012. Isolation and identification of actinomycetes from termite's gut against human pathogen. Asian J. Anim. Vet. Adv., 7: 68-73.

Lechevalier, M.P. and H. Lechevalier, 1970. Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Evol. Microbiol., 20: 435-443.

Mellouli, L., R.B. Ameur-Mehdi, S. Sioud, M. Salem and S. Bejar, 2003. Isolation, purification and partial characterization of antibacterial activities produced by a newly isolated *Streptomyces* sp. US24 strain. Res. Microbiol., 154: 345-352.

Muharram, M.M., M.S. Abdelkader and S.I. Alqasoumi, 2013. Antimicrobial activity of soil actinomycetes isolated from Alkharj, KSA. Int. Res. J. Microbiol., 4: 12-20.

NTP, 1989. Numerical taxonomy of *Streptomyces* species program (PIB WIN) (*Streptomyces* species). J. Gen. Microbiol., 1989: 13512-13533.

Nitsch, B. and H.J. Kutzner, 1969. Egg-yolk agar as a diagnostic medium for streptomycetes. Cell. Mol. Life Sci., 25: 220-221.

Pridham, T.G. and D. Gottlieb, 1948. The utilization of carbon compounds by some *Actinomycetales* as an aid for species determination. J. Bacteriol., 56: 107-114.

Pridham, T.G., P. Anderson, C. Foley, L.A. Lindenfelser, C.W. Hesseltine and R.G. Benedict, 1957. A section of media for maintenance and taxonomic study of *Streptomycetes*. Antibiotics Ann., 1: 947-953.

Raja, A., P. Prabakaran and P. Gajalakshmi, 2010. Isolation and screening of antibiotic producing psychrophilic actinomycetes and its nature from rothang hill soil against viridans *Streptococcus* sp. Res. J. Microbiol., 5: 44-49.

Sambrook, J., E.F. Fritsch and T.A. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. 2nd Edn., Cold Spring Harbor Laboratory Press, New York, USA., ISBN-13: 9780879695774, Pages: 397.

Sanger, F., S. Nicklen and A.R. Coulson, 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U. S. A., 74: 5463-5467.

Sekiguchi, J.I., T. Miyoshi-Akiyama, E. Augustynowicz-Kopec, Z. Zwolska and F. Kirikae *et al.*, 2007. Detection of multidrug resistance in *Mycobacterium tuberculosis*. J. Clin. Microbiol., 45: 179-192.

Shirling, E.B. and D. Gottlieb, 1966. Methods for characterization of *Streptomyces* species. Int. J. Syst. Evol. Microbiol., 16: 313-340.

Ubukata, M., N. Shiraishi, K. Kobinata, T. Kudo and I. Yamaguchi *et al.*, 2007. RS-22A, B and C: New macrolide antibiotics from *Streptomyces violaceusniger*. I. Taxonomy, fermentation, isolation and biological activities. J. Antibiot (Tokyo), 48: 289-292.

Umezawa, H., 1977. Recent advances in bioactive microbial secondary metabolites. Jpn. J. Antibiot., 30: 138-163.

Williams, S.T. and F.L. Davies, 1965. Use of antibiotics for selective isolation and enumeration of actinomycetes in soil. J. Gene. Microbiol., 38: 251-261.

Williams, S.T., 1989. Bergey's Manual of Systematic Bacteriology. Vol. 4, Williams and Williams, Baltimore, London.

Zamanian, S., G.H. Shahidi Bonjar and I. Saadoun, 2005. First report of antibacterial properties of a new strain of *Streptomyces plicatus* (strain 101) against *Erwinia carotovora* from Iran. Biotechnology, 4: 114-120.

Zhang, L., K. Yan, Y. Zhang, R. Huang and J. Bian *et al.*, 2007. High-throughput synergy screening identifies microbial metabolites as combination agents for the treatment of fungal infections. Proc. Natl. Acad. Sci. USA., 104: 4606-4611.

Zhang, L.J., Z.H. Jin,, X.G. Chen, Q.C. Jin and M.G. Feng, 2012. Glycine feeding improves pristinamycin production during fermentation including resin for in situ separation. Bioprocess Biosyst. Eng., 35: 513-517.