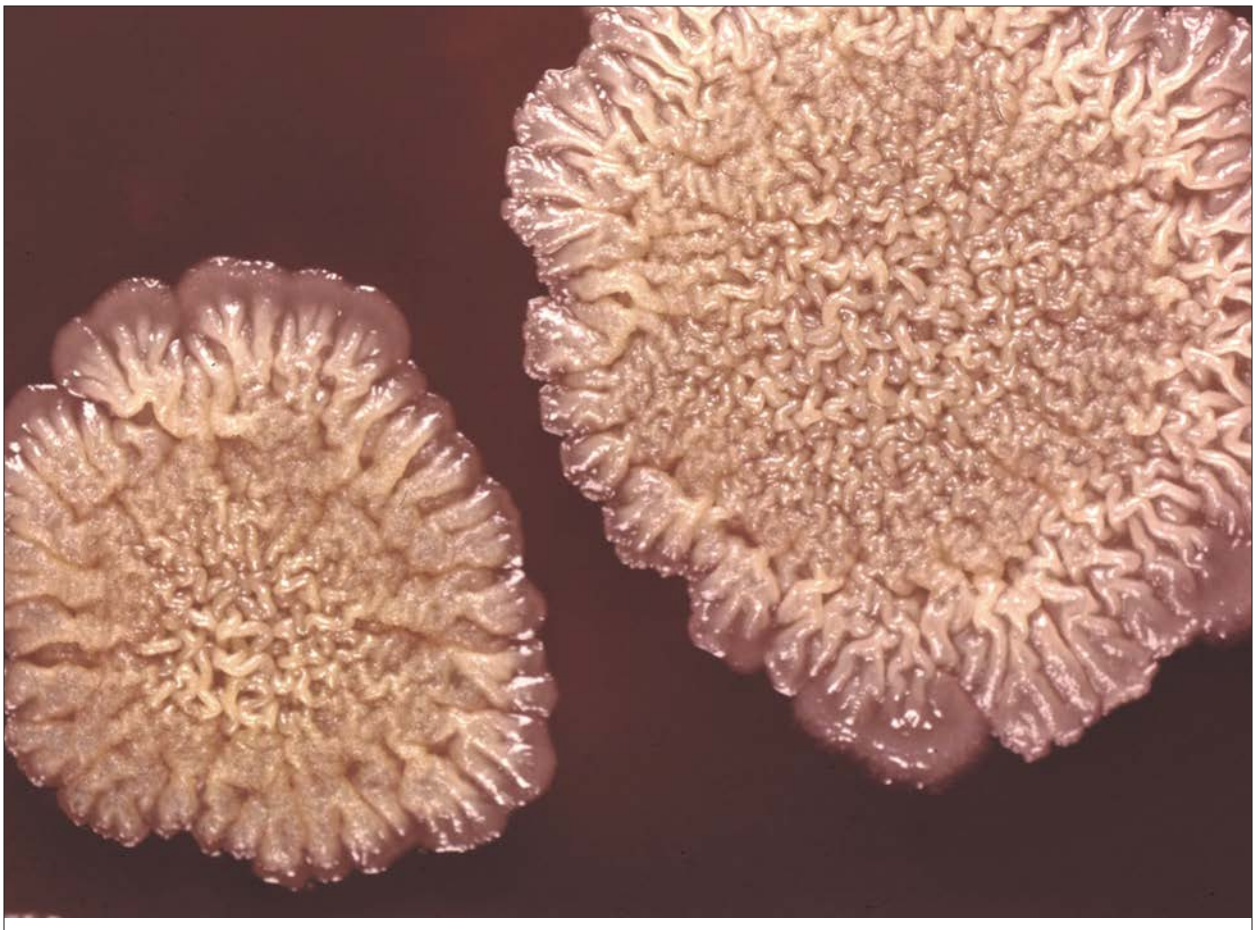

Bacteriology

Journal

ISSN 2153-0211

Academic
Journals Inc.

www.academicjournals.com

Bacteriology Journal 2 (3): 46-65, 2012
ISSN 2153-0211 / DOI: 10.3923/bj.2012.46.65

Biosynthesis of Hygromycin-B Antibiotic by *Streptomyces crystallinus* AZ151 Isolated from Assuit, Egypt

[1,2]M.M. Afifi, [3,4]H.M. Atta, [1]A.A. Elshanawany, [1]U.M. Abdoul-raouf and [1]A.M. El-Adly
[1]Department of Botany and Microbiology, Faculty of Science, Al-Azhar University, Assuit 71524, Egypt
[2]Department of Medical Science, Faculty of Applied Medical Science, King Khalid Uiversity, Bisha 551, Saudi Arabia
[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, Taif University, KSA

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

ABSTRACT

Hygromycin B is an aminoglycoside antibiotic that kills bacteria, fungi and higher eukaryotic cells by inhibiting protein synthesis. It is biosynthesized in this research by *Streptomyces* sp. AZ151, isolated from soil sample in Assuit governorate and chosen among 194 actinomycete isolates. Morphological, physiological and biochemical culture characteristics as well as 16S rRNA nucleotide analysis confirmed strain AZ151 as *Streptomyces crystallinus*. An optimum environmental and nutritional condition in culture medium of this strain showed strong antibacterial and antifungal activities. Using organic solvent extraction, silica gel column chromatography and TLC, a high active metabolite, Hygromycin-B, was separated and identified as a suggested empirical formula of $C_{15}H_{30}N_2O_{10}$ and confirmed a biological efficiency.

Key words: Hygromycin-B antibiotic, *Streptomyces crystallinus*, 16S rRNA, extraction, identification, antimicrobial activities

INTRODUCTION

The genus *Streptomyces* is a particularly fruitful source of antifungals, antibiotics (antibacterials) and chemotherapeutic (anticancer) drugs (Raja and Prabakarana, 2011). The bacterial genus *Streptomyces* is a high G+C content, Gram-positive filamentous soil bacteria with a complex life cycle that includes morphological differentiation and spore formation (Del-Sol *et al*., 2007). Medical geography is the influence of environmental (natural and human) factors on biological system of human, plant and animals which is even recognized as an effective and basic factor in mortality (Esmaeili and Moshiri, 2011). Also, it has long been appreciated as a rich source for the production of various secondary metabolites including many pharmaceutically valuable compounds such as antibiotics, anti-cancer agents, immunosuppressants and enzyme inhibitors (Hindra and Elliot, 2010; Myles, 2003). Based on 16S rDNA sequence and phylogenetic tree analysis, Khucharoenphaisan *et al*. (2012) assigned *Streptomyces* strain FSPNRU 102 and reported that this a new isolate belong to the *Streptomyces niveoruber*. It is well documented that the biosynthesis of *Streptomyces* secondary metabolites is typically regulated via multiple regulatory pathways operating with several layers of complicated control systems (Chen *et al*., 2010; Lee *et al*., 2005). The rRNA sequences is a particularly powerful tool in streptomycete taxonomy. In addition,

rRNA sequence has also been useful for answering questions concerning the horizontal transfer of genes within the genus *Streptomyces* (Huddleston *et al*., 1997).

The crude antibiotic was tested by using pre coated Thin-Layer Chromatography (TLC) plates and purified by column chromatography using silica gel (Augustine *et al*., 2005). The antibacterial activity of Hygromycin A (HA) arises from protein synthesis inhibition and is dependent upon a methylenedioxy bridged-aminocyclitol moiety. Selective gene deletions and chemical complementation in *Streptomyces hygroscopicus* NRRL 2388 showed that the hyg18 and hyg25 gene products, proposed to generate a *myo*-inositol intermediate, are dispensable for HA biosynthesis but contribute to antibiotic yields (Palaniappan *et al*., 2009). Antibacterial compound was purified from the filtrate by solvent extraction method. A comparative study on the total antibiotic sensitivity of the free cells and immobilized cells showed that the immobilized strains were found to be effective against the tested microorganisms. The immobilized cell of actinomycetes was found to be more efficient for the production of secondary metabolites with batch fermentation (Dhananjeyan *et al*., 2010). The morphological character showed the variety of aerial hyphae and spore forming in each strain. The dendrogram was constructed based on biodegradation activity of tested strains (Khucharoenphaisan *et al*., 2011).

This research has first confirmed the isolation *Streptomyces* strain, AZ151 out of 194 isolates from 44 soil samples at upper Egypt (Assuit governorate). Then it confirmed the identity of this strain, by morphological, physiological and biochemical characteristics as well as 16S rRNA, as *Streptomyces crystallinus*. Optimum environmental and nutritional requirements were carried out to obtain the highest yield of antibiotic. An antimicrobial compound was separated and purified from the culture broth of this strain through organic solvent extraction, column chromatography and TLC purification. It possesses strong inhibitory activity toward fungal and bacterial strains tested which give a chance to be applied in medical and other fields.

MATERIALS AND METHODS

Microorganism: The actinomycete AZ151 was isolated from soil sample collected from Assuit governorate. It was purified using the soil dilution plate technique described by Williams and Davies (1965).

Screening for antimicrobial activity: The anti-microbial activity was determined according to Kavanagh (1972).

Taxonomic studies of actinomycete isolate (AZ151)

Morphological characteristics: Morphological characteristics of aerial hyphae, spore mass, spore surface, color of aerial and substrate mycelia and soluble pigments production were conducted by growing the organism on starch-nitrate agar medium and Yeast extract-malt extract agar medium (Atta *et al*., 2011).

Physiological and biochemical characteristics: Lecithinase was detected using egg B yolk medium according to the method of Nitsch and Kutzner (1969), Lipase (Elwan *et al*., 1977), Protease (Chapman, 1952), Pectinase (Hankin *et al*., 1971), α-amylase (Ammar *et al*., 1998) and Catalase Test (Jones, 1949). Melanin pigment (Pridham *et al*., 1956). Esculin broth and xanthine have been done according to Gordon *et al*. (1974). Nitrate reduction was performed according to the method of Gordon (1966). Hydrogen sulphide production was carried out according to Cowan and Steel (1974). The utilization of different carbon and nitrogen sources was carried out

according to Pridham and Gottlieb (1948). Determination of Diaminopimelic acid (DAP) and sugar pattern was carried out according to Becker *et al.* (1964) and Lechevalier and Lechevalier (1970).

Color characteristics: The ISCC-NBS Color Name Charts illustrated with centroid detection of the aerial, substrate mycelia and soluble pigments (Kenneth and Deane, 1955).

DNA isolation and manipulation: The locally isolated actinomycete strain was grown for 6 days on a starch agar slant at 30°C. Two milliliters of a spore suspension were inoculated into the starch-nitrate broth and incubated for 4 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 as described by 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.-ACGTGTGCAG CCCAAGACA-3. and Strep R; 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 16 S rRNA gene was sequenced on both strands via the dideoxy chain termination method (Sanger *et al.*, 1977).

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 evaluated using BioEdit software (Hall, 1999).

Parameters controlling antimicrobial agent biosynthesis: These included; incubation period and temperature; agitation and aeration; pH values; carbon source and nitrogen sources; vitamins, $MgSO_4.7H_2O$ and K_2HPO_4 concentrations; inoculum age and size, amino acids; and medium kinds. All these parameters have been determined by the standard methods.

Fermentation and purification of antibacterial agent

Fermentation: The *Streptomyces crystallinus*, AZ151 inoculum was introduced aseptically into each sterile flask containing the following ingredients (g L^{-1}): Arabinose, 20; $NaNO_3$, 2.0; K_2HPO_4, 0.8; $MgSO_4.7H_2O$, 0.7; vitamin H, ppm and KCl, 0.5. The pH was adjusted at 8.0 before sterilization. After 10 days of incubation at 35°C filtration was carried out through filter paper Whatman No. 1 and followed by centrifugation at 5000 rpm for 15 min. Only clear filtrates were tested for their antimicrobial activities.

Extraction: The clear filtrate was adjusted at different pH values (4-9) and extraction process was carried out using different solvents separately at the level of 1:1 (v/v). The organic phase was concentrated to dryness under vacuum using a rotary evaporator.

Precipitation: The precipitation process of the crude compound was carried out using petroleum ether (b.p. 60-80°C) followed by centrifugation at 5000 r.p.m for 15 min. The precipitate was tested for its antimicrobial activities.

Separation: 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: The purification of the antimicrobial compound was carried out using silica gel column (2.5×50) chromatography. Chloroform and Methanol 8:2 (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 antibacterial activities.

Physico-chemical properties of antimicrobial agent

Elemental analysis: The elemental analysis of 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 assay (Kavanagh, 1972).

Characterization of the antibacterial agent: The antibiotic produced by *Streptomyces crystallinus*, AZ151 was identified according to the recommended international references of Umezawa (1977) and Berdy (1974, 1980).

RESULTS

Isolation, purification and bioactivity of actinomycete isolates: Isolation and purification of actinomycete colonies (the broadest source of antibiotics) from 40 soil samples collected from various Egyptian localities e.g. (Assiut, Luxor and El-Minia governorates). The highest number of isolates (84) out of 194 (43.2%) were isolated on starch nitrate agar medium followed by 55 isolates (28.3%) on both starch casein agar and glycerol asparagine agar (data not shown). Screening test for 194 actinomycete isolates, against certain bacteria, fungi and yeast, confirmed that the highest percentage (38%) 74 active isolates was obtained against *Staphylococcus aureus* 90.5% (67) followed by *Aternaria alternata*, 43.2% (32) and *Klebsiella pneumoniae*, 41.8% (31) while the lowest percentage was obtained against *Fusarium verticillioides* 21.6% (16 isolates), *Salmonella typhi* 20.2% (15 isolates), *Escherichia coli* 9.4% (7 isolates), *Aspergillus fumigatus* 9.4% (7 isolates), *Saccharomyces cerevisiae* 8.1% (6 isolates) and *Aspergillus flavus* 6.7% (5 isolates) (data not shown).

Characterizations of the actinomycete isolate, AZ151

Morphological characteristics: Spore chains are spiral and rectiflexibiles, spore masses are medium red and reddish gray, spore surfaces are smooth and reverse color light yellow to light brown while, diffusible pigment production is moderate yellowish brown to deep brown (Plate 1).

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

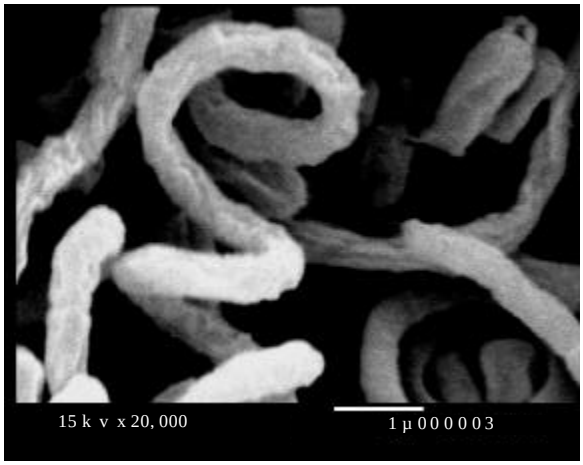

Plate 1: Scanning electron micrograph of the actinomycete isolate AZ-A151 growing on starch nitrate agar medium showing spiral spore were, rectiflexibiles and had a smooth surface. Neither sclerotic granules, sporangia nor flagellated spores were observed (X20, 000)

Physiological and biochemical characteristics: The actinomycete isolate AZ151 could hydrolyze starch and protein, whereas lipid, pectin and catalase are negative, Melanin pigment is positive, degradation of xanthin, esculin, production of H_2S, decomposition of urea, utilization of citrate and KCN are positive but nitrate reduction is negative.

The isolate under study utilizes; D-mannose, D-glucose, D-galactose, mannitol, meso-inositol, raffinose and trehalose but do not utilize, D- xylose, sucrose, L-rhamnose, L-arabinose, lactose, maltose and ribose; whereas, doubtful result was obtained with D-fructose. Good growth on L-glycine, L-asparagine and L-lysine. No growth on L-valine, L-leucine L-histidine, L-phenyl alanine and L-methionine. Moreover, no growth in the presence of up to 5% NaCl. The growth is not inhibited in the presence of 0.1% (w/v) phenol and at 451°C but inhibited in the presence of 0.01% (w/v) sodium azide. The actinomycete isolate is resistant to Ampicillin (25 μg mL^{-1}), Nalidixic acid (30 μg mL^{-1}), Cefoperazone (75 μg mL^{-1}) and Fusidic acid (10 μg mL^{-1}), whereas not resistant to Polymyxin (30 μg mL^{-1}), Gentamicin (10 μg mL^{-1}) and Kanamycin (30 μg mL^{-1}) (Table 1).

Color and culture characteristics: Data recorded on AZ151 declared that, the growth of this strain was disappeared in ISP-1, moderate in ISP-2, 4, 5, 6 and 7 and good growth on SNA and ISP-3. While, the aerial mycelium appeared red on all media used except SNA medium, it was reddish gray. Substrate mycelium light brown on all media used except ISP-5 has shown light yellow. Almost all media used didn't induce any diffusible pigments except SNA shown moderate yellowed brown and ISP-6, 7 showed deep brown pigments (data not shown).

Molecular phylogeny of the selected isolate: The nucleotide sequence of the 16S rRNA gene (1.5 kb) of the actinomycete isolates AZ151 and the phylogenetic tree (as displayed by the Tree View program) revealed 92% similarity with *Streptomyces crystallinus* (Fig. 2). Multiple sequence alignment was conducted the sequences of the 16S rDNA gene of *Streptomyces crystallinus* and the sequencing product was determined as 1141 bp (Fig. 1).

Table 1: The morphological, physiological and biochemical characteristics of the actinomycete isolate AZ151

Characteristic	Result	Characteristic	Result
Physiological and biochemical properties		**Utilization of carbon sources**	
Starch hydrolysis	+	D-xylose	-
Protein hydrolysis	+	D-mannose	+
Lipid hydrolysis	-	D-glucose	+
Pectin hydrolysis	-	D-galactose	+
Catalase test	-	Sucrose	-
Production of melanin pigment on		L- rhamnose	-
Peptone yeast- extract iron agar	+	Raffinose	++
Tryptone yeast extract broth	-	Mannitol	+++
Xanthin degradation	+	L- arabinose	-
Esculin degradation	+	Meso-inositol	+++
H_2S production	+	Lactose	-
Nitrate reduction	-	Maltose	-
Citrate utilization	+	Trehalose	++
Urea test	+	D- ribose	-
KCN test	+	D-fructose	±
Morphological characteristics		**Utilization of amino acids**	
Spore chains	Spiral and rectiflexibiles	L-glycine	+
Spore mass	Medium red-reddish gray	L-valine and L-leucine	-
Spore surface	Smooth	L-histidine	-
Color of substrate mycelium	Light brown- deep brown	L-phenylalanine	-
Diffusible pigment	Yellowish brown	L-asparagine	+
Motility	Non-motile	L-methionine	-
Cell wall hydrolysate		**Growth with (% w/v)**	
Diaminopimelic acid (DAP)	LL-DAP	Sodium azide (0.01)	-
Sugar pattern	Not-detected	Phenol (0.1)	+
Growth at different concentration of NaCl (%)		**Growth at different temperatures (EC)**	
1	+	10 -	
3	+	30 ++	
5	+	35 ++	
7	-	40 ++	
9	-	45 ++	
11	-	50 "	
13	-	55 -	
15	-		
Resistance to			
Ampicillin (25 μg mL^{-1}), Nalidixic acid (30 μg mL^{-1}), Cefoperazone (75 μg mL^{-1}) and Fusidic acid (10 μg mL^{-1})	+	Polymyxin (30 μg mL^{-1}), Gentamicin (10 μg mL^{-1}) and Kanamycin (30 μg mL^{-1})	-

-: Negative and +: Positive. ±: doubtful results, ++: Moderate growth, +++: Good growth

Identification of actinomycete isolate, AZ151: This was performed basically according to the recommended international Key's viz. (Buchanan and Gibbons, 1974; Williams, 1989) and Numerical taxonomy of *Streptomyces* species program (PIB WIN). On the basis of above collected data and in view of the comparative study of the recorded properties of AZ151 in relation to the most closest reference strain, viz., *Streptomyces crystallinus*, it could be concluded that it is identical on the basis of spore mass is medium red or reddish gray, spore chain is spiral and rectiflexibiles

Table 2: A comparative study of the characteristic properties of AZ151 in relation to reference strain, *Streptomyces crystallinus*

Characteristics	AZ-A151	Reference strain Streptomyces crystallinus
Morphological characteristics		
-Spore mass	Medium red/ reddish gray	Red
-Spore surface	Smooth	Smooth
-Reverse color	Light yellow/ light brown	Light to dark brown
-Spore chain	Spiral and Rectiflexibiles	Rectiflexibiles
-diffusible pigment	Brown	Brown
-Motility	Non-motile	Non-motile
Cell wall hydrolysate		
-Diaminopimelic acid (DAP)	LL-DAP	LL-DAP
-Sugar pattern		
Utilization of carbon sources		
L-arabinose	-	ND
D-fructose	-	ND
D-galactose	-	ND
D-glucose	-	ND
Meso-inositol	+	+
D-mannitol	-	ND
Raffinose	-	ND
Sucrose	-	ND
D-xylose	-	ND

ND: Melanin pigment not-detected

```
GG    1 GGGCGTGC TAAACACATG CAAGTCGAAG GCATGAACCA CTTCGGTGGG ATTAGTGGCG
61    AACGGGTGAG TAACACGTGG GCTTTCCTGC CTTCACTCTG GGACAAGCCC TGGTTTCGGC
121   CTCTAATACC GGATACGAGG TGGAAGCGCA TGCTTCCGGG TGGTTTGCTC CGGCGGTGTT
181   GGATGAGGGG GCGGCCTATC AGCAAGTTGG TCCCCTAATG GCCTACCAAG GCGACGACCC
241   CTAGCCGGCC TGAGAGGGCG ACCGGCCACA CTCCCACTGA GACACGGCCC AGACTCCTAC
301   GGGACCCAGC AGTGCCGAAT ATTGCACAAT GGGCGTTTGC CTGATGCAGC GACGCCGCGT
361   GAGGGATGAC GGCCTTCGGG TTGTAAACCT CTTTCAGCAG GGAAGAAGCG TTTGTGACGG
421   TACCTGCAGA AGTTGCGCCG GCTTTCTACG TGCCAGCAGC CGCGGTAATA CGTAGGGCGC
481   AAGCGTTGTC CGGAATTATT GGGCGTTTAG AGCTCGTAGG CGGCTTGTCA CGTCGGATGT
541   GAAAGCCCGG GGCTTAACCC CGGGTCTGCA TTCGATACGG GCTAGCTAGA GTGTCCTAGG
601   GGAGATCGTT ATTCCTGGTG TAGCGGTGAA ATGCGCAGAT ATCAGGAGGA ACACCGGTGG
661   CGAAGGCGGA TCTCTGGGCC ATTACTGACG CTGAGGAGCG TTTGCGTGGG GAGCGTTCAG
721   GATTAGATAC CCTGGTAGTC CACGCCGTTT TCGTTGGGAA CTAGGTGTTG GCGACATTCC
781   ACGTCGTCGG TGCCGCAGCT AACGCATTAA GTTCTCCGCC TGGGGAGTAC GGCCGCTTGG
841   CTAAAACTCT TTGGAATTGA CGCCGGCCCG CACAAGCAGC GGAGCATGTG GCTTCATTCG
901   ACGCAACGCG AAGAACCTTA CCAAGGCTTG ACATATACCG GAAAGCATTA GAGATAGTGC
961   CCCCCTTGTG GTCGGTATAC AGGTGGTGCA TGGCTGTCGT CAGCTCGTGT CGTGAGATGT
1021  TGGGTTAAGT CCCGCAACGA GCGCAACCCT TGAACTGTGT TGCCAGCATG CCCTTCCCCC
1081  TGATGGGGAC TCACAGGAGA CTGCCGCCCT CAACTCCCAG GAAGGTGGGG ACGACGTCAA
1141  GTCATCATGC CCCTTATGTC TTGGGCTGCA CACGTGCTAC AATGGCCGGT
```

Fig. 1: The sequence alignment was conducted the sequences of the 16S rDNA gene of *Streptomyces crystallinus*

and non motile spores. Cell wall hydrolysate contains LL-diaminopimelic acid and sugar pattern not detected. Melanin pigments are produced. Utilization of D-mannose, D-glucose, D-galactose, mannitol, meso-inositol, raffinose and trehalose but do not utilize D-xylose, sucrose, rhamnose, L-arabinose, lactose, maltose and Ribose, whereas, doubtful with D-fructose. In view of all the previous characteristics of AZ151, it could be stated that it is suggestive of being belonging to *Streptomyces crystallinus* (Table 2).

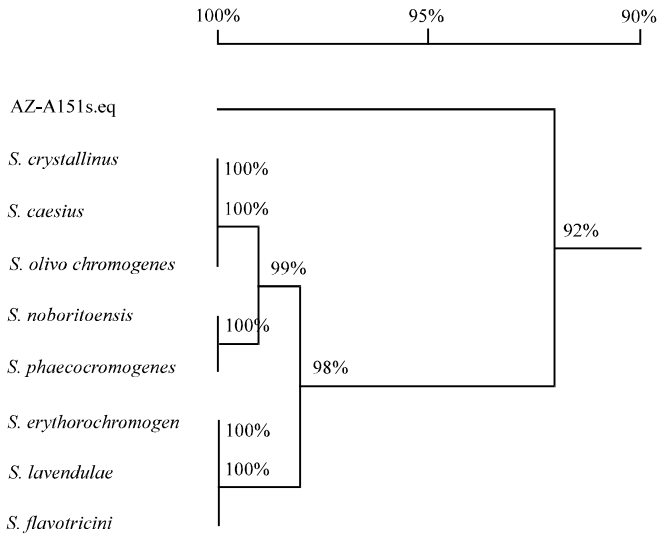

Fig. 2: The phylogenetic position of the local *Streptomyces* sp. strain among neighboring species. The phylogenetic tree was based on the pairwise comparisons of 16S rDNA sequences

Parameters controlling antimicrobial agent biosynthesis: The effect of different environmental and nutritional factors on the antimicrobial activity indicated that the maximum activities was obtained in starch nitrate broth medium at, incubation period (10 days), incubation temperature (35°C), agitation and aeration (160 rpm), pH value (8.0), carbon source (Starch), nitrogen source ($NaNO_3$), water soluble vitamin (vitamin H), inoculum age (12 days), inoculum size.

Fermentation and isolation of antimicrobial agent: The *Streptomyces crystallinus*, AZ151 inoculum was introduced aseptically into each sterile flask containing the following ingredients (g L^{-1}): Arabinose, 20; $NaNO_3$, 2.0; K_2HPO_4, 0.8; $MgSO_4.7H_2O$, 0.7; vitamin H, ppm and KCl, 0.5. The pH was adjusted at 8.0 before sterilization. After 10 days of incubation at 35°C filtration was carried out through filter paper Whatman No. 1 and followed by centrifugation at 5000 rpm for 15 min. Only clear filtrate was tested for its antimicrobial activity.

The clear filtrate containing the active metabolite, was adjusted at pH 7.0 then extraction process was carried out using Ethyl acetate at the level of 1:1 (v/v). The organic phase was collected and evaporated under reduced pressure using rotary evaporator. The residual material was dissolved in least amount of DMSO and filtered. The filtrates were test for their antibacterial activities. The antimicrobial agent was precipitated by petroleum ether (b.p. 60-80 EC) and centrifuged at 4000 r.p.m for 15 min. The fraction was test for antimicrobial activities. 8-10% (v/v), amino acid (Asparagine), $MgSO_4.7H_2O$ concentration (0.7 g L^{-1}), K_2HPO_4 (0.8 g L^{-1}), inoculum size (8-10 % (v/v) and at inoculum culture age (12 days) (Table 3).

Separation of antimicrobial agent into individual components was carried out by thin-layer chromatography using a solvent system composed of chloroform and methanol (24:1, v/v). Among three bands developed, only one band at R_f 0.55 showed antibacterial activity. The purification process through column chromatography packed with silica gel, indicated that maximum activity was at fractions Nos. 14-23 (Fig. 3).

Physicochemical characteristics of the antimicrobial agent: The purified antimicrobial agent produced by *Streptomyces crystallinus*, AZ151 produces characteristic odour, its melting

Table 3: The environmental conditions and nutritional requirements affecting the biosynthesis of antimicrobial agent by *Streptomyces crystallinus*, AZ151

Parameter	Mean diameter of inhibition zone (mm) against				Parameter	*Mean diameter of inhibition zone (mm) against			
	S. aureus	*K. pnumoniae*	*A. alternata*	*S. cerevisiae*		*S. aureus*	*A. alternata*	*K. pnumoniae*	*S. aureus*
Incubation periods (day)					**Nitrogen source (2 g L^{-1})**				
0	0.0	0.0	0.0	0.0	Ammonium nitrate	29.8±0.6	18.5±1.4	15.5±0.0	4.0±0.0
2	0.0	0.0	0.0	0.0	Casein	26.5±1.0	17.0±0.8	13.8±0.5	12.5±0.0
4	15.0±0.0	11.5±0.0	9.8±0.2	8.5±0.4	Sodium nitrate	34.5±0.6	24.8±0.5	21.0±0.0	16.5±1.0
6	20.5±0.6	15.0±0.2	13.0±0.0	12.0±0.0	Potassium nitrate	30.0±0.0	19.3±0.5	17.0±0.0	15.0±0.0
8	26.0±0.0	17.0±0.4	14.5±0.0	15.0±0.0	Magnesium nitrate	16.8±0.0	12.0±0.0	10.5±0.0	0.0
10	35.0±0.0	24.5±0.0	20.5±0.0	16.8±0.0	Ammonium sulphate	31.0±0.8	20.8±1.0	17.5±1.0	15.0±0.5
12	35.0±0.2	24.5±0.6	20.3±0.2	16.8±0.6	Ammonium carbonate	23.5±0.0	15.5±0.0	12.0±0.0	10.0±0.0
14	30.0±0.0	22.0±0.0	18.0±0.8	16.0±0.1	**Amino acids (2 g L^{-1})**				
16	29.0±0.2	22.0±0.0	18.0±0.0	16.0±0.2	L-phenylalanine	0.0	0.0	0.0	0.0
18	29.0±0.9	21.0±0.4	18.0±0.0	16.0±0.1	DL-cystine	24.5±0.4	17.3±0.5	14.0±0.0	10.5±0.0
20	28.0±0.7	20.5±0.4	17.0±0.0	15.0±0.0	DL-methonine	0.0	0.0	0.0	0.0
Different pH values					Lysine	22.5±0.0	16.5±0.0	13.5±0.0	10.0±0.0
4	13.0±0.0	0.0	0.0	0.0	L-leucine	0.0	0.0	0.0	0.0
5	19.0±0.0	12.5±0.0	13.5±1.0	11.5±0.6	Glycine	0.0	0.0	0.0	0.0
6	24.5±1.0	17.5±0.4	18.0±0.0	14.0±0.0	Trptophane	28.0±0.4	20.3±0.3	16.5±0.6	12.0±0.2
7	30.0±0.0	21.5±1.0	21.0±0.0	16.5±0.6	Asparagine	30.0±0.4	22.3±0.3	18.5±0.6	14.0±0.2
8	34.5±0.0	24.0±0.0	18.8±1.5	14.5±0.6	Proline	19.5±0.0	13.0±0.0	0.0	0.0
9	30.8±1.5	21.0±0.0	15.5±0.0	12.0±0.0	L-asparatic acid	0.0	0.0	0.0	0.0
10	0.0	0.0	0.0	0.0	L-serine	0.0	0.0	0.0	0.0
11	0.0	0.0	0.0	0.0	Sodium nitrate (control)	34.5±0.2	24.5±0.4	21.2±0.6	16.8±0.5
Temperature (°C)					**Carbon sources**				
15	0.0	0.0	0.0	0.0	Starch	38.0±2.0	26.0±0.0	23.0±0.8	20.5±0.6
20	17.3±0.5	12.0±0.0	0.0	0.0	Galactose	29.0±0.0	18.5±0.0	17.0±0.0	11.5±0.6
25	25.5±1.0	19.3±0.5	16.0±0.0	12.8±0.5	Glycerol	30.5±0.6	24.8±1.0	15.5±0.4	15.0±0.0
30	33.0±0.0	24.5±0.6	20.5±1.7	16.5±0.6	Glucose	27.0±0.8	17.5±0.0	12.0±0.0	10.0±0.0
35	37.5±1.9	28.0±0.0	23.5±0.6	19.5±0.8	Mannose	28.5±1.0	19.3±0.5	13.0±0.0	11.5±1.0
40	29.3±0.5	21.5±1.0	17.0±0.0	14.5±0.6	Mannitol	31.0±0.0	20.5±1.0	19.5±0.0	16.0±0.0
45	22.0±0.0	16.8±1.0	13.5±0.0	12.0±0.8	Cellulose	30.5±1.7	21.3±0.5	17.0±0.0	13.5±0.0
50	16.5±0.6	12.5±0.6	11.5±0.0	9.5±0.6	Meso-inositol	32.0±0.0	20.0±0.0	19.0±0.0	12.5±0.0
55	0.0	0.0	0.0	0.0	Raffinose	32.5±0.6	21.8±0.5	18.0±0.0	14.0±0.4

Table 3: Continue

Parameter	Mean diameter of inhibition zone (mm) against *S. aureus*	*K. pnumoniae*	*A. alternata*	*S. cerevisiae*	Parameter	*Mean diameter of inhibition zone (mm) against *S. aureus*	*A. alternata*	*K. pnumoniae*	*S. aureus*
Different shaking speed (rpm)					**Media used ****				
0	22.0±1.0	14.0±0.0	11.5±0.0	0.0	(SN)	35.8±0.4	24.5±0.6	20.3±0.2	16.8±0.2
40	25.0±0.0	16.5±1.0	13.0±0.0	10.5±0.4	(SC)	27.5±0.6	15.0±0.0	14.5±0.6	12.5±0.0
80	30.5±2.5	22.0±0.0	17.8±1.0	12.5±0.0	(YEME)	22.0±0.0	11.5±1.4	12.0±1.0	0.0
120	33.3±0.5	24.0±0.0	19.5±0.6	14.0±0.0	(GA)	25.5±0.6	13.0±0.0	14.0±0.0	11.5±0.7
160	36.5±0.6	25.5±0.6	21.5±0.4	18.8±0.5	(ISS)	34.8±0.8	24.0±4.3	20.0±0.6	14.0±0.0
200	35.0±0.0	25.5±0.0	20.5±0.0	14.5±0.6	(TYE)	0.0	0.0	0.0	0.0
Inoculums age (days)					**Ioculum sizes % (v/v)**				
3	18.5±1.0	13.0±0.0	12.0±0.0	0.0	2	30.5±0.0	21.5±0.6	17.0±0.0	12.3±0.5
6	25.0±0.0	17.5±0.6	15.0±0.0	11.0±0.0	4	36.0±0.0	26.0±0.0	19.5±0.6	14.0±0.0
9	31.5±1.9	22.0±0.0	19.0±0.0	14.5±1.0	6	40.5±1.7	29.5±1.7	22.5±1.0	16.0±0.0
12	36.5±3.0	26.0±0.8	22.5±0.0	18.5±0.0	8	42.5±0.6	31.0±0.8	25.5±1.0	22.5±0.6
15	35.0±0.0	25.5±1.7	21.0±0.0	15.5±0.0	10	44.0±0.0	32.0±0.0	22.0±0.0	15.0±0.0
18	31.5±1.0	22.0±1.6	18.5±0.6	14.0±0.0	12	41.5±1.7	30.0±0.0	19.5±0.0	13.5±0.6
21	26.0±0.0	18.0±0.0	15.0±0.0	11.5±0.0	14	38.0±0.6	27.5±1.0	17.0±0.0	11.5±0.6
24	19.0±0.0	14.0±0.0	12.0±0.0	9.0±0.0	16	33.5±1.0	21.0±0.8	13.0±0.0	0.0
27	12.5±0.0	9.5±0.6	0.0	0.0	18	27.0±2.0	15.5±0.0	0.0	0.0
30	0.0	0.0	0.0	0.0	20	21.0±0.0	10.5±0.6	0.0	0.0
K_2HPO_4 conc.(%, w/v)					**$MgSO_4.7H_2O$ Conc. (g L^{-1}, w/v)**				
0.00	19.5±0.0	15.0±0.0	14.5±0.0	10.5±0.4	0.0	0.0	0.0	0.0	0.0
0.02	22.5±0.4	17.0±0.0	16.0±0.0	11.5±0.0	0.1	19.5±0.6	12.0±0.0	9.5±0.5	0.0
0.04	25.0±0.8	18.5±0.9	18.5±0.0	13.0±0.4	0.3	23.0±2.0	14.5±0.4	11.5±0.5	9.5±0.6
0.06	29.5±0.0	21.0±0.0	23.0±0.0	19.5±0.0	0.5	35.5±0.6	24.3±0.5	20.5±0.0	17.0±0.0
0.08	37.0±1.9	26.0±0.0	20.5±0.6	14.0±0.0	0.7	36.0±0.0	25.5±0.0	21.5±0.5	18.0±0.0
0.10	31.5±0.8	22.0±0.0	18.0±0.0	12.8±0.8	1.0	26.0±1.2	18.5±0.6	14.5±0.4	10.0±0.0
0.20	24.5±0.0	16.5±0.4	13.8±0.7	9.0±0.0	2.0	16.5±0.0	13.0±0.0	11.3±0.5	0.0
0.30	19.0±0.0	12.5±0.4	10.3±0.0	0.0					
Different Vitamins and their concentrations (ppm)					**Different Vitamins and their concentrations (ppm)**				
B12					Vitamin H				
50	35.0±0.0	24.5±0.0	20.5±0.6	14.0±0.0	50	34.5±0.0	24.5±0.6	20.0±1.4	14.3±0.5
100	39.5±1.0	28.0±0.0	23.0±0.0	17.0±0.0	100	40.5±0.6	29.0±0.8	24.0±0.8	18.0±0.0
200	39.5±0.6	28.0±0.0	23.5±1.0	17.0±0.0	200	42.5±0.0	30.5±1.0	24.5±1.0	18.5±0.6

Table 3: Continue

Parameter	Mean diameter of inhibition zone (mm) against *S. aureus*	*K. pnumoniae*	*A. alternata*	*S. cerevisiae*
Different Vitamins and their concentrations (ppm)				
D2				
50	33.5±1.0	23.0±0.8	19.5±0.6	13.5±0.6
100	37.5±3.1	26.0±0.0	22.0±0.8	15.5±0.0
200	41.0±2.4	29.0±1.2	24.5±0.0	17.0±0.4
D-pantothenic acid				
50	36.5±1.0	25.5±0.6	21.0±0.0	15.0±0.8
100	36.5±0.6	25.0±0.0	23.5±0.6	17.0±0.0
200	36.0±0.0	25.5±0.6	23.0±0.0	17.0±0.0
Folic acid				
50	37.0±1.4	26.0±0.0	22.5±0.6	16.0±0.4
100	39.5±0.6	27.5±1.0	24.0±0.0	18.0±0.0
200	39.5±0.6	27.5±0.6	24.0±0.0	17.5±0.6

Parameter	*Mean diameter of inhibition zone (mm) against *S. aureus*	*A. alternata*	*K. pnumoniae*	*S. aureus*
Different Vitamins and their concentrations (ppm)				
Riboflavine				
50	35.5±0.6	25.0±0.0	21.5±0.6	15.0±1.4
100	38.0±0.0	27.5±0.6	23.5±1.0	17.0±0.8
200	38.0±0.0	27.0±0.8	25.0±0.0	19.0±0.0
Thiamin				
50	41.5±0.6	27.5±0.6	23.0±0.8	17.0±0.0
100	41.0±0.0	27.5±0.6	23.0±0.0	17.0±0.0
200	41.0±0.4	27.0±0.4	23.0±0.5	17.0±0.0
Control (no vitamin)				
	32.0±0.0	21.8±0.5	18.0±0.8	12.5±0.0

*Mean values of triplicate determinations were calculated. **SN: Starch nitrate medium, SC: Starch casein medium , YEME: Yeast extract malt extract medium, GA: Glycerol asparagine medium, ISS: Inorganic salt starch medium and TYE: Tryptone yeast extract medium

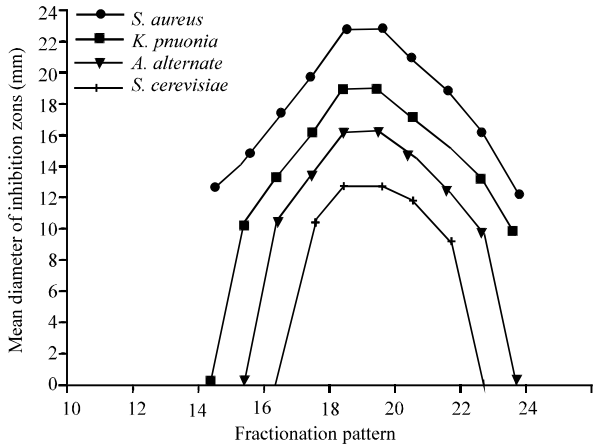

Fig. 3: Antimicrobial activity of fractions for antimicrobial agent

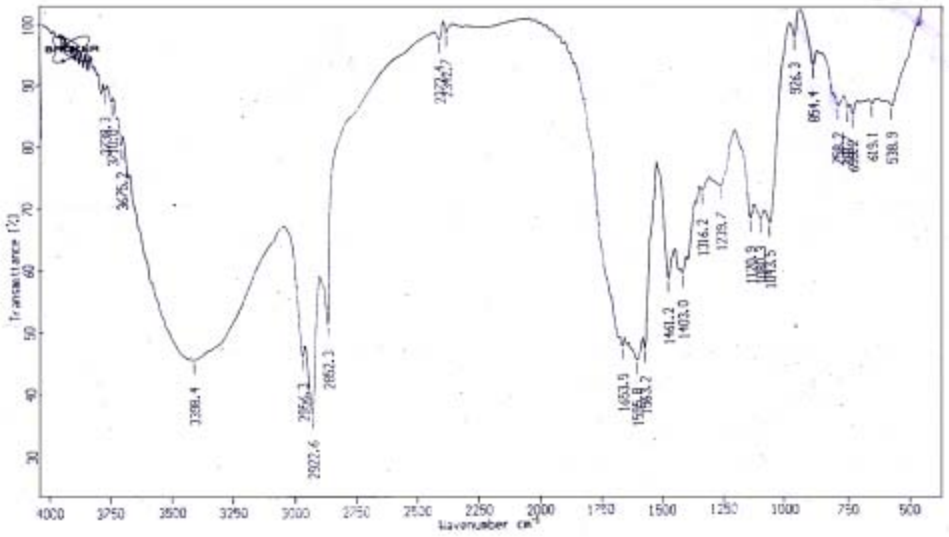

Fig. 4: IR spectrum of antimicrobial agent

points is 160°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, hexane and benzene.

Elemental analysis: The elemental analytical data of the antibacterial agent revealed the following data: C = 46.43; H = 7.46; N = 6.81; O = 39.43 and S = 0.0. This analysis indicates a suggested calculated empirical formula of $C_{15}H_{80}N_2O_{10}$.

Spectroscopic characteristics: The spectroscopic analysis of purified antimicrobial agent produced by *Streptomyces crystallinus,* AZ151 have been determined. The infrared (IR) spectrum showed characteristic band corresponding to 21 peaks (Fig. 4), the ultraviolet (UV) spectrum recorded a maximum absorption peak at 225 NM (Fig. 5) and the Mass spectrum indicate that the molecular weight was 432.36 (Fig. 6).

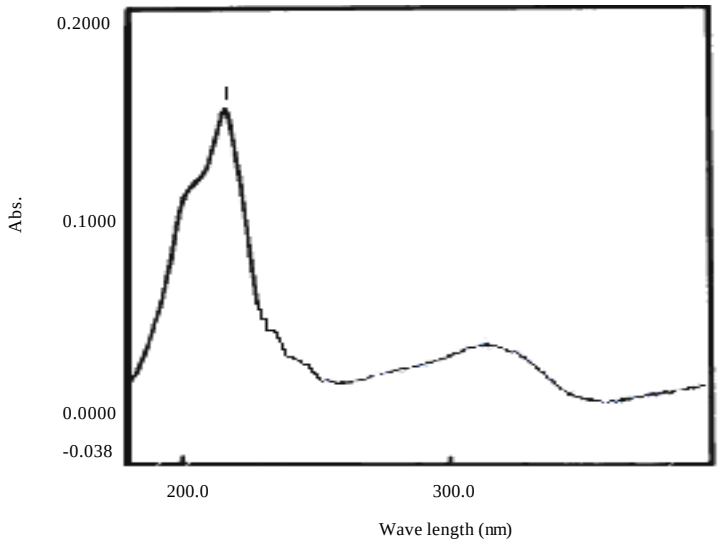

Fig. 5: Ultraviolet absorbance of antimicrobial agent

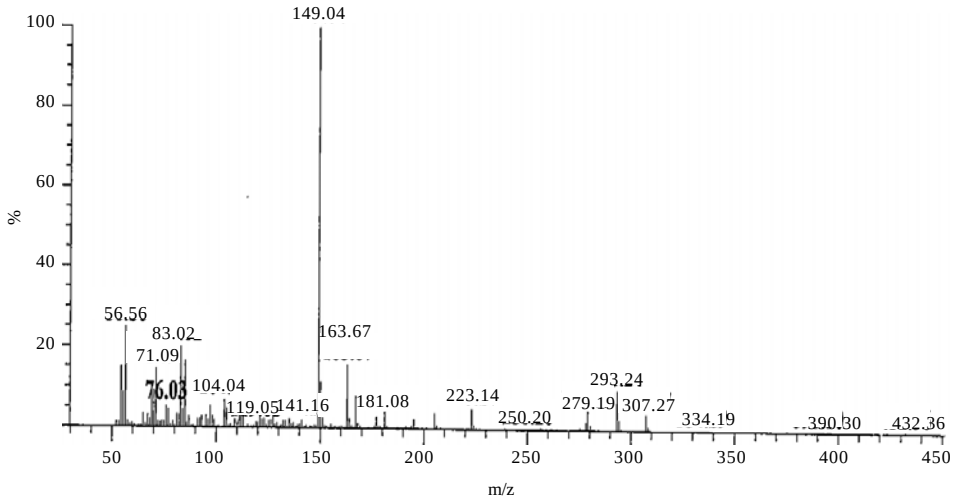

Fig. 6: Mass spectrum of antimicrobial agent

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 antimicrobial agent is suggestive of being belonging to Hygromycin-B antibiotic (Table 4).

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 and unicellular and filamentous fungi. The Minimum Inhibitory Concentration (MIC) of antibiotic produced by *Streptomyces crystallinus,* AZ151 was determined and results showed that MIC (μg mL^{-1}) against *Staphylococcus aureus* (1.73), *Klebsiella pneumonia* (3.9), *Escherichia coli* and

Table 4: A comparative study of characteristic properties of antimicrobial agent in relation to Reference antibiotic (Hygromycin-B)

Characteristic	Purified antibiotic	Standard antibiotic
Melting point	160°C	160-180°C
Molecular weight	432.36	436
Chemical analysis		
C	46.43	46.40
H	7.46	7.48
N	6.81	6.82
O	39.3	39.3
Ultra violet	225	No characteristic absorbance $C_{15}H_{30}N_2O_{10}$
Formula	$C_{15}H_{30}N_2O_{10}$	Active against Gram positive, Gram negative bacteria, unicellular and filamentous fungi
Active against	Active against Gram positive, Gram negative bacteria, unicellular and filamentous fungi	

Table 5: Minimum inhibitory concentration of the antimicrobial agent

	MIC ($\mu g\ mL^{-1}$) concentration
Test organism	Antimicrobial agent produced by AZ-A151
S. aureus	1.73
E. coli	7.80
K. pneumonia	3.90
S. typhi	7.80
S. cerevisiae	62.50
A. flavus	31.25
A. alternate	31.25

Salmonella typhi (7.8), for *Aspergillus flavus* and *Alternaria alternata* (31.25) and *Saccharomyces cerevisiae* (62.5) (Table 5).

DISCUSSION

The aminoglycosides such as Hygromycin B are a large and diverse class of antibiotics that characteristically contain two or more aminosugars linked by glycosidic bonds to an aminocyclitol component. The cyclitol is 2-deoxystreptamine-streptomycin which has a streptidine moiety (Umezawa and Hooper, 1982). Soil, in particular, is an intensively exploited ecological inhabitant, of which, produce many useful natural products, including clinically important antibiotics (Waksman, 1961; Waksman, 1975). Among soil inhabitants, actinomycetes and more specifically streptomycetes, are of practical importance because they produce most of the useful natural antibiotics for medical use. Nevertheless, selective isolation of soil actinomycetes is important for understanding their ecological properties and for finding novel strains which can produce useful bioactive secondary metabolites. Therefore, various media and techniques have been developed for selective isolation of actinomycetes (Hozzein *et al*., 2008). A total 6 actinomycetes were isolated from the soil sample through crowded plate technique were subjected to primary screening and identified as *Intrasporangium* sp., *Dactyl sporangium* sp., *Micromonospora* sp., *Streptoverticillium* sp. and two *Streptomyces* sp. (Raja *et al*., 2010).

In the course of searching for new actinomycetes producing antibiotics in our study, screening program has been conducted. The data presented in this study gave detail information on the isolation and identification of actinomycetes from various Egyptian localities e.g. (Assiut, Luxor and

El-Minia governorates). The results indicated the highest percentage of isolation (43.2%), 84 isolates out of 194, were isolated on Starch Nitrate Agar (SNA) medium followed by (28.3%), 55 isolates on both starch casein agar and glycerol asparagine agar (data not shown).

The most active isolates, in terms of mean diameter of inhibition zone (mm), 74 (38%) was obtained against *Staphylococcus aureus* 90.5% (67) followed by *Aternaria alternata* 43.2% (32) and *klebsiella pneumoniae*, 41.8% (31) while the lowest percentage was obtained against *Fusarium verticillioides* 21.6% (16 isolates), *Salmonella typhi* 20.2% (15 isolates), *Escherichia coli* 9.4% (7 isolates), *Aspergillus fumigatus* 9.4% (7 isolates), *Saccharomyces cerevisiae* 8.1% (6 isolates) and *Aspergillus flavus* 6.7% (5 isolates).

Many other percentages were also found 100% (14) against *Phytophthora megasperma*, followed by *Alternaria solani* and *Alternaria alternata* 78.5% (11) while the lowest percentage was obtained against *Fusarium solani* and *Saccharomyces cerevisiae* 14.2% (2) and 7.1% (1), respectively (Aghighi *et al*., 2004). In addition, streptomycetes isolates appear to be highly active against Gram-positive bacteria (Hamdi *et al*., 1980; Hussein *et al*., 1981; Saadoun *et al*., 1998).

Among our strains (194), an actinomycete isolates (AZ151) showed high potencies against all microorganisms tested. Its morphological, physiological and biochemical characteristics as well as, its color and culture characteristics have been investigated. A similar study found that the highest percentage of active isolates was red and gray series and the lowest in the green and white ones (Saadoun *et al*., 1998). However, most antimicrobial producing species of streptomycetes were found in the gray and yellow series of no chromomeric type and no antibiotic produced by white and green series chromomeric type (Aria *et al*., 1976). Antimicrobial sensitivity testing of these strains was done by Kirby-Bauer disc diffusion method (Khan *et al*., 2011). Minimum Inhibitory Concentration (MIC) of the extracts was determined by the micro broth dilution against 14 clinical and standard strains of methicillin resistant and sensitive of *Staphylococcus aureus* (Dadgar *et al*., 2006). Over 110 soil actinomycetes isolates were screened among which one isolate showed high level of activity in Agar disk and Well diffusion methods against *E. carotovora* subsp. *carotovora* and identified as a new strain of *Streptomyces plicatus* (strain 101) (Zamanian *et al*., 2005).

The species 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). Over 110 soil actinomycetes isolates were screened among which one isolate showed high level of activity in Agar disk and Well diffusion methods against *E. carotovora* subsp. *carotovora* and identified as a new strain of *Streptomyces plicatus* (strain 101) (Zamanian *et al*., 2005).

The extract from culture filtrate of endophytic *Streptomyces* sp. ST8 by ethyl acetate has activity against *S. mutans* ATCC25175 and 104B. The extract at such concentrations (0.05-5 mg mL^{-1}) showed the inhibition of bacterial adherence on glass surfaces and saliva-coated hydroxyapatite. The crude extract also decreased the activity of glucosyltransferase and glucan-binding lectin from both strains (Taechowisan *et al*., 2008).

Besides, selective isolation of soil actinomycetes is important for understanding their ecological properties and for finding novel strains which can produce useful bioactive secondary metabolites. Therefore, various media and techniques have been developed for selective isolation of actinomycetes (Hozzein *et al*., 2008).

In this study, we found the nucleotide sequence of the 16S rRNA gene (1.5 kb) of the actinomycete isolate, AZ151 evidenced a 92% similarity with *Streptomyces crystallinus* 16S rRNA genes. In addition, multiple sequence alignment was conducted the sequences of the 16S rDNA

gene of *Streptomyces crystallinus*, (CHR and SNG). The sequencing product was determined as 1141 bp2). On the basis of theses data and in view of the comparative study of the recorded properties of AZ151 in relation to the most closest reference strain, viz. *Streptomyces crystallinus*, it could be stated that it is suggestive of being belonging to *Streptomyces crystallinus* which can produce a broad-spectrum antibiotic.

In addition, 16S rRNA sequence data have proved invaluable in Streptomycetes systematics, in which they have been used to identify several newly isolated *Streptomyces* (Mehling *et al*., 1995). This finding is in agreement with that noticed of antibiotic phenazine derivatives and their formation pathways in a new *Streptomyces* strain P510, where culture characteristics and 16S rRNA nucleotide analysis confirmed strain P510 as *Streptomyces griseoluteus* (Wang *et al*., 2011).

Several methods have been developed to identify *Streptomyces* species. These include, culturing methods using the selective plating technique (Kuster and Williams, 1964), construction of genetic marker systems (Wipat *et al*., 1991), a combination of chemical markers, the presence of LL diaminopimelic acid and the absence of characteristic sugars in the cell wall (Lechevalier and Lechevalier, 1970; Atta *et al*., 2011). Also, sensitivity to antibiotics and phages, serological reactions and ecological properties has also been used for the classification of *Streptomyces* spp. (Shirling and Gottlieb, 1966; Lechevalier and Lechevalier, 1980).

The highest antimicrobial activity was achieved at optimum environmental and nutritional conditions in *Streptomyces crystallinus* AZ151 culture. Several studies have shown the optimization of nutritional and environmental conditions for antibiotic production (Kumar and Satyanarayana, 2007; Latifian *et al*., 2007; Gupta *et al*., 2008; Kagliwal *et al*., 2009; Atta *et al*., 2011). San *et al*. (2010) worked on two venoms of *Calloselasma rhodostoma* and *Ophiophagus hannah* and determined (MIC) with *Staphylococcus aureus* ATCC25923, ATCC29213 and ATCC43300. The MIC values obtained for *Calloselasma rhodostoma* were 125 μg mL^{-1} when tested against *S. aureus* ATCC25923 and ATCC43300 while it was 250 μg mL^{-1} when tested against *S. aureus* ATCC29213. MIC values obtained for *Ophiophagus hannah* were 250 μg mL^{-1} when tested against all three strains.

In fact, the active metabolites were extracted by ethyl acetate (Criswell *et al*., 2006; Sekiguchi *et al*., 2007; Augustine *et al*., 2005). The extract from culture filtrate of endophytic *Streptomyces* sp. ST8 by ethyl acetate has activity against *S. mutans* ATCC25175 and 104 B. The extract at such concentrations (0.05-5 mg mL^{-1}) showed the inhibition of bacterial adherence on glass surfaces and saliva-coated hydroxyapatite. The crude extract also decreased the activity of glucosyltransferase and glucan-binding lectin from both strains (Taechowisan *et al*., 2008).

The organic phase was collected and evaporated under reduced pressure using rotary evaporator. Moreover, the purification process indicated that maximum activity was recorded between fraction Nos. 14-23. Many workers used a column chromatography packed with silica gel and the same situation was observed (Hitchens and Kell, 2003; Criswell *et al*., 2006; Sekiguchi *et al*., 2007).

CONCLUSION

In this study we determined the physico-chemical characteristics of the purified antimicrobial agents produced by *Streptomyces crystallinus* AZ151 and indicated that, it produces characteristic odour, its melting points is 160°C, infrared (IR) spectrum showed characteristic band corresponding to 21 peaks, a maximum absorption UV peak recorded at 225 NM and Mass spectrum confirmed that the molecular weight is at 432.36. Consequently, the elemental analytical data are find to be:

C = 46.43; H = 7.46; N = 6.81 and O = 39.3, indicate a suggested empirical formula of $C_{15}H_{30}N_2O_{10}$ and being belonging to Hygromycin-B antibiotic. Physico-chemical characteristics of many antibiotics were determined (Koshiyama *et al.*, 1969; Singh and Gurusiddaiah, 1984; Omura *et al.*, 1987; Uyeda *et al.*, 2001; Yanai and Murakami, 2004; Atta *et al.*, 2011).

REFERENCES

Aghighi, S., G.H.S. Bonjar, R. Rawashdeh, S. Batayneh and I. Saadoun, 2004. First report of antifungal spectra of activity of Iranian actinomycetes strains against *Alternaria solani, Alternaria alternate, Fusarium solani, Phytophthora megasperma, Verticillium dahliae* and *Saccharomyces cerevisiae*. Asian J. Plant Sci., 3: 463-471.

Ammar, M.S., M. El-Esawey, M. Yassin and Y.M. Sherif, 1998. Hydrolytic enzymes of fungi isolated from certain Egyptian antiquities objects while utilizing the industrial wastes of Sugar and Integrated Industries Company (SIIC). Egypt. J. Biotechnol., 3: 60-90.

Aria, T., S. Kuroda and Y. Mikami, 1976. Classification of Actinomycetes with Reference to Antibiotic Production. In: Actinomycetes: The Boundary Microorganisms, Aria, T. (Ed.). Topan Company Ltd., Tokyo and Singapore, pp: 261-276.

Atta , H.M., B.M. Haroun and M.A. Khalifa, 2011. Physico-chemical characteristics of vernamycin: A antibiotic biosynthesis by *Streptomyces* SP-AZ-SH-29. J. Saudi Chem. Soc., 15: 247-255.

Augustine, S.K., S.P. Bhavsar and B.P. Kapadnis, 2005. A non-polyene antifungal antibiotic from *Streptomyces albidoflavus* PU 23. J. Biosci., 30: 201-211.

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 development of antibiotic research and classification of antibiotic according to chemical structure. Adv. Appli. Microbiol., 14: 309-406.

Berdy, J., 1980. Recent advances in and prospects of antibiotics research. Process Biochem., 15: 28-35.

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

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

Chen, Y., M. Yin, G.P. Horsman, S. Huang and B. Shen, 2010. Manipulation of pathway regulation in *Streptomyces globisporus* for overproduction of the enediyne antitumor antibiotic C-1027. J. Antibiot. (Tokyo), 63: 482-485.

Cowan, S.T. and K.J. Steel, 1994. Cowan and Steel`s Manual for the Identification of Medical Bacteria. 2nd Edn., Cambridge, University Press, Cambridge, UK., ISBN-13: 9780521203999, Pages: 238.

Criswell, D., V.L. Tobiason, J.S. Lodmell and D.S. Samuels, 2006. Mutations conferring aminoglycoside and spectinomycin resistance in *Borrelia burgdorferi*. Antimicrob. Agents Chemother., 50: 445-452.

Dadgar, T., M. Asmar, A. Saifi, M. Mazandarani and H. Bayat *et al.*, 2006. Antibacterial activity of certain Iranian medicinal plants against methicillin-resistant and sensitive. Asian J. Plant Sci., 5: 861-866.

Del-Sol, R., I. Armstrong, C. Wright and P. Dyson, 2007. Characterization of changes to the cell surface during the life cycle of *Streptomyces coelicolor*: Atomic force microscopy of living cells. J. Bacteriol., 189: 2219-2225.

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.

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.

Esmaeili, P. and R. Moshiri, 2011. Geographical factors in medicine and human settlements. J. Applied Sci., 11: 212-218.

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

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

Gupta, S., M. Kapoor, K.K. Sharma, L.M. Nair and R.C. Kuhad, 2008. Production and recovery of an alkaline exo-polygalacturonase from *Bacillus subtilis* RCK under solid-state fermentation using statistical approach. Biores. Technol., 99: 937-945.

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.

Hamdi, Y.A., A.M. Al-Taj and A. Dewwdar, 1980. Genera and species of actinomycetes isolated from Iraqi soils. Egypt. J. Microbiol., 15: 6-21.

Hankin, L., M. Zucker and D.C. Sands, 1971. Improved solid medium for the detection and enumeralion of proteolytic bacteria. Applied Microbiol., 22: 205-509.

Hindra, P.P. and M.A. Elliot, 2010. Regulation of a novel gene cluster involved in secondary metabolite production in *Streptomyces coelicolor*. J. Bacteriol., 192: 4973-4982.

Hitchens, G.D. and D.B. Kell, 2003. On the effects of thiocyanate and venturicidin on respiration-driven proton translocation in *Paracoccus denitrificans*. Biochim. Biophys. Acta, 766: 222-232.

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.

Huddleston, A.S., N. Cresswell, M.C. Neves, J.E. Beringer, S. Baumberg, D.I. Thomas and E.M. Wellington, 1997. Molecular detection of streptomycin-producing streptomycetes in Brazilian soils. Applied Environ. Microbiol., 63: 1288-1297.

Hussein, A.M., A.M. Rajab, A.A. Elgammal, F.A. Mansour, E. Sami, M. Helmy and N.E. Sheheta, 1981. Taxonomy of gray pigmented *Streptomyces* spp. isolated from Egyptian soil. Egypt. J. Bot., 23: 9-16.

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.

Kagliwal, L.D., S.A. Survase and R.S. Singhal, 2009. A novel medium for the production of cephamycin C by *Nocardia lactamdurans* using solid-state fermentation. Bioresour. Technol., 100: 2600-2606.

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 DC., USA.

Khan, F., I. Shukla, M. Rizvi, T. Mansoor and S.C. Sharma, 2011. Detection of biofilm formation in *Staphylococcus aureus*. Does it have a role in Treatment of MRSA Infections? Trends Med. Res., 6: 116-123.

Khucharoenphaisan, K., U. Puangpetch, K. Puttaraksa and K. Sinma, 2011. Grouping of actinomycetes isolated from termites using biochemical character. J. Biol. Sci., 11: 314-319.

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.

Koshiyama, H., H. Tsukiura, K. Fujisawa, M. Konishi and M. Hatori, 1969. Studies on cirramycin A1. I. Isolation and characterization of cirramycin A1. J. Antibiot., 22: 61-64.

Kumar, P. and T. Satyanarayana, 2007. Optimization of culture variables for Improving glucoamylase production by alginate-entrapped Thermomucor indicae-seudaticae using statistical methods. Bioresour. Technol., 98: 1252-1259.

Kuster, E. and S.T. Williams, 1964. Selection of media for isolation of streptomycetes. Nature, 202: 928-929.

Latifian, M., Z. Hamidi-Esfahani and M. Barzegar, 2007. Evaluation of culture conditions for cellulase production by two *Trichoderma reesei* mutants under solid-state fermentation conditions. Bioresour. Technol., 98: 3634-3637.

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.

Lechevalier, M.P. and H.A. Lechevalier, 1980. The Chemotaxonomy of Actinomycetes. In: Actinomycete Taxonomy, Dietz, A. and D.W. Thayer (Eds.). Special Publication No. 6. Society for Industrial Microbiology, Arlington, VA., USA., pp: 227-294.

Lee, E.J., N. Karoonuthaisiri, H.S. Kim, J.H. Park, C.J. Cha, C.M. Kao and J.H. Roe, 2005. A master regulator δB governs osmotic and oxidative response as well as differentiation via a network of sigma factors in *Streptomyces coelicolor*. Mol. Microbiol., 57: 1252-1264.

Mehling, A., U.F. Wehmeier and W. Piepersberg, 1995. Nucleotide sequences of *Streptomyces* 16S ribosomal DNA: Towards a specific identification system for Streptomycetes using PCR. Microbiology, 141: 2139-2147.

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.

Myles, D.C., 2003. Novel biologically active natural and unnatural products. Curr. Opin. Biotechnol., 14: 627-633.

Nitsch, B. and H.J. Kutzner, 1969. Egg-Yolk agar as a diagnostic medium for Streptomyces. Experientia, 23: 220-221.

Omura, S., A. Nakagawa, T. Fujimoto, K. Saito, K. Otoguro and J.C. Walsh, 1987. Hygromycin A, an antitreponemal substance. I. Screening method and therapeutic effect for *Treponema hyodysenteriae*-caused infection in CF-1 mice. J. Antibiot., 40: 1619-1626.

Palaniappan, N., V. Dhote, S. Ayers, A.L. Starosta, D.N. Wilson and K.A. Reynolds, 2009. Biosynthesis of the aminocyclitol subunit of hygromycin A in *Streptomyces hygroscopicus* NRRL 2388. Chem. Biol., 16: 1180-1189.

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. Hesselting and R.G. Benedict, 1956. A section of media for maintenance and taxonomic study of *Streptomycetes*. Antibiotics Ann., 1: 947-953.

Raja, A. and P. Prabakarana, 2011. Actinomycetes and drug-An overview. Am. J. Drug Discovery Dev., 1: 75-84.

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.

Saadoun, I., M.J. Mohammad, F. Al-Momani and M. Meqdam, 1998. Diversity of soil streptomycetes in Northern Jordan. Actinomycetes, 9: 52-50.

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

San, T.M., J. Vejayan, K. Shanmugan and H. Ibrahim, 2010. Screening antimicrobial activity of venoms from snakes commonly found in Malaysia. J. Applied Sci., 10: 2328-2332.

Sanger, F., S. Nicklen and A.R. Coulson, 1977. DNA sequencing with chainterminating inhibitors. Proc. Natl. Acad. Sci. USA., 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.

Singh, S.K. and S. Gurusiddaiah, 1984. Production, purification and characterization of chandramycin, a polypeptide antibiotic from *Streptomyces lydicus*. Antimicrob. Agents Chemother., 26: 394-400.

Taechowisan, T., A. Sitthipanya, A. Wanbanjob and P. Tantiwachwuttikul, 2008. Inhibitory effects of endophytic *Streptomyces* sp. ST8 on the growth, adherence and glucosyltransferase of *Streptococcus mutans*. J. Boil. Sci., 8: 43-51.

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

Umezawa, H. and I.R. Hooper, 1982. Aminoglycoside Antibiotics. Springer, Berlin, ISBN: 9783540115328, Pages: 368.

Uyeda, M., M. Mizukami, K. Yokomizo and K. Suzuki, 2001. Pentalenolactone I and hygromycin A, immunosuppressants produced by *Streptomyces filipinensis* and *Streptomyces hygroscopicus*. Biosci. Biotechnol. Biochem., 65: 1252-1254.

Waksman, S.A., 1961. The Actinomycetes, Classification, Identification and Description of Genera and Species. Vol. 2, The Williams and Wilkins Company, Baltimore, pp: 61-292.

Waksman, S.A., 1975. The Antibiotic Era. The Waksman Foundation of Japan Inc., Tokyo, pp: 39-53.

Wang, Y., Q. Luo, X. Zhang and W. Wang, 2011. Isolation and purification of a modified phenazine, griseoluteic acid, produced by *Streptomyce griseoluteus* P510. Res. Microbiol., 162: 311-319.

Williams, S.T. and F.L. Davies, 1965. Use of antibiotics for selective isolation and enumeration of actinomycetes in soil. J. Gene. Microbiol., 38: 251-262.

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

Wipat, A., E.M. Wellington and V.A. Saunders, 1991. Streptomyces marker plasmids for monitoring survival and spread of *Streptomycetes* in soil. Applied Environ. Microbiol., 57: 3322-3330.

Yanai, K. and T. Murakami, 2004. The kanamycin biosynthetic gene cluster from *Streptomyces kanamyceticus*. J. Antibiot., 57: 351-354.

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.