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ISOLATION AND CHARACTERIZATION OF ACTINOMYCETE STRAIN RELATED TO GENUS STREPTOMYCES AND STUDY THE ACTIVE SUBSTANCE THAT PRODUCED

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Abstract

An actinomycete culture could be isolated from a soil sample collected from Bani-Swife governorate, Egypt. The isolate AZ- B2 produced a wide spectrum antibiotic (anti-Gram-positive and Gram-negative bacteria). The isolate AZ-B2 matches with ***Streptomyces griseoviridis*** in the morphological, physiological and biochemical characters. Thus, it was given the suggested name *Streptomyces griseoviridis*. The parameters controlling the biosynthetic process of antibacterial agent formation including different pH values, different temperatures, different incubation period, and different carbon and nitrogen sources were fully investigates.

The separation of the active ingredient and its purification was performed using both thin layer chromatography (TLC) and column chromatography (CC) techniques. The physico-chemical characteristics of the purified antibiotic viz. color, melting point, solubility, elemental analysis, spectroscopic characteristics and chemical reactions have been investigated. This analysis indicates a suggested imperical formula of $C_{18}H_{36}N_4O_{11}$. The minimum inhibition concentrations "MICs" of the purified antibiotic were also determined. The collected data emphasized the fact that the purified antibiotic compound was suggestive of being belonging to Aminocyclitol group (Kanamycin A antibiotic) produced by *Streptomyces griseoviridis*, AZ- B2

Introduction

Kanamycin (Km), one of the most commercially successful antibiotics, is an aminocyclitol produced by *Streptomyces kanamyceticus* (Yanai, 2004). Kanamycin a very important class of antibiotics is bactericidal in action, and in general, its bacteriological spectrum is very similar to that of neomycin (Rodger *et al.,* 1991) It has a wide range of action against Gram-negative and some Gram-positive bacteria (Davis *et al.,* 1974). Aminoglycosides work by binding to the bacterial 30S ribosomal subunit, causing misreading of t-RNA, leaving the bacterium unable to synthesize proteins vital to its growth. (Gale *et al.,* 1981; JezDowska-Bojczuk *et al.,* 1998 and Mikkelsen *et al.,* 1999). Interactions between ribonucleic acid molecules and aminoglycosides underlie many of the above effects. (Tanaka *et al.,* 1984). The

strength of these interactions are determined by electrostatics between negatively charged backbone phosphates and amino groups localized on the aminosugar or aminocyclitol rings of aminoglycoside molecules, which are positively charged at physiological pH (Hehl *et al.,* 1999). The results of recent studies introduce aminoglycosides as a novel family of stimulators of reactive oxygen species (ROS) formation (Greenwald, 1985). Kanamycin molecule consists of the central 2-deoxystreptamine ring, to which sugar moieties of 3-D-glucosamine (kanosamine) and 6-D-glucosamine are attached by glycosidic bonds. The crystal structure of kanamycin A shows that all three rings in the molecule assume the chair conformation (Micera and Koz³owski 1995). Its four amino functions, together with the neighboring hydroxyl groups, offer several potential binding sites for metal ions, such as copper(II), which was previously demonstrated to form stable complexes with related aminoglycosides. (Hehl *et al.,* 1999). Aminosugars bind Cu(II) effectively. The anchoring of the cupric ion to their molecules is accomplished through amine functions (Onaolapo, 1994). The stability and stoichiometry of the resulting complexes depend, however, on the formation of chelate rings using appropriate hydroxyl groups of the ligand (Nakajima *et al.*, 1984).

Materials and Methods

Microorganism:

The actinomycete AZ- B2 was isolated from soil sample collected from Bani-Swife governorate. It was purified using the soil dilution plate technique described by Williams and Davis (1965).

Screening for antimicrobial activity:

The anti- microbial activity was determined according to (Kavanagh, 1972).

Taxonomic studies of actinomycete isolate (AZ- B2)*: -*

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.

Physiological and biochemical characteristics:

Lecithinase was detected using egg–yolk medium according to the method of Nitsh 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.*, 1956-57). 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 (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 Lechevaier (1968).

Color characteristics:

The ISCC-NBS color –Name Charts illustrated with centroid detection of the aerial, substrate mycelia and soluble pigments (Kenneth and Deane, 1955) was used.

Parameters controlling antibacterial agent

These included incubation period, pH values, incubation temperatures; different carbon and nitrogen sources and have been determine by the standard methods.

Fermentation and purification of antibacterial agent:

Fermentation*: Streptomyces griseoviridis*, AZ- B2 was inoculated into 250 ml Erlenmeyer flasks containing 75 ml of liquid starch nitrate medium. The flasks were incubated on a rotary shaker (200 rpm) at 30 ^{0}C for 7 days. Twenty-liter total volume was filtered through Whatman No.1 filter paper, followed by centrifugation at 5000 r.p.m for 20 minutes. The clear filtrates were tested for their activities against test organisms.

Extraction: The clear filtrate was adjusted at different pH values (4 to 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 antibacterial activities.

Separation: Separation of the antibacterial 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 antibacterial compound was carried out using silica gel column (2.5 X 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-ml 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.

Bioautography of the purified antibacterial agent:

Twenty microliters of the purified antibacterial compound were loaded on paper chromatographic strips and the R_f values were calculated upon application of different solvent systems according to Bilinov and Khoklov, (1970). After development, the paper strips (Whatman No.1) were air-dried and placed on agar plates seeded with *Micrococcus luteus*, ATCC 9341; *Staph. aureus*, NCTC 7447 and *Escherichia coli*, NCTC 10416 as the test bacterial strain.

Physico-Chemical properties of antibacterial agent:

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

2- Spectroscopic analysis: The IR, UV, Mass spectrum, NMR spectrum and HPLC-spectrum were determined at the microanalytical center of Cairo University, Egypt.

3- Reaction of the antibacterial agent with certain chemical test: For this purpose, the following reactions were carried out: Molish's, Fehling, Sakaguchi, Ninhydrin, Ehrlish, Nitroprusside, Ferric chloride, and Mayer reactions.

4-Biological activity: The minimum inhibitory concentration (MIC) has been determined by the cup method assay (Kavanagh, 1972).

5-Characterization of the antibacterial agent: The antibiotic produced by *Streptomyces griseoviridis*, AZ- B2 was identified according to the recommended international references of Umezawa (1977), Berdy (1974, 1980a, b, c).

Results

Characterizations of the actinomycete isolate, AZ- B2:
Morphological characteristics:

Spore chain are spiral, spore mass are red; spore surfaces are smooth and the reverse is grayish yellow and light yellowish brown; diffusible pigment production is grayish red orange (Plates 1, 2).

Cell wall hydrolysate:

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

Physiological and biochemical characteristics

The actinomycete isolate AZ-B2 could hydrolyze protein, starch, lipid, cellulose, pectin and lecithin and catalase are negative. Melanin pigment is negative, degradation of xanthine, esculine; production of H_2S; decomposition of urea; nitrate reduction and utilization of citrate are positive but coagulation of milk are negative.

The isolate under study utilizes L-arabinose, D- mannose, D- glucose, rhamnose, mannitol, *meso*-inositol, lactose, maltose, raffinose, D-meticitose and trehalose, 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-lysine, L-valine, L-glutamic acid, L-Hydroxyproline and L- phenyl alanine. Growth in the presence of up to (7 %) NaCl . The growth is not inhibited in the presence of sodium azide, thallous acetate and phenol. The actinomycete isolate not resistance to Pencillin G; Amoxicillin, Gentamycin and Cephalosporin "Keflex" whereas; resistunce to Augmentin Carbenicillin, Cefadroxil, Cephardine Ceptrine, Chloramphinicol, Cloxacillin, Doxycycline, Neomycin Erythromycin, Rifampicin and Noroxin. The isolate AZ-B2 is active against Gram positive and Gram negative bacteria (*Staph. aureus*, NCTC 7447; *B.subtilis*, NCTC 1040; *M. luteus*, NCTC 1089; *E. coli*, NCTC 10416 and *K. pneumonia* NCIMB 9111) (Table 1).

Color and culture characteristics :

The isolate AZ-B2 exhibited good growth on starch-nitrate agar medium, 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-5). Moderate growth was detected on Oat-meal agar medium (ISP-3), aerial mycelium medium red, substrate mycelium light grayish 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 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 (2).

Identification of actinomycete isolate, AZ- B2:

This was performed basically according to the recommended international Key's viz. (Buchanan and Gibsons, 1974; Williams, 1989; and Hensyl, 1994) and Numerical taxonomy of *Streptomyces* species program (PIB WIN) (*Streptomyces* species J. Gen Microbiol. 1989 13512-133 lang. On the basis of the previously collected data and in view of the comparative study of the recorded properties of AZ- B2 in relation to the most closest reference strain, viz. *Streptomyces griseoviridis* it could be stated that actinomycetes isolate, AZ- B2 is suggestive of being likely belonging to *Streptomyces griseoviridis,* AZ- B2. Table (3).

Screening for the antimicrobial activities:

The active metabolites produced by *Streptomyces griseoviridis,* AZ- B2 exhibited various degrees of activities against Gram positive and Gram negative bacteria (Table 4).

Parameters controlling the biosynthesis of the antibiotic:

Incubation period:

Maximum biosynthesis of the antibacterial agents could be record within an incubation period of 7 days.

pH value:

The biosynthesis of the antibacterial agents reached its maximum yield in the production medium adjusted at pH 7.0.

Incubation temperature:

Maximum production of the antibacterial agents could be recorded at incubation temperature 30ºC.

Carbon source:

Lactose was found the best carbon source for the antibacterial agent production.

Different lactose concentrations:

The best concentration of lactose for the biosynthesis of the antibacterial agent is 3.0 g/100 ml.

Nitrogen source:

The nitrogen sources exhibited an increase in the level of antibacterial agent production by *Streptomyces griseoviridis*, AZ- B2 where $NaNO_3$ was found to be the best nitrogen source for the antibacterial agent production.

Sodium nitrate concentration:

The application of different concentrations of $NaNO_3$ indicated that optimum concentration of $NaNO_3$ for maximum antibiotic production by *Streptomyces griseoviridis*, AZ- B2 is 2.5 g/l.

Fermentation extraction, separation and purification:

The fermentation process was carried out for six days at 30°C using liquid starch nitrate medium as production medium. Filtration was conducted followed by centrifugation at 4000 r.p.m. for 15 minutes.

The clear filtrates containing the active metabolite (20 liters), was adjusted to 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 antibacterial agent was precipitated by petroleum ether (b.p. 60-80°C) and centrifuged at 5000 r.p.m for 15 minute. Its color is grayish red orange. The fraction was test for antibacterial activities (Table 5).

Separation of antibacterial 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 0.80 showed antibacterial activity. The purification process through column chromatography packed with silica gel, indicated that maximum activity was at fractions Nos. 23 and 24. Fig. (1)

Bioautography of the purified antibacterial agent:

Data of the antibacterial agent are summarizing in table (6). It is evident that only one band exhibited an activity against test organisms at different developing solvent system.

Physicochemical characteristics of the antibacterial agent:

The purified antibacterial agent is soluble in chloroform, n-Butanol, acetone, carbon tetrachloride, ethanol, DMSO and methanol but insoluble in petroleum ether, hexane and benzene with a melting point of 260 °C.

Elemental analysis:

The elemental analytical data of the antibacterial agent revealed the following data: **C**=44.94; **H**=7.59; **N**= 11.37, **O** = 36.1 and **S**= 0.0. This analysis indicates a suggested calculated imperical formula of $\boldsymbol{C_{18}H_{36}N_4O_{11}}$.

Spectroscopic characteristics:

The infrared (IR) spectrum of the antibacterial agent showed characteristic band corresponding to 17 peaks (Fig.2).The ultraviolet (UV) absorption spectrum of the antibacterial agent recorded a maximum absorption peak at 242 and 444 nm (Fig. 3). The HPLC- spectrum showed that maximum peak at 2.45 (Fig.4). The Mass spectrum revealed that the molecular weight 482 (Fig.5). The NMR-Spectrum could be also determined (Fig.6).

Biochemical reaction of the antibacterial agent:

The reactions revealed the detection of certain groups in the investigated molecule. The antibacterial agent exhibited positive results with ninhydrin, ferric chloride and Mayer tests and negative results with nitroprusside, molish's, fehling, sakaguchi, and ehrlish reactions (Table 7).

Biological activities of the antibacterial agent:

Data of the antibacterial agent spectrum indicated that the agent is active against Gram-positive and Gram-negative bacterial strains (Table 8).

Identification of the antibacterial 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 antibacterial agent, it could be stated that the antibacterial agent is suggestive of being belonging to Aminocyclitol group (Kanamycin-A antibiotic) (Table 9).

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Plate (1a). A photograph of the actinomycete isolate AZ-B2 growing on starch nitrate agar medium showing spore chain spiral shape (X1600).

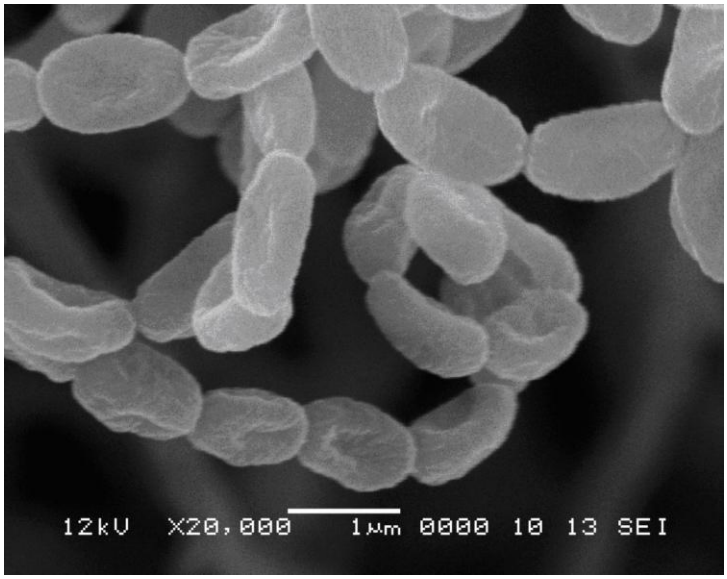

Plate (1b). Scanning electron micrograph of the actinomycete isolate AZ-B2 growing on starch nitrate agar medium showing spore chain spiral shape and spore surfaces smooth (X20,000).

Table (1): The morphological, physiological and biochemical characteristics of the actinomycete isolate AZ-B2.

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	Maltose	+
Motility	Non-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 of:		L-Valine	-
Protein; Starch , cellulose, Lipid	+	L-Histidine	+
Egg-yolk (lecithin**)** and Pectin	+	L-Phenylalanine	-
Catalase test	-	L-Arginine	+
Resistance of different antibiotics		Lysine	-
Pencillin G;,Amoxicillin, Gentamycin and Cephalosporin (Keflex)	-	L-Hydroxproline	-
		L-Glutamic acid	-
		Growth inhibitors:	
Augmentin Carbenicillin, Cefadroxil, , Cephardine Ceptrine, Chloramphinicol, Cloxacillin, Doxycycline, Neomycin Erythromycin, Rifampicin and Noroxin	+	Thallous acetate (0.001)	+
		Sodium azide (0.01)	+
		Phenol (0.1)	+
		Growth at different temperatures (˚C):	
		10	-
Production of melanin pigment on:		20 - 45	+
eptone yeast- extract iron agar (ISP-6)	-	50	-
Tyrosine agar medium(ISP-7)	-	**Growth at different pH values:**	
Tryptone – yeast extract broth (ISP-1)	-	4	-
		5-9	+
Degradation of:		10	-
Xanthin	+	**Growth at different concentrations of NaCl (%)**	
Esculin	+	1-7	+
H_2S Production	+	10	-
Nitrate reduction	+	**Active against of :**	
Citrate utilization	+	*Staph. aureus,* NCTC 7447 *B.subtilis,* NCTC 1040; *M. luteus,* NCTC 1089*; E. coli,* NCTC 10416. and *K. pneumonia* NCIMB 9111.	+
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 (2): Cultural characteristics of the actinomycete isolate AZ-B2.

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

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

Table (3): Numerical taxonomy of *Streptomyces* species program (PIB WIN) (*Streptomyces* species) J. Gen Microbiol. 1989 13512-133 lang.

Characteristic	AZ-B2	*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		
1-Peptone yeast extract-iron agar medium (ISP-6)	-	-
2-Tyrosine agar medium (ISP-7)	-	-
Active against of:		
1- B. subtilis and M.luteus	+	+
2- C. albicans and A.niger	-	-
Lecithinase activity	+	+
Lipolysis activity	+	+
Pectin hydrolysis	+	+
Nitrate reduction	+	+
H_2S production	+	+
Degradation of Xanthin	+	+
Resistance of:		
Neomycin	-	-
Rifampicin	+	+
Pencillin G.	+	+
Growth at 45°C	+	+
Growth at NaCl 7% (w/v)	+	+
Growth inhibitors		
Phenol (0.1 % w/v)	+	+
Thallus acetate (0.001 % w/v)	+	+
Utilization of		
L- Cysteine	+	+
L- Valine	-	-
L- phenylalanine	-	-
L- Histadine	+	+
L-Hydroxproline	-	-
Sucrose	-	-
meso-Inositol	+	+
Rhamnose	+	+
Raffinose	+	+
Melezitose	+	±

No.	Key	Source	Identification	ID Score
1	AZ-B2	Bani-Swife governorate	*Streptomyces griseoviridis*	0.99963

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

Table (4): Mean diameters of inhibition zones (mm) caused by 100µl of the antimicrobial activities produced by *Streptomyces griseoviridis*, AZ-B2 in the agar plate diffusion assay (The diameter of the used cup assay was 10 mm).

Test organism		Mean diameters of inhibition zone (mm)
A-Bacteria		
1-Gram Positive		
Staphylococcus aureus,	NCTC 7447	32.0
Bacillus subtilis,	NCTC 1040	32.0
Bacillus pumilus,	NCTC 8214	31.0
Micrococcus luteus,	ATCC 9341	33.5
2-Gram Negative		
Escherichia coli,	NCTC 10416	29.0
Klebsiella pneumonia,	NCIMB 9111	28.0
Pseudomonas aeruginosa,	ATCC 10145	27.0
B- Fungi		
1-Unicellular fungi		
Candida albicans,	IMRU 3669	0.0
Saccharomyces cervicea		0.0
2-Filamentous fungi		
Aspergillus niger,	IMI 31276	0.0
Aspergillus fumigatus		0.0
Aspergillus flavus,	IMI 111023	0.0
Fusarium oxysporum		0.0
Penicillium chrysogenum		0.0

Table (5): Fermentation, Isolation and Separation steps of antibacterial agent produced by *Streptomyces griseoviridis* AZ-B2.

Step	* Mean values of inhibition zones (mm) against:		
	***Micrococcus luteus*, ATCC 9341**	***Staph. aureus*, NCTC 7447**	***Escherichia coli*, NCTC 10416**
1-Fermentation	35.0	34.0	30.0
2-Extraction	33.5	32.5	28.5
3-Precipitatio	33.0	32.0	28.0

- ***Mean values of 4 determinations*.**

Table (6): The R_f values of the bioautography of antibacterial agent produced by *Streptomyces griseoviridis,* AZ-B2.

Developing solvent systems and symbols	R_f value
1- Petroleum ether	0.1
2- Chloroform	0.8
3- n-Butanol	0.3
4- Ethyl acetate	1.0
5- Chloroform - Ethyl acetate (1:1,v/v)	0.9
6- Chloroform - n-Butanol (1:1, v/v)	0.7
7- Methanol	0.8
8- Acetone	0.7
9- Distilled water	0.0
10- Ammonium chloride (3%)	0.0
11- n-Butanol : Pyridine : water (2.0.0.6:1.0, v/v)	0.5
12- n-Butanol : acetic acid : water (1:1:1, v/v)	0.3

Table (7): Biochemical reactions of the purified antibacterial agent produced by *Streptomyces griseoviridis,* AZ-B2.

Chemical test	Result	Remark
Molish's reaction	-	Absent of sugar moiety
Fehling test	-	Absence of free aldehyde or keto sugar
Ninhydrin test	+	Presence of free-NH_2 group
Sakaguchi reaction	-	Absence of Arginine
Nitroprusside reaction	-	Absence of Sulfur
Ferric chloride reaction	+	Presence of Di-ketons group
Ehrlish rection	-	Absence of indolic acid
Mayer reaction	+	Presence of nitro group

Table (8): Biological activities (MIC) of the antibacterial agent by cup method assay.

Test organisms		MIC (µg/ml) concentrations
A- Bacteria		
a. Gram positive cocci		
Staph. aureus	NCTC 7447	7.8
Micrococcus luteus	ATCC 9341	7.8
b. Gram positive bacilli		
Bacillus pumilus	NCTC 8214	15.6
Bacillus subtilis	NCTC 10400	11.7
c. Gram negative bacteria		
Escherichia coli	NCTC 10416	23.43
Klebsiella pneumonia	NCTC 9111	31.25
Salmonella typhi	ATCC 10416	46.87
Pseudomonas aeruginosa	ATCC 10415	62.50

Table (9): A comparative study of the characteristic properties of AZ-B2 antibiotic in relation to Reference antibiotic (Kanamycin-A) (C.F. Umzawa ,1977 and Berdy, 1980,Vol.II).

Characteristic	Kanamycin-A	Purified antibacterial agent
1- Melting point	263-268 °C	260 ^{0}C
2- Molecular weight	483	482
3- Chemical analysis:		
C	44.8	44.84
H	7.5	7.59
N	11.3	11.37
O	ND	36.1
S	0.0	0.0
4- Ultra violet	End absorbance	242 and 444 nm
5- Formula	$C_{18}H_{36}N_4O_{11}$	$C_{18}H_{36}N_4O_{11}$
6- Active against	Gram-Positive and Gram-Negative Bacteria,	Gram-Positive and Gram-Negative Bacteria

ND=No Data and -=Negative results.

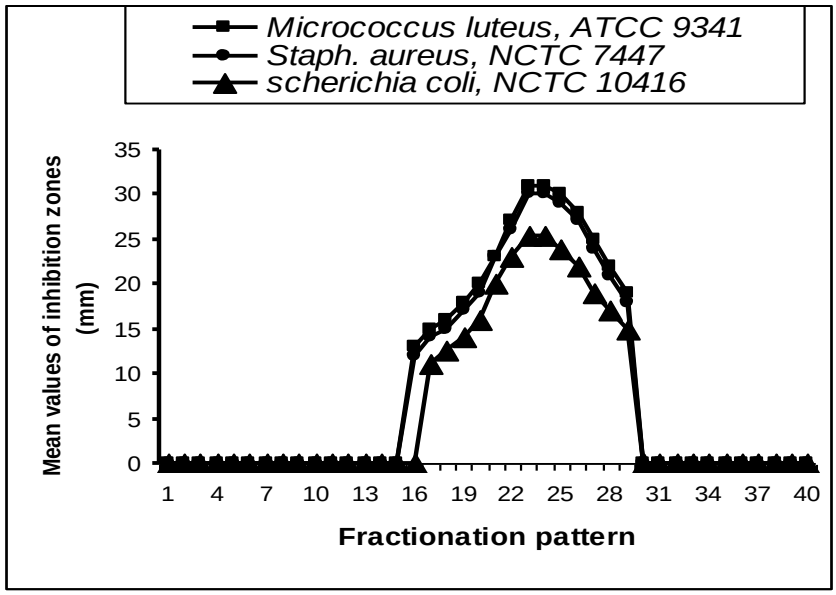

Fig. (1): Effect of fractionation patterns of antibacterial agent produced by *Streptomyces griseoviridis,* AZ-B2.

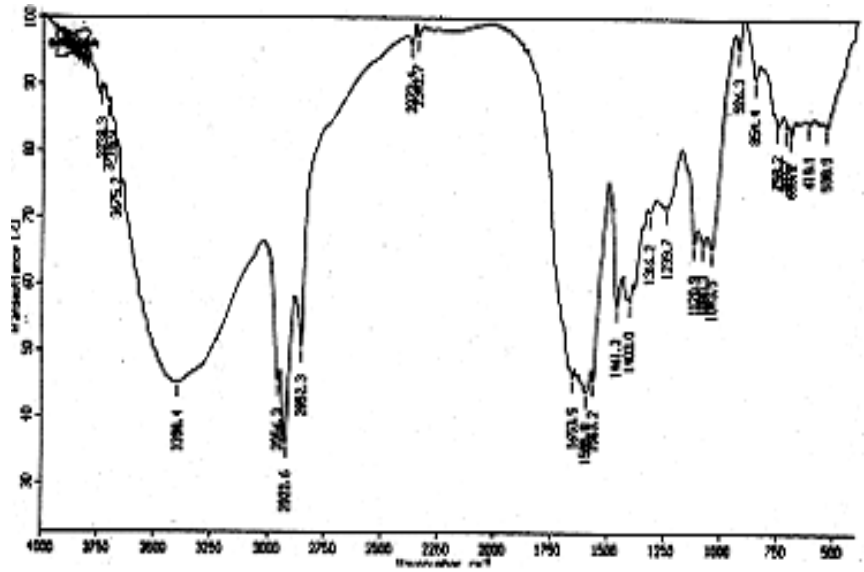

Figure 2: Infrared spectrum of the antibacterial agent.

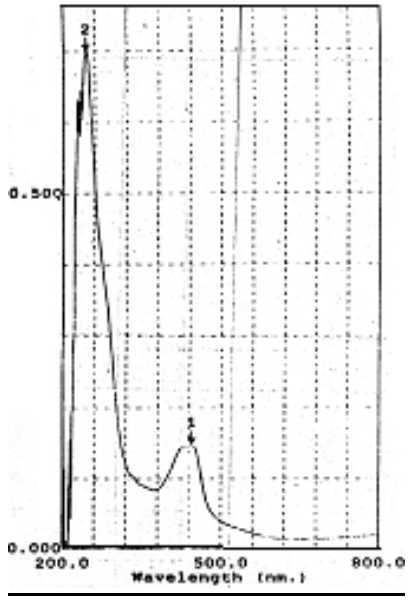

Figure 3: Ultraviolet absorbance of antibacterial agent

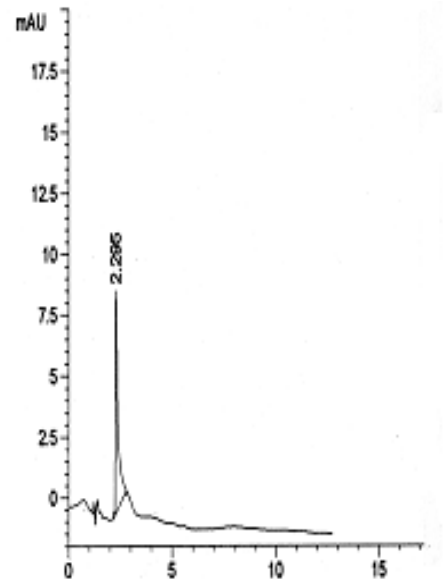

Figure 4: HPLC spectrum of antibacterial agent

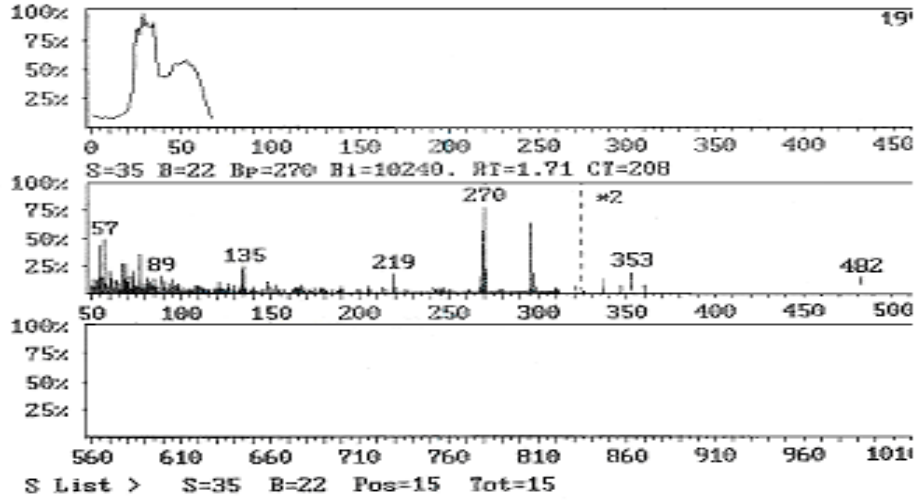

Figure 5: Mass Spectrum of antibacterial agent.

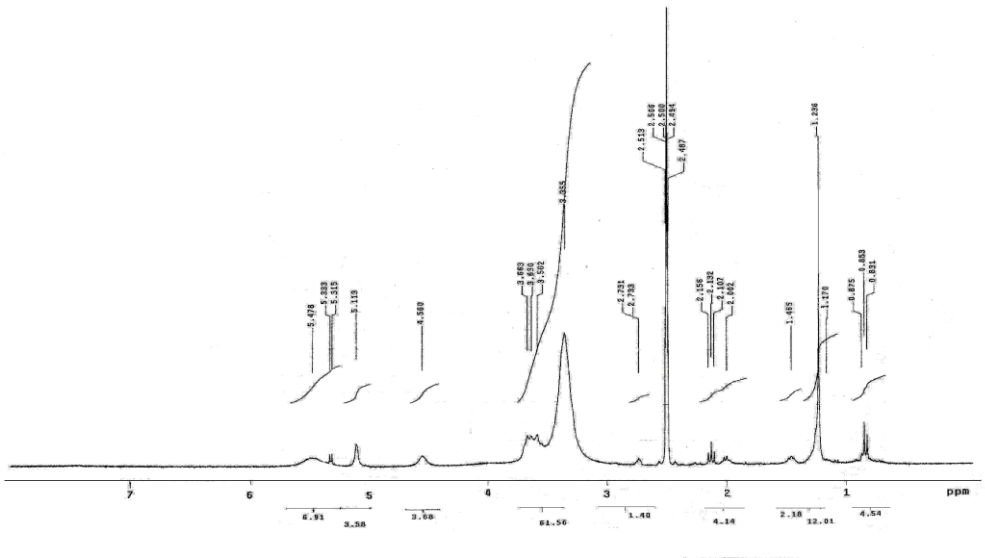

Figure 6: NMR- Spectrum of antibacterial agent.

Discussion

The actinomycete isolate, AZ- B2 was isolated from Bani-Swife governorate, Egypt. The isolate was growing on starch nitrate agar medium for investigating its potency to produce antimicrobial agents. The actinomycete isolate exhibited antibacterial activities.

Identification process has been carried out according to the Key's given in Bergey's Manual Of Determinative Bacteriology 8th edition (Buchanan and Gibbsons, 1974), Bergey's Manual Of systematic Bacteriology, vol. 4 (Williams, 1989) and Bergey's Manual Of Determinative Bacteriology, 9th edition (Hensyl, 1994) and Numerical taxonomy of *Streptomyces* species program (PIB WIN) (*Streptomyces* species J. Gen Microbiol. 1989 13512-133 lang. For the purpose of identification of actinomycete isolate, 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 grayish yellow, and diffusible pigment grayish red orange. The results of physiological, biochemical characteristics (table 1) and cell wall hydrolysate of actinomycetes isolate, exhibited that the cell wall containing LL-diaminopimelic acid (DAP) and sugar pattern of cell wall hydrolysate could not detected. These results emphasized that the actinomycetes isolate related to a group of *Streptomyces*.

In view of all the previously recorded data, the identification of actinomycete isolate AZ- B2 was suggestive of being belonging to *Streptomyces griseoviridis*, AZ- B2, which can produce a broad-spectrum antibiotic.

For optimizing the biosynthesis of the antibiotic from *Streptomyces griseoviridis*, AZ-B2, different environmental conditions such as pH, temperature, and incubation period, effect of different carbon and nitrogen sources was studied. The maximum biosynthesis was achieved at the end of an incubation period at 7 days for the antibacterial agent production. Similar results have been recorded by various workers; Ichikawa (1958) Marie-Françoise *et al.* (2002) and Kharel *et al.* (2004).

The fact that maximum yield of antibacterial agent occurred at the end of an incubation temperature of 30ºC was in complete accordance with those reported by Gourevitch, *et al.* (1958) Yanai (2004) and Jantaj (2004)

Data of the effect of different carbon and nitrogen sources on the production of the antibacterial agent indicated that *Streptomyces griseoviridis*, AZ-B2, require lactose and sodium nitrate at concentrations 3.0% and 0.25% respectively. Similar results have been recorded by various workers: Aharonowitz and Demain (1978 and

1979) Basak, and Majumdar (1993); Howells *et al.* (2002) and Criswell *et al.* (2006).

The active metabolites were extracted by ethyl acetate at pH 7. Similar results were obtained by Takagi *et al.*(1981), Yoram, *et al.* (2006) Criswell *et al.* (2006) and Sekiguchi, *et al.*, (2007).

The organic phase was collected and evaporated under reduced pressure using rotary evaporator. The extract was concentrated and treated with petroleum ether (b.p. 60-80°C) for precipitation process, where only one fraction was obtained in the form of grayish red orange powder, and then tested for antibacterial activities. The purification process through a column chromatography packed with silica gel and an eluting solvents composed of chloroform and methanol (8:2, v/v), indicated that maximum activity was recorded at fraction Nos. 23 and 24. Many workers used a column chromatography packed with silica gel. Similar results were obtained by Hitchens and Kell (2003), Criswell *et al.* (2006) and Sekiguchi, *et al.*, (2007).

The physico-chemical characteristics of the purified antibiotic revealed that, melting point is 260^0C; and soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and DMSO, but insoluble in petroleum ether, hexan and benzene. Similar results were recorded by Yanai (2004) and Jantaj (2004)

A study of the elemental analysis of the antibacterial agent lead to an imperical formula of*:* $C_{18}H_{36}N_4O_{11}$.The spectroscopic characteristics of the antibacterial agent under study revealed the presence of the maximum absorption peak in UV. at 242 and 444 nm, infra-red absorption spectrum represented by 17 peaks. HPLC-spectrum has emphasized that a high peak at 2.45 and NMR-spectrum was determined. The biochemical tests of the antibacterial agent gave positive reaction with ninhydrin, Mayer and Ferric chloride reactions. Similar results were recorded by Bastian, *et al.* (2001); Kruuner, *et al.* (2003); Criswell *et al.* (2006) and Sekiguchi, *et al.* (2007).

The biological activities (MIC) of the antibacterial agent emphasized that the antibiotic are active against Gram-positive and Gram-negative bacteria. Similar studies were conducted by Gourevitch, *et al.* (1958); Misumi and Tanaka (1980); Onaolapo (1994) Criswell *et al.* (2006) and Sekiguchi, *et al.* (2007).

Identification of antibiotic according to recommended international keys indicated that the antibiotic is suggestive of being likely belonging to Aminocyclitol group (Kanamycin-A antibiotic).

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" عزل وتوصيف سلالة أكتينوميسيتية تتبع جنس أستربتوميسس ودراسة المادة الفعالة التى تنتجها"

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[2] ميكروبيولوجيست فى مركز البيوتكنولوجى وقطاع الأبحاث والتطوير والجودة بشركة إيبيكو للأدوية.

تم عزل مزرعة اكتينوميسيتية من عينة تربة جمعت من محافظة بنى سويف بمصر. والعزلة أشير أليها بالرمز أ زد–بى 2 وقد وجد أن لتلك العزلة نشاط ضد إحيائي على البكتيريا الموجبة والسالبة لصبغة جرام. وبدراسة الصفات المورفولوجية والفسيولوجية والبيوكيميائية لتلك العزلة وجد أنها تماثل الخواص المعروفة للسلالة استربتوميسيس جريزيوفيريديس, لذلك تم إعطائها الاسم المقترح استربتوميسيس جريزيوفيريديس, أ زد–بى – 2.

تمت دراسة العوامل المؤثرة على التخليق الأحيائى للمضاد البكتيرى حيث اشتملت الدراسة علي , تأثير الأس الهيدروجيني , تأثير درجات الحرارة المختلفة, تأثير فترات التحضين المختلفة, تأثير المصادر الكربونية والنيتروجينية

تم استخلاص النواتج الأيضية النشطة باستخدام خلات الأيثيل. كما تم فصل المواد الفعالة وتنقيتها باستخدام الفصل اللونى بنوعية الرقائق الورقية وعمود الفصل اللونى .

ولمزيد من الدراسة تم إجراء الصفات الفيزيائية والكيميائية للمركب النقي ذات نشاط ضد بكتيري والتي اشتملت على اللون, درجة الانصهار, الذوبانية والتحليل الكيميائي للعناصر والخواص الطيفية والكمية والتفاعلات الكيميائية. وقد تبين من إجراء كافة الاختبارات أن الصيغة الكيميائية للمركب هي $C_{18}\ H_{36}\ N_4O_{11}$. كما تم أيضا دراسة الأنشطة البيولوجية للمركب النقي مثل تحديد التركيز الأدنى المثبط للمادة الفعالة النقية (MIC).

وبمقارنة خواص وصفات المادة الفعالة الناتجة من استربتوميسيس جريزيوفيريديس وجد أنها تماثل المضاد الحيوي كاناميسين–أ (Kanamycin-A); الذي ينتمي إلى مجموعة المضادات Aminocyclitol group .