

**Antifungal agent from *Streptomyces* sp: Taxonomy, Fermentation, Purification and Biological Activities****Houssam M. Atta<sup>1\*</sup> and Ahmed M. Reyad<sup>2</sup>**<sup>1</sup> Botany and Microbiology Department, Faculty of Science (Boys), Al-Azhar University, Cairo, Egypt. The present address: Biotechnology Department, Faculty of Science and Education, Taif University, Al-Khurmah branch – KSA.<sup>2</sup> Botany Department, Faculty of Science, Beni-Suef University, Egypt. The present address: Department of Biology, Faculty of Sciences, Jazan University, KSA.\*[houssamatta@yahoo.com](mailto:houssamatta@yahoo.com) and [houssamatta@hotmail.com](mailto:houssamatta@hotmail.com); Tel: 00966506917966

**ABSTRACT:** An actinomycete culture was isolated from a soil sample collected from Al-Khurmah governorate, KSA. This actinomycete isolate, KSA-818 was found to be active against unicellular and filamentous fungi viz. *Saccharomyces cerevisiae*, ATCC 9763; *Candida albicans*, IMRU 3669; *Aspergillus niger*; *Aspergillus fumigatus*; *Aspergillus flavus*; *Aspergillus terreus*; *Fusarium solani*; *Fusarium oxysporum*; *Fusarium moniliforme*; *Alternaria alternata*; *Botrytis cinerea*; *Penicillium chrysogenum* and *Rhizoctonia solani*. The nucleotide sequence of the 16S rRNA gene (1.5 Kb) of the most potent strain evidenced a 90% similarity with *Streptomyces fimbriatus*. From the taxonomic features, the actinomycetes isolate KSA-818 matched with *Streptomyces fimbriatus* in the morphological, physiological and biochemical characters. Thus, it was given the suggested name *Streptomyces fimbriatus*, KSA-818. The active metabolite was extracted using n-butanol (1:1, v/v) at pH 7.0. 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 antifungal agent viz. color, melting point, solubility, elemental analysis and spectroscopic characteristics have been investigated. This analysis indicates a suggested empirical formula of C<sub>26</sub> H<sub>36</sub> O<sub>9</sub> N<sub>2</sub>. The minimum inhibition concentrations "MICs" of the purified antifungal agent were also determined. In conclusion, the collected data emphasized the fact that the purified antifungal agent was suggestive of being belonging Blastmycin antibiotic produced *Streptomyces fimbriatus*, KSA-818.

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**1. INTRODUCTION**

Actinomycetes are the most economically and biotechnologically valuable prokaryotes able to produce wide range of bioactive secondary metabolites, such as antibiotics, antitumor agents, immunosuppressive agents and enzymes [Deepa *et al.*, 2013]. These metabolites are known to possess antibacterial, antifungal, anticancer, antialgal, antimalarial and anti-inflammatory activities [Ravikumar *et al.*, 2011]. Deepa *et al.*, 2013, says that the Actinomycete have the capacity to synthesize many different biologically active secondary metabolites such as cosmetics, vitamins, nutritional materials, herbicides, antibiotics, pesticides, anti-parasitic and enzymes like cellulose and xylanase used in waste treatment [Ogunmwonyi *et al.*, 2010]. They are free living, saprophytic bacteria, and a major source for production of antibiotics [Atta *et al.*, 2009].

The Blastmycin antibiotic consists of 9- membered dilactone ring which bears a long alkyl side chain and O-acyl group, linked via an amide bond to a 3-formamino-salicylic acid residue [Berdy, 1980]. Blastmycin producing *Streptomyces* sp e.g *S. blastomyceticus*, *S. kitazawaensis*, *S. griseus* and *S. fimbriatus* are commonly recognized in the screening programs directed toward the isolation of antifungal

compounds other than polyenes. A wide variety of saprophytic and pathogenic fungi & yeast are highly sensitive to Blastmycin and used as antitumor [Umezawa, 1977; Berdy, 1980 and Jacobi, *et al* 2000].

In the present work we describe the isolation of an actinomycete strain from Al-Khurmah governorate, KSA, which generates an antifungal compound. The identification of this strain, based on the cultural, morphology, physiology and biochemical characteristics, as well as 16s rRNA methodology, is also reported. The primary bioactive substance was isolated, purified and biological activities were determined.

**2. MATERIALS AND METHODS****2.1. Microorganism**

The actinomycete strain was isolated from soil sample collected from Al-Khurmah governorate, KSA. It was purified using the soil dilution plate technique described by [Williams and Davis, 1965].

**2.2. Media Used****Growth media (g/L)**

Starch, 20.0; NaNO<sub>3</sub>, 2.0; K<sub>2</sub>HPO<sub>4</sub>, 1.0; MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.5; KCl, 0.5; FeSO<sub>4</sub> 0.01; Yeast extract 2.0 agar, 20.0 distilled water up to 1000 ml. The pH was

adjusted at 7.2 before sterilization.

### 2.3. Test organisms

#### 2.3.1. Bacteria

##### 2.3.1.1. Gram Positive

*Micrococcus kristinae*, ATCC 27570; *Staphylococcus aureus*, NCTC 7447; *Staphylococcus haemolyticus*, NCTC 29968; *Bacillus subtilis*, NCTC 1040, *Bacillus pumilus*, NCTC 8214 and *Sarcina maxima*, ATCC 33910.

##### 2.3.1.2. Gram Negative

*Escherichia coli*, NCTC 10416; *Klebsiella pneumoniae*, NCIMB, 9111 and *Pseudomonas aeruginosa*, ATCC 10145.

#### 2.3.2. Fungi

##### 2.3.2.1. Unicellular fungi

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

##### 2.3.2.2. Filamentous fungi

*Aspergillus niger*; *Aspergillus fumigatus*; *Aspergillus flavus*; *Aspergillus terreus*; *Fusarium solani*; *Fusarium oxysporum*, *Fusarium moniliforme*, *Alternaria alternata*, *Botrytis cinerea*, *Penicillium chrysogenum* and *Rhizoctonia solani*.

### 2.4. Identification of actinomycete isolate, KSA-818:

#### 2.4.1. Morphological characteristics

Purified isolates of actinomycetes were identified using morphological and cultural characteristics by the methods as described in the International *Streptomyces* Project (ISP) [Shirling and Gottlieb, 1966]. The morphology of the spore bearing hyphae with the entire spore chain, the structure and arrangement of the spore chain with the substrate and aerial mycelium of the actinomycetes were examined using slide culture technique and identified [Williams *et al.*, 1989]. After growth, the slide cultures were examined under light microscope [Pridham, 1965].

#### 2.4.2. Physiological and biochemical characteristics

Lecithinase was conducted on Egg-Yolk medium according to the method of [Nitsh and Kutzner, 1969]; Lipase [Elwan, *et al.*, 1977]; Protease [Ammar, *et al.*, 1991]; Pectinase [Ammar, *et al.*, 1995b];  $\alpha$ -amylase [Ammar, *et al.*, 1998] and Catalase Test [Jones, 1949]. Degradation of Esculin and xanthine has been done according to [Gordon, *et al.*, 1974]. Nitrate reduction was performed according to the methods of [Gordon, 1966]. Hydrogen sulphide production was carried out according to [Cowan, 1974]. The utilization of different carbon and nitrogen sources were carried out according to [Shirling and Gottlieb, 1966]. Determination of Diaminopimelic acid (DAP) and sugar pattern was carried out according to [Becker, *et al.*, 1964 and Lechevalier and Lechevaier, 1968].

#### 2.4.3. Color characteristics

The ISCC-NBS color –Name Charts illustrated with centroid detection of the aerial, substrate mycelium and diffusible pigment [Kenneth and Deane, 1955] was

used.

### 2.5. Screening for antimicrobial activity

The anti- microbial activity was determined by cup method assay according to [Kavanagh, 1972].

### 2.6. Taxonomic studies of actinomycete isolate

Morphological characteristics of the most potent produce strain KSA-818 grown on starch nitrate agar medium at 30 °C for 5 days was examined under scanning electron microscopy (JEOL Technics Ltd.,).

### 2.7. DNA isolation and manipulation

The locally isolated actinomycete strain was grown for 5 days on a starch agar slant at 30°C. Two ml of a spore suspension were inoculated into the starch-nitrate broth and incubated for 5 days on a shaker incubator at 200 rpm and 30°C to form a pellet of vegetative cells (pre-sporulation). The preparation of total genomic DNA was conducted in accordance with the methods described by [Sambrook *et al.*, 1989].

### 2.8. Amplification and sequencing of the 16s rRNA gene

PCR amplification of the 16s rRNA gene of the local actinomycete strain was conducted using two primers, StrepF; 5.-ACGTGTGCAGCCCAAGACA-3. and Strep R; 5.ACAAGCCCTGGAACGGGGT-3., in accordance with the method described by [Edwards *et al.*, 1989]. The PCR mixture consisted of 30 pmol of each primer, 100 ng of chromosomal DNA, 200  $\mu$ M dNTPs, and 2.5 units of Taq polymerase, in 50  $\mu$ l of polymerase buffer. Amplification was conducted for 30 cycles of 1 min at 94°C, 1 min of annealing at 53°C, and 2 min of extension at 72°C. The PCR reaction mixture was then analyzed via agarose gel electrophoresis, and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The 16s rRNA gene was sequenced on both strands via the dideoxy chain termination method, as described by [Sanger *et al.*, 1977]. The 16s rRNA gene (1.5 kb) sequence of the PCR product was acquired using a Terminator Cycle Sequencing kit (ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA).

### 2.9. Sequence similarities and phylogenetic analysis

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

### 2.10. Fermentation

The *Streptomyces fimbriatus*, KSA-818 cultivated into a 500 ml flask containing 75 ml of liquid medium composed of Starch, 20.0; NaNO<sub>3</sub>, 2.0; K<sub>2</sub>HPO<sub>4</sub>, 1.0; MgSO<sub>4</sub> .7H<sub>2</sub>O, 0.5; KCl, 0.5; FeSO<sub>4</sub> 0.01; Yeast extract 2.0 agar, 20.0 distilled water up to 1000 ml. The pH was adjusted at 7.2 before sterilization. After incubation at 30 °C for 5 days, filtration was carried out through cotton wool and followed by centrifugation at 5000

r.p.m. for 20 minutes. The clear filtrates were tested against test organisms.

### 2.11. Extraction

The clear filtrate was adjusted at pH 7.0 and extraction process was carried out using different solvents to be added to fermentation broth at the level of 1:1 (v/v) respectively. The organic phase was concentrated to dryness under vacuum by using a rotary evaporator.

### 2.12. Precipitation

The precipitation process of the antifungal agent was carried out using petroleum ether. The compound precipitate was centrifuged at 5000 rpm for 15 min. The antibiotic powder was tested for its antifungal activity by using paper disk method.

### 2.13. Separation

Separation of the antifungal agent into its individual components has been tried by thin layer chromatography using a solvent system composed of chloroform and methanol (24: 1, v/v).

### 2.14. Purification

The purification of the antibiotic was carried out by using silica gel column chromatography. A column of 2.5 X 50 cm was used for this purpose. Chloroform and Methanol 8:2 (v/v), was used as an eluting solvent. The column was left for over night until the silica gel (BDH – 60- 120 mesh) was completely settled. One-ml crude extract to be fractionated was added on the silica column surface and the extract was adsorbed on top of silica gel. Fifty fractions were collected (each of 5 ml). Antifungal activities were performed for each separate fraction.

### 2.15. Physico-chemical properties of antifungal antibiotic

#### 2.15.1. Elemental analysis

The elemental analysis C, H, O, N and S was carried out by the Microanalytical Center of Cairo University, Egypt.

#### 2.15.2. Spectroscopic analysis

The IR, UV and Mass spectrum were determined at the Microanalytical Center of Cairo University, Egypt.

#### 2.15.3. Reaction of the antifungal agent with certain chemical test

For this purpose the following reactions were carried out: Molish's, Fehling, Sakaguchi, Ninhydrin, Ehrlich, Nitroprusside, Ferric chloride, and Mayer reactions

#### 2.16. Biological activity

The minimum inhibitory concentration (MIC) has been determined by cup method assay [Kavanagh, 1972].

#### 2.17. Characterization of the antifungal antibiotic

The antibiotic produced by *Streptomyces fimbriatus*, KSA-818 was tried to be identified according to the recommended international references of [Umezawa, 1977 and Berdy, 1974, 1980 a, b & c].

## 3. RESULTS

### 3.1. Screening for the antimicrobial activities

The actinomycete isolate, KSA-818 exhibited various degrees of activities against unicellular and filamentous fungi: *Saccharomyces cerevisiae*, ATCC 9763; *Candida albicans*, IMRU 3669; *Aspergillus niger*; *Aspergillus fumigatus*; *Aspergillus flavus*; *Aspergillus terreus*; *Fusarium solani*; *Fusarium oxysporum*, *Fusarium moniliforme*, *Alternaria alternata*, *Botrytis cinerea*, *Penicillium chrysogenum* and *Rhizoctonia solani* (Table 1).

### 3.2. Identification of the actinomycete isolate

#### 3.2.1. Morphological characteristics

The actinomycete isolate KSA-818, Spore chain are spiral, Spore surfaces are smooth and Spore mass is light gray, substrate mycelium is Light gray- yellowish brown (ISP 2, 4 & 5) and deep yellowish brown (ISP 6 & 7) while, diffusible pigment was produced on ISP-media Nos. 6 & 7 (plate 1). Neither both sclerotic granules and sporangia nor flagellated spores were observed.

#### 3.2.2. Cell wall hydrolysate:

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

#### 3.2.3. Physiological and biochemical characteristics

Tests for protein, starch, lipid, and pectin are positive whereas and Egg-yolk (lecithin) and Catalase test are negative. Melanin pigment, production of H<sub>2</sub>S and nitrate reduction are positive, and degradation of esculin, xanthine, utilization of citrate, and decomposition of urea are positive, whereas KCN test is negative.

The isolate KSA-818 utilizes, xylose, mannose, glucose, galactose, sucrose, mannitol, raffinose, *meso*-insoitol, arabinose, lactose, rhamnose, maltose, fructose, trehalose, ribose, L-phenylalanine, L-arginine, L-glutamic acid, L-cysteine L-valine and L-histidine. Good growth could be detected within a temperature range of 20 to 40 °C. Growth in the presence of NaCl up to 5% was recorded and growth at different pH values from 5 to 8 was also recorded and finally no growth in the presence of growth inhibitors Sodium azide, phenol and thallos acetate (Table 2).

#### 3.2.4. Color and culture characteristics

For the purpose of investigating the cultural characteristics of KSA-818, the ISCC-NBS color- name charts illustrated centroid color detection of aerial and substrate mycelia and diffusible pigment was used for this purpose [Kenneth and Deane, 1955].

As shown in Table (3), the KSA-818 was allowed to grow on the ISP-media [Shiriling and Gottlieb, 1966], the isolate KSA-818 exhibited no growth on tryptone- yeast extracts broth (ISP-1). Moderate growth on yeast extract-malt extract agar medium (ISP-2), the aerial mycelium is light gray, substrate mycelium is

light gray yellowish brown and the diffusible pigment is moderate yellowish brown. Good growth was detected on oat-meal agar medium (ISP-3), aerial mycelium is light gray, substrate mycelium is moderate yellowish brown and diffusible pigment is pinkish white. Good growth was detected on inorganic salts-starch agar medium (ISP-4), aerial mycelium is light gray, substrate mycelium is light gray-yellowish brown and diffusible pigment is yellowish gray. Poor growth was detected on glycerol-asparagine agar medium (ISP-5), aerial mycelium is light gray, substrate mycelium light gray-yellowish brown and no diffusible pigment are produced. Moderate growth was detected on peptone yeast extract-iron agar medium (ISP-6), aerial mycelium is light gray, substrate mycelium is deep yellowish brown, and diffusible pigment brown black. Moderate growth was detected on tyrosine agar medium (ISP-7), aerial mycelium is light gray, substrate mycelium is deep yellowish brown, and diffusible pigment is moderate deep brown. Good growth on Starch-nitrate agar medium, aerial mycelium is light gray, substrate mycelium is moderate yellowish brown and the diffusible pigment is dark grayish yellowish brown (Table 3).

### 3.2.5. Taxonomy of actinomycete isolate

This was performed basically according to the recommended international Key's viz. [Buchanan and Gibsons, 1974; Williams *et al.*, 1989 and Hensyl, 1994]. On the basis of the previously collected data and in view of the comparative study of the recorded properties of KSA-818 in relation to the most closest reference strain, viz. *Streptomyces fimbriatus*, Table (4), it could be concluded that both isolates are identical on the basis of Spore chain spiral, spore mass light gray, spore surface smooth (Plate 1) and spore non-motile. Cell wall hydrolysate contains LL-diaminopimelic acid, and sugar pattern not detected. Melanin pigment is produced. Utilization of xylose, sucrose, *meso*-inositol, L-arabinose, D-fructose, D-galactose, D-glucose, D-mannitol, raffinose and L-rhamnose. In view of all the recorded characteristics of KSA-818, it could be stated that KSA-818 is suggestive of being belonging to *Streptomyces fimbriatus*, KSA-818.

### 3.2.6. Amplification of the 16S rRNA gene

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the universal primers. The primers that was used to 16S rRNA sequencing were 16F357 of the sequence strepF; 5'-ACGTGTGCAGCCCAAGACA-3' and strpR; 5'-ACAAGCCCTGGAAACGGGGT-3', the product of the PCR was analyzed on 1.5% ethidium bromide gel.

### 3.2.7. Molecular phylogeny of the selected isolate

The 16S rRNA sequence of the local isolate was compared to the sequences of *Streptomyces* spp. In order to determine the relatedness of the local isolate to these *Streptomyces* strains. The phylogenetic tree (as

displayed by the Tree View program) revealed that the locally isolated strain is closely related to *Streptomyces* sp., rather related to *Streptomyces* sp., rather than to *Streptomyces fimbriatus* (Fig. 1). Multiple sequence alignment was conducted the similarly revealed that the 16S rRNA sequence was 90% identical *Streptomyces fimbriatus* (Fig. 1).

### 3.3. Fermentation, Extraction and Purification

The *Streptomyces fimbriatus*, KSA-818 was inoculated into a 500 ml flask containing 75 ml of liquid medium composed of Starch, 20.0; NaNO<sub>3</sub>, 2.0; K<sub>2</sub>HPO<sub>4</sub>, 1.0; MgSO<sub>4</sub> .7H<sub>2</sub>O, 0.5; KCl, 0.5; FeSO<sub>4</sub> 0.01; Yeast extract 2.0 distilled water up to 1000 ml. The pH was adjusted at 7.2 before sterilization. After incubation 30 °C for 5 days with 3 discs (protein content 0.036 mg. protein/ disc). The culture broth (18 liters) harvested at 5 days of fermentation. Filtration and centrifugation was performed at 5000 rpm for 20 min. Only clear filtrates (supernatant) were tested for their antimicrobial activity. The clear filtrate was adjusted at pH 7.0 then extraction process was carried out n-Butanol was added to fermentation broth at the level of 1:1 (v/v). The organic phase was collected, evaporated under reduced pressure using a rotary evaporator. The residual syrup was dissolved in least amount of DMSO and filtered. The filtrates were tested for their antimicrobial activity. Only one fraction was obtained with petroleum ether (b.p. 40-60°C) by centrifugation at 5000 rpm for 15 minute. Separation of the antifungal agent(s) into individual components has been carried out by thin layer chromatography (TLC). The obtained results revealed that there is one compound exhibited obvious inhibitory effects against the growth of the fungal organisms.

### 3.4. Physicochemical characteristics

The physical characteristics such as melting point are 173-175°C and soluble in chloroform, n-Butanol, carbon tetra chloride, ethanol, DMSO and methanol but insoluble in petroleum ether, hexane and benzene were investigated.

### 3.5. Elemental analysis

The elemental analytical data of the antifungal agent produced by *Streptomyces fimbriatus*, KSA-818 showed the following: C=60.0; H=6.9; N= 5.4; O= 27.7 and S= 0.0. This analysis indicates a suggested empirical formula of: C<sub>26</sub> H<sub>36</sub> O<sub>9</sub> N<sub>2</sub>.

### 3.6. Spectroscopic characteristics

The ultraviolet (UV) absorption spectrum of the antifungal agent recorded a maximum absorption peak at 230 and 320 nm (Fig. 2). The infra red (IR) spectrum of the antifungal agent showed characteristic band corresponding to 28 peaks (Fig.3). The Mass spectrum of antifungal agent showed that the molecular weight at 520.6 (Fig.4).

### 3.7. Biochemical reaction of antifungal agent

The reactions revealed the detection of certain groups in the molecule investigated. The antifungal

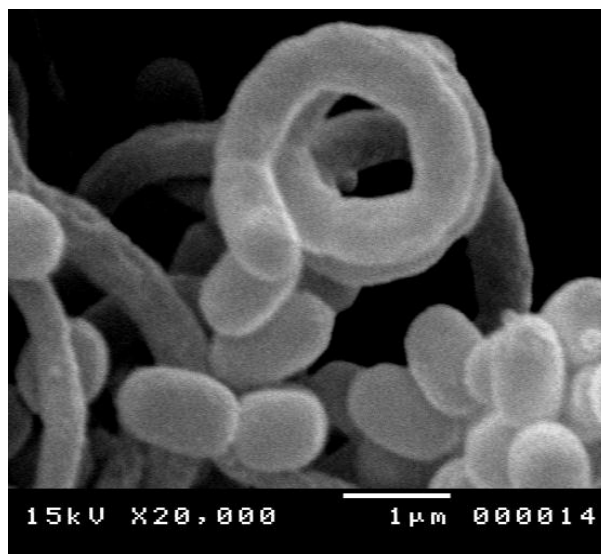
agent showed positive result with Mayer reaction, and Molish's test and negative result with nitroprusside reaction, Ninhydrin test Fehling test, Sakaguchi reaction, Ferric chloride and Ehrlich reactions (Table 5).

### 3.8. Biological activities

Data of the antimicrobial spectrum of antibiotic indicated that the antibiotic is fairly active against unicellular and filamentous fungi (Table 6).

### 3.9. Identification of the antifungal 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 antibiotics, it could be stated that the antifungal agent is suggestive of being belonging to dilactone (Blastmycin antibiotic) (Table 7).



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

**Table 1.** Mean diameters of inhibition zones (mm) caused by 100µl of the antimicrobial agent KSA-818 in the agar plate diffusion assay (The diameter of the used cup assay was 10 mm).

Test organism	Mean diameters of inhibition zone (mm)
<b>A-Bacteria</b>	
<b>1-Gram Positive:</b>	
<i>Micrococcus kristinae</i> , ATCC 27570	0.0
<i>Staphylococcus aureus</i> , NCTC 7447	0.0
<i>Staphylococcus haemolyticus</i> , NCTC 29968	0.0
<i>Bacillus subtilis</i> , NCTC 1040	0.0
<i>Bacillus pumilus</i> , NCTC 8214	0.0
<i>Sarcina maxima</i> , ATCC 33910	0.0
<b>2-Gram Negative</b>	
<i>Escherichia coli</i> , NCTC 10416	0.0
<i>Klebsiella pneumonia</i> , NCIMB 9111	0.0
<i>Pseudomonas aeruginosa</i> , ATCC 10145	0.0
<b>B- Fungi:</b>	
<b>1-Unicellular fungi</b>	
<i>Saccharomyces cerevisiae</i> , ATCC 9763	25.0
<i>Candida albicans</i> , IMRU 3669	25.0
<b>2-Filamentous fungi</b>	
<i>Aspergillus niger</i> , IMI 31276	24.0
<i>Aspergillus fumigatus</i> , ATCC 16424	21.0
<i>Aspergillus flavus</i> , IMI 111023	20.0
<i>Fusarium solani</i>	23.0
<i>Aspergillus terreus</i>	0.0
<i>Fusarium oxysporum</i>	22.0
<i>Botrytis cinerea</i>	21.0
<i>Botrytis fabae</i>	20.0
<i>Fusarium moniliforme</i>	21.0
<i>Alternaria alternata</i>	19.5
<i>Penicillium chrysogenum</i>	21.5
<i>Rhizoctonia solani</i>	22.0

**Table 2. The morphological, physiological and biochemical characteristics of the actinomycete isolate KSA-818**

Characteristic	Result
<b>Morphological characteristics:</b>	
Spore chains	spiral
Spore mass	light gray
Spore surface	Smooth
Color of substrate mycelium	moderate yellowish brown & Light gray- yellowish
Diffusible pigment	deep brown & brown black (ISP 6&7)
Motility	Non-motile
<b>Cell wall hydrolysate:</b>	
Diaminopimelic acid (DAP)	LL-DAP
Sugar Pattern	Not-detected
<b>Physiological and biochemical properties:</b>	
<b>Hydrolysis of:</b>	
Starch , Protein , Lipid & Pectin	+
Egg-yolk (lecithin)	-
Catalase test	-
<b>Production of melanin pigment on:</b>	
Peptone yeast- extract iron agar	+
Tyrosine agar medium	+
Tryptone – yeast extract broth	-
<b>Degradation of:</b>	
Xanthin	+
Esculin	+
H <sub>2</sub> S Production	+
Nitrate reduction	+
Citrate utilization	+
Urea test	+
KCN test	-
<b>Utilization of:</b>	
D-Xylose	+
D- Mannose	++
D- Glucose	++
D- Galactose	++
Sucrose	+
L- Rhamnose	+
Raffinose	++
Mannitol	+++
L- Arabinose	++
meso-Inositol	++
Lactose	+
Maltose	+
Trehalose	++
D- Ribose	+
D-Fructose	++
L-Cysteine	+
L-Valine	+
L-Histidine	++
L-Phenylalanine	+
L-Arginine	+
L-Glutamic acid	+
<b>Growth inhibitors:</b>	
Sodium azide ( 0.01); Phenol (0.1) and Thallous acetate (0.001)	-
<b>Growth at different temperature (°C):</b>	
10	-
20-40	+
45	±
50	-
<b>Growth at different pH values:</b>	
3-4	-
5-8	+
8.5-12	-
<b>Growth at different concentration of NaCl (%)</b>	
1-5	+
7	-

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

**Table 3. Culture characteristics of the actinomycete isolate KSA-818.**

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigments
1-Starch nitrate agar medium	Good	264-1. gray light gray	77-m.ybr moderate yellowish brown	81-d.gy-ybr dark grayish yellowish brown
2-Tryptone yeast extract broth (ISP-1)	No growth	-	-	-
3-Yeast extract malt extract agar medium (ISP-2)	moderate	264-1. gray light gray	79-I-gy-.yBr Light gray- yellowish brown	77-m.ybr moderate yellowish brown
4-Oatmeal agar medium (ISP-3)	Good	264-1. gray light gray	77-m.ybr moderate yellowish brown	9-pk.White Pinkish white
5-Inorganic salts starch agar medium (ISP-4)	Good	264-1. gray light gray	79-I-gy-.yBr Light gray- yellowish brown	23-y. gray yellowish gray
6-Glycerol – asparagine agar medium (ISP-5)	Poor	264-1. gray light gray	79-I-gy-.yBr Light gray- yellowish brown	-
7-Peptone yeast extract iron agar medium (ISP-6)	moderate	264-1. gray light gray	78-dybr deep yellowish brown	65 -br-Black Brown black
8-Tyrosine agar medium (ISP-7)	moderate	264-1. gray light gray	78-dybr deep yellowish brown	59-d.br deep brown

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

**Table 4. A comparative study of the characteristics of actinomycete isolate, KSA-818 in relation to reference strain *Streptomyces fimbriatus***

Characteristics	KSA-818	Williams(1989) <i>Streptomyces fimbriatus</i>
<b>Morphological characteristics:</b>		
Color of aerial mycelium	Light gray	gray
Color of substrate mycelium	moderate yellowish brown, Light gray- yellowish brown	ND
Spore mass	light gray	gray
Spore chain	spiral	spiral
Spore surface	Smooth	Smooth
<b>Cell wall hydrolysate:</b>		
- Diaminopimelic acid (DAP)	LL-DAP	LL-DAP
- Sugar pattern	Not-detected	Not- detected
Melanin pigment	+	+
<b>Hydrolysis of:</b>		
lipid	+	ND
Gelatin	+	ND
Pectin	+	ND
Starch	+	ND
Egg-Yolk	-	ND
Motility	Non-Motile	Non-Motile
<b>Utilization of:</b>		
Sucrose	+	ND
D- Mannitol	+++	+
meso-Inositol	++	+
L- Rhamnose	+	+
Raffinose	++	+
L-Arabinose	++	+
D-Xylose	+	+
D-Fructose	++	+
D-Galactose	++	+
D-Glucose	++	+

+ =Positive, ND =No data

**Table 5. Summarizes the response of the antifungal agent to certain biochemical reactions**

Chemical test (reaction)	Result	Remark
Molish's reaction	+	Presence of sugar moiety
Fehling test	-	Absence of free aldehyde or keto sugar
Ninhydrin test	-	Absence of free-NH <sub>2</sub> group
Sakaguchi reaction	-	Arginin is absent
Nitroprusside reaction	-	Absence of Sulfur
Ferric chloride reaction	+	Presence of Di-ketons group
Ehrlich reaction	-	Absence of indolic acid
Mayer reaction	+	Presence of nitro group

**Table 6. Antifungal spectrum of the Purified antibiotic by applying the cup method assay.**

Test organism	MIC (µg/ml) concentration
<b>1-Unicellular:</b>	
<i>Saccharomyces cerevisiae</i> ATCC 9763	15.6
<i>Candida albicans</i> IMRU 3669	15.6
<b>2-Filamentous</b>	
<i>Fusarium solani</i>	23.6
<i>Fusarium oxysporum</i>	23.6
<i>Rhizoctonia solani</i>	23.6
<i>Aspergillus niger</i> IMI 31276	15.6
<i>Aspergillus fumigatus</i> ATCC 16424	31.6
<i>Aspergillus flavus</i> IMI 111023	41.6
<i>Botrytis cinerea</i>	31.6
<i>Alternaria alternata</i>	41.6
<i>Penicillium chrysogenum</i>	31.6

**Table 7. A comparative study of the characteristic properties of antifungal agent in relation to Reference antibiotic (Blastmycin).**

Characteristic	Blastmycin	Purified antifungal agent
1- Melting point	174.5-175°C	173-175 °C
2- Molecular weight	520.56	520.6
3- Chemical analysis:		
C	59.99	60.0
H	6.97	6.9
N	5.38	5.4
O	27.66	27.7
S	ND	O.O
4- Ultra violet	228 & 328	230 & 320
5- Formula	C <sub>26</sub> H <sub>36</sub> O <sub>9</sub> N <sub>2</sub>	C <sub>26</sub> H <sub>36</sub> O <sub>9</sub> N <sub>2</sub>

ND=No Data



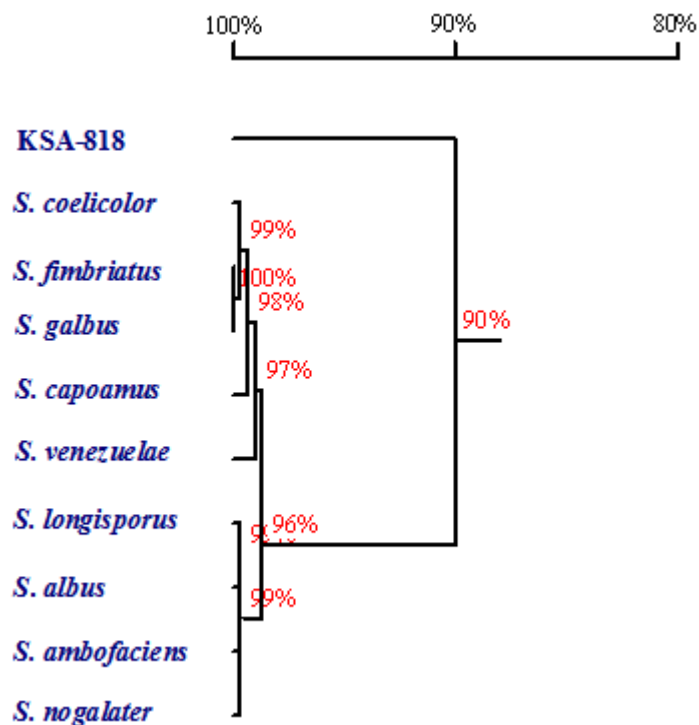


Fig. 1. The phylogenetic position of the local *Streptomyces* sp. strain among neighboring species. The phylogenetic tree was based on the pairwise comparisons of 16s rRNA sequences.

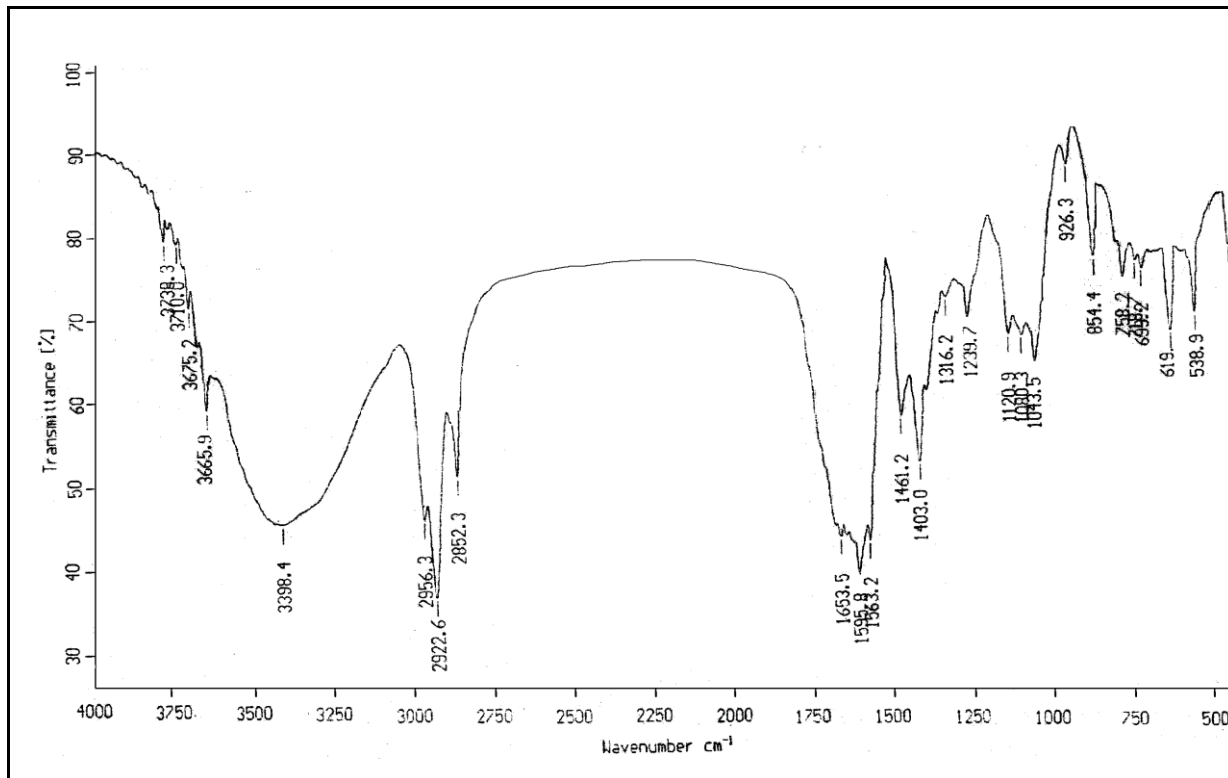


Fig. 2. IR spectrum of antifungal agent produced by *Streptomyces fimbriatus*, KSA-818.

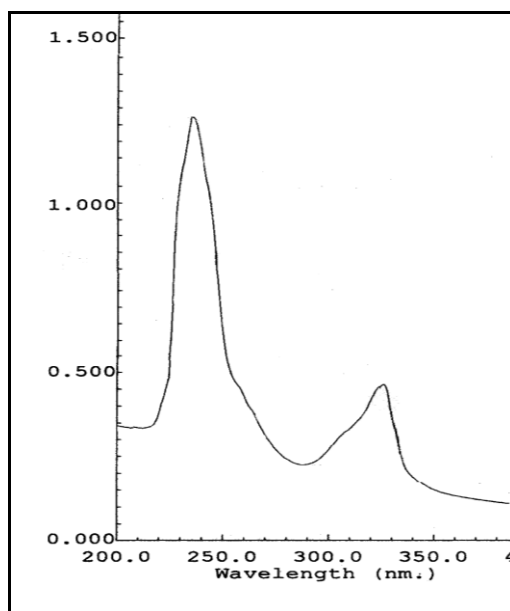


Fig. 3. Ultraviolet absorbance of antifungal agent produced by *Streptomyces fimbriatus*, KSA-818.

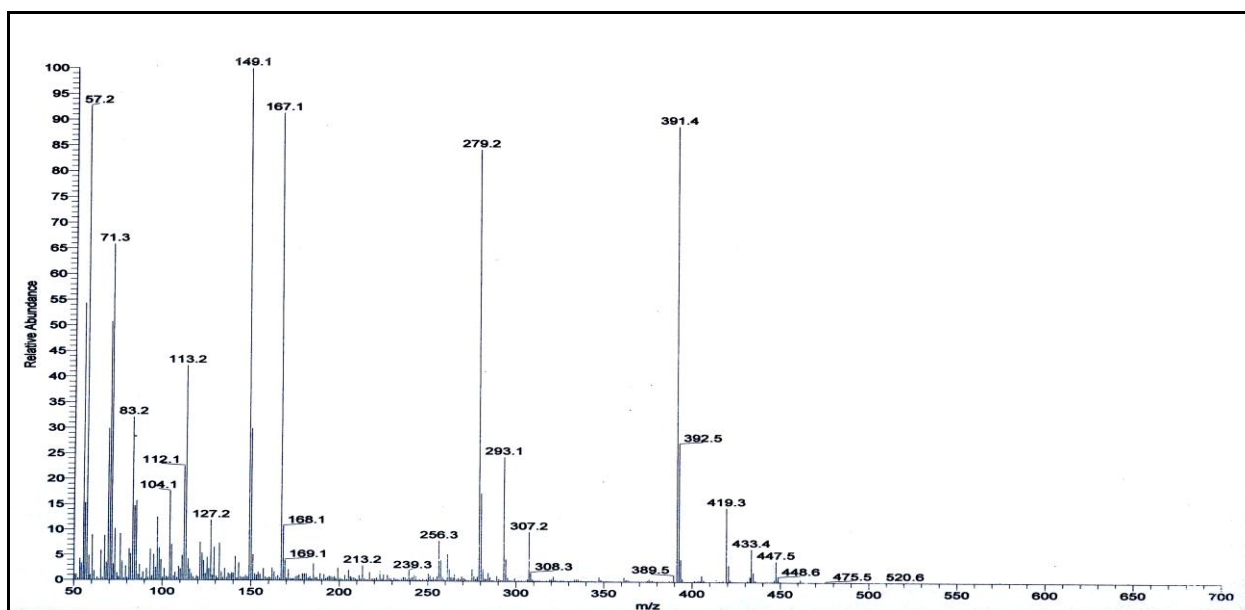


Fig. 4. Mass spectrum of antifungal agent produced by *Streptomyces fimbriatus*, KSA-818.

#### 4. DISCUSSION

The *Streptomyces fimbriatus* was isolated from Al-Khurmah governorate, KSA. The isolate KSA-818 was growing on starch nitrate agar medium for investigating its potency to produce antimicrobial agents. The actinomycete isolate, exhibited a wide spectrum antifungal agent [Kavanagh, 1972]. Identification process has been carried out according to [Williams *et al.*, 1989, Hensyl, 1994 and Numerical taxonomy program, 1989]. For the purpose of

identification of actinomycete isolate, the morphological characteristics and microscopic examination emphasized that the spore chain is spiral. Spore surfaces are smooth and Spore mass is light gray, substrate mycelium is light gray- yellowish brown (ISP 2, 4 & 5) and deep yellowish brown (ISP 6 & 7) and diffusible pigment was produced on ISP-media Nos. 6 & 7. The results of physiological, biochemical characteristics 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. Moreover, spore non-motile, Melanin pigment is produced and utilization of xylose, sucrose, meso-inositol, L- arabinose, D- fructose, D- galactose, D- glucose, D- mannitol, raffinose and L-rhamnose. 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 was suggestive of being belonging to *Streptomyces fimbriatus*. The phylogenetic tree (diagram) revealed that the local isolate is closely related *Streptomyces fimbriatus*, similarity matrix is 90%.

The active metabolites were extracted by n-Butanol at pH 7.0 [Atta *et al.*, 2010].

The organic phase was collected and evaporated under reduced pressure using a rotary evaporator. The extract was concentrated and treated with petroleum ether (b.p. 40-60°C) for precipitation process where only one fraction was obtained in the form of yellowish ppt. and then tested for their antifungal activity. Separation of antifungal agent into individual components has been tried by thin-layer chromatography using a solvent system composed of chloroform and methanol (24:1, v/v) as developing solvent [Zhang *et al.*, 2007 and Atta *et al.*, 2009]. For the purpose of purification process, the antifungal agent were allowed to pass through a column chromatography packed with silica gel and eluting solvent was composed of chloroform and methanol (8:2 v/v), fifty fractions were collected and tested for their activities. The maximum activity was recorded at fraction No. 21. Similarly, many workers used a column chromatography packed with silica gel and eluting solvents composed of various ratios of chloroform and methanol [Criswell *et al.* 2006 and Sekiguchi, *et al.*, 2007]. The physico-chemical characteristics of the purified antibiotic revealed that, melting point is 173-175°C, soluble in chloroform, n-butanol, methanol, ethanol, DMSO, carbon tetra chloride, but insoluble in petroleum ether, hexane and benzene; similar results were recorded by [Lotfi *et al.*, 2003; El-Tayeb *et al.*, 2004c and Atta *et al.*, 2010].

A study of the elemental analysis of the antibiotic showed the following C=60.0; H=6.9; N= 5.4; O= 27.7 and S= 0.0. lead to an empirical formula of: C<sub>26</sub>H<sub>36</sub>N<sub>2</sub>O<sub>9</sub>. The spectroscopic characteristics of antibiotic revealed the presence of the maximum absorption peak in UV. at 230 and 320 nm, infra-red absorption spectrum represented by 28 peaks and Mass-spectrum showed that the molecular weight is 520.6. The biochemical tests of antibiotic gave positive reaction with Ferric chloride, Mayer and Molish's reactions [Yutaka *et al.*, 2001]. The MIC of antibiotic under study exhibited fairly active against unicellular

and filamentous fungi: *Saccharomyces cerevisiae*, ATCC 9763; *Candida albicans*, IMRU 3669; *Aspergillus niger*; *Aspergillus fumigatus*; *Aspergillus flavus*; *Aspergillus terreus*; *Fusarium solani*; *Fusarium oxysporum*, *Fusarium moniliform*, *Alternaria alternata*, *Botrytis cinerea*, *Penicillium chrysogenum* and *Rhizoctonia solani*. Similar investigations and results were attained by [Imnagaki *et al.*, 2006; Sekiguchi, *et al.*, 2007 and Atta, 2010]. Identification of antifungal agent according to recommended international keys indicated that the antibiotic is suggestive of being belonging to Dilactones (Blastmycin) antibiotic [Umezawa, 1967 and 1977 and Berdy, 1979 and 1980a, b & c].

## 5. CONCLUSION

The present study mainly involved in the isolation of Actinomycetes based on the cultural, morphology, physiology and biochemical characteristics, as well as 16s rRNA methodology. Further work should be focused in most potent *Streptomyces* isolate for production the antifungal activities against unicellular and filamentous Fungi. The bioactive substance was suggestive of being belonging to Blastmycin antibiotic.

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## 7. REFERENCES

1. **Ammar, M.S.; El-Louboudy, S.S and Abdul-Raouf, U.M. 1991.** Purification and Properties of mesophilic protease(s) produced by *Bacillus anthracis*, S-44. Isolated from a temple in Aswan. Al- Azhar Bull. Sci., Vol. 2(1): 325-338.
2. **Ammar, M.S.; El-Gamal, M.S.; El-Louboudy, S.S. and Ibrahim, A.M. 1995b.** Fermentation of mesophilic proteases by *B.subtilis*, S-19 in relation to certain nutritional factors under solid state fermentation in relation to vitamins, amino acids, heavy metals and certain plant growth regulators.<sup>1</sup> Intern. Confr. (Sci. and developm.) Cairo 20-23, March (1995).
3. **Ammar, M.S.; EL- Esawey, M.; Yassin, M. and Sherif, Y.M. 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., Vol. 3. Jan. 1998: PP. 60 - 90.
4. **Atta, H. M. 2010.** Production, Purification,

- Physico-Chemical Characteristics and Biological Activities of Antifungal Antibiotic Produced by *Streptomyces antibioticus*, AZ-Z710. American-Eurasian Journal of Scientific Research. 5 (1): 39-49, 2010.
5. **Atta, H. M.; A. T. Abul-hamd and H. G. Radwan, 2009.** Production of Destomycin-A antibiotic by *Streptomyces* sp. using rice straw as fermented substrate. Comm. Appl. Biol. Sci, Ghent University, 74 (3) : 879-897, 2009.
  6. **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. APPL. Microbiol., 12: 421 – 423.
  7. **Berdy, J. 2005.** Bioactive microbial metabolites. J Antibiot (Tokyo) 58: 1-26.
  8. **Berdy, J. 1974.** Recent development of antibiotic research and classification of antibiotic according to chemical structure. Adv. App. Microbiol., 14: 309-406.
  9. **Berdy, J. 1980a.** Recent advances in and prospects of antibiotics research. Proc. Biochem., 15: 28-35.
  10. **Berdy, J. 1980b.** CRC Handbook of antibiotic compounds. Vol I. CRC Press, Boca Raton, Florida.
  11. **Berdy, J. 1980c.** CRC Handbook of antibiotic compounds. Vol II. CRC Press, Boca Raton, Florida.
  12. **Buchanan, R. E. and N. E. Gibbson, 1974.** Bergey's Manual of Determinative bacteriology 8th edition. The Williams & Wilkins company/ Baltimore.
  13. **Chapman, G.S. 1952.** A simple method for making multiple tests on a microorganism. J. Bacteriol. 63:147.
  14. **Cowan, S .T. 1974.** Cowan and Steel' s Manual For The Identification Of Medical Bacteria 2<sup>nd</sup>. Edition Cambridge, Univ. Press.
  15. **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.
  16. **Deepa, S.; Kanimozhi, K.and Panneerselvam, A. 2013.** 16S rDNA Phylogenetic Analysis of Actinomycetes Isolated from Marine Environment Associated with Antimicrobial Activities. Hygeia.J.D.Med.5 (2) October 2013, 43-50.
  17. **Edwardss, U.; T. Rogall; H. Bocker; M. Emade and E. Bottger, 1989.** Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16s ribosomal DNA. *Nucleic Acid Res.* 17: 7843-7853.
  18. **El-Tayeb, O.M.; A. Salama, M. Hussein and H.F. El-Sedawy, 2004c.** Optimization of industrial production of rifamycin B by *Amycolatopsis mediterranei* I. The role of colony morphology and nitrogen sources in productivity. Afr. J. Biotechnol. 3: 266-272.
  19. **Elwan, S .H.; M. R. El-Nagar and M. S. Ammar, 1977.** Characteristics of Lipase(s) in the growth filtrate dialystate of *Bacillus stearotherophilus* grown at 55 °C using a tributryin- cup plate assay. Bull. Of the Fac. of Sci ., Riyadh Univ ., vol .8 : 105 – 119.
  20. **Gordon, R.E. 1966.** Some Criteria for The Recognition of *Nocardia madura* (Vincent) Blanchord. J. General Microbiology, 45:355-364.
  21. **Gordon, R.E.; D.A. Barnett; J.E. Handehan and C.H. Pang, 1974.** *Nocardia coeliaca* , *Nocardia autotrophica* and *Nocardia* Strain. International Journal of Systematic Bacteriology. 24:54-63.
  22. **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.
  23. **Hankin, L.; M. Zucker and D.C. Sands, 1971.** Improved solid medium for the detection and enumeration of proteolytic bacteria. Appl. Microbiol., 22:205-509.
  24. **Hensyl, W. R. 1994.** Bergey's Manual of Systematic Bacteriology 9<sup>th</sup> Edition. John. G. Holt and Stanley, T. Williams (Eds.) Williams and Wilkins, Baltimore, Philadeiphia, Hong kong, London, Munich.
  25. **Imnagaki, T.; K. Kaneda; Y. Suzuki; H. Hirai; E. Nomura; T. Sakakibara; Y. Yamauchi; L.H. huang; M. Norcia; L.M. Wondrack and N. Kojima, 2006.** CJ-12, 373, a novel topoisomerase II inhibitor: Fermentation, isolation, structure, elucidation and biological activities. J. of Antibiotics, 51 :( 2): 112-116.
  26. **Jacobi, P.A.; Coutts, L.D.; Guo, J.; Hauck, S.I.; Leung, S. 2000.** New Strategies for the Synthesis of Biologically Important Tetrapyrroles. The 'BC + D + A' Approach to Linear Tetrapyrroles," *J. Org. Chem.* 65, 205
  27. **Jones, K. 1949.** Fresh isolates of actinomycetes in which the presence of sporogenous aerial mycelia is a fluctuating characteristics. J. Bacteriol., 57: 141-145.
  28. **Kavanagh, F. 1972.** Analytical Microbiology. Vol. 2, Acad. Press, New York.
  29. **Kenneth, L.K. and B.J. Deane, 1955.** Color universal language and dictionary of names. United States Department of Commerce. National Bureau of standards. Washington, D.C., 20234.
  30. **Lechevalier, M.P and H.A. Lechevalier, 1968.** Chemical composition as a criterion in the classification of aerobic actinomycetes. J. Systematic Bacteriology . 20 : 435-443.
  31. **Lotfi, M. O.; B. Raoudha; A. Ameer-Mehdi; S. Samiha; S. B. Mansour and B. Samir, 2003.**

- Isolation, purification and partial characterization of antibacterial activities produced by a newly isolated *Streptomyces* sp. US24 strain. *Research in Microbiology* 154, 345–352.
32. **Nitsh, B. and H.J. Kutzner, 1969.** Egg-Yolk agar as diagnostic medium for *Streptomyces* sp., 25:113.
  33. **Numerical taxonomy program 1989.** Numerical taxonomy of *Streptomyces* species program (PIB WIN) (*Streptomyces* species J. Gen Microbiol. 1989 13512-133.
  34. **Ogunmwoyi, I.H.; Mazomba, N.; Mabinya, L.; Ngwenya, E.; Green, E. and Akinpelu, D.A. 2010.** Studies on the cultural marine actinomycetes isolated from the Nahoon beach in the Eastern Cape Province of South Africa. *Afr. J Microbiol Res* 2010; 4(21): 2223-2230.
  35. **Pridham, T. G. 1965.** Colour and streptomycetes. Report of an international workshop on determination of colour of streptomycetes. *Appl. Microbiol.* 1965. 13: 43–6.
  36. **Pridham, T.G.; P. Anderson; C. Foley; L.A. Lindenfelser; C.W. Hesselting and R.G. Benedict, 1957.** A section of media for maintenance and taxonomic study of *Streptomycetes*. *Antibiotics Ann.* pp. 947-953.
  37. **Ravikumar, S.; Inbaneson, S.J.; Uthiraselvam, M.; Priya, S.R.; Ramu, A. and Banerjee, M.B. 2011.** Diversity of endophytic actinomycetes from Karangkadu mangrove ecosystem and its antibacterial potential against bacterial pathogens. *J Pharm Res* 2011;4(1): 294-296.
  38. **Sambrook, J.; E. F. Fritsch and T. Maniatis, 1989.** *Molecular cloning. A laboratory Manual* Cold Spring Harbor Laboratory press, Cold Spring Harbor, New York, USA.
  39. **Sanger, F.; S. Nicklen, and A.R. Coulson, 1977.** DNA sequencing with chain terminator inhibitors. *Proc. Natl. Acad. Sci.* 74: 5463-5467.
  40. **Sekiguchi, M.; N. Shiraish; K. Kobinata; T. Kudo; I. Yamaguchi; H. Osada and K. Isono, 2007.** RS-22A and C: new macrolide antibiotics from *Streptomyces violaceusniger*; Taxonomy, fermentation, isolation and biological activities. *Journal of Antibiotics* 48(4): 289-292.
  41. **Shirling, E. B. and D. Gottlieb, 1966.** Methods for characterization of *Streptomyces* species. *Int. J. Syst Bacteriol.* 16: 313-340.
  42. **Umezawa, H. 1977.** Recent advances in bio-active microbial secondary metabolites. *Jap. J. Antibiotic. Suppl.*, 30: 138-163.
  43. **Williams, S. T.; Sharpe, M. E. and Holt, J. G. 1989.** *Bergey's Manual of Determinative Bacteriology*, vol. 4. Williams and Wilkins co., Baltimore, 1989.
  44. **Williams, S.T. and F. L. Davies, 1965.** Use of antibiotics for selective isolation and enumeration of actinomycetes in soil. *J. Gen. Microbiol.*, 38:251-262.
  45. **Yutaka, N., Eisaku, T., Yukiko, Y., Kazutoshi, S., Yasuhisa, T., Shigetada, F., Reiko, O., Toru, K., Motohiro, H., and Seiji, H. 2001.** Fr198248, a new anti-Influenza agent isolated from *Aspergillus terreus* No. 13830.I- Taxonomy, Fermentation, Isolation, Physico-Chemical properties and Biological activates. *J. of antibiotics*, 54(2): 136-143.
  46. **Zhang, L.; K. Yan; Y. Zhang; R. Huang; J. Bian; C. Zheng; H. Sun; Z. Chen; N. Sun; R. An; F. Min; W. Zhao; Y. Zhuo; J. You; Y. Song; Z. Yu; Z. Liu; K. Yang; H. Gao; H. Dai; X. Zhang; J. Wang; C. Fu; G. Pei; J. Liu; S. Zhang; M. Goodfellow; Y. Jiang; J. Kuai; G. Zhou; and X. Chen, 2007.** High-throughput synergy screening identifies microbial metabolites as combination agents for the treatment of microbial infections. *Proc. Natl. Acad. Sci. USA* 104: 4606-4611.

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