International Journal of Life Sciences Vol. 4. No. 4. 2015. Pp. 260-269

Full Length Research Paper

Antimicrobial Activities of a *Streptomyces microflavus* Isolated From KSA: Taxonomy, Fermentation, Extraction and Biological activities

Houssam M. Atta*[1,2] and Ayman M. Yassen[2]

[1.] *Botany and Microbiology Department, Faculty of Science (Boys), Al-Azhar University, Cairo, Egypt.* ***Present address:*** *[3] Biology Department. Faculty of Education and Science, Taif University, Al-Khurmah branch - KSA.*

[2] *Biology Department, Faculty of Education and Science, Taif University, Al-Khurmah branch - KSA.*

**Corresponding author: Houssam M. Atta*

Abstract

This work was carried out for the biosynthesis of antimicrobial substance that demonstrated inhibitory effects against pathogenic microorganisms from Streptomyces sp. It is active in vitro against some microbial pathogenic viz: Staphylococcus aureus, NCTC 7447; Bacillus subtilis, NCTC 1040; Bacillus pumilus, NCTC 8214; Micrococcus luteus, ATCC 9341; Aspergillus flavus, IMI 111023; Aspergillus fumigatous, ATCC 16424and Penicillium chrysogenium. The KSA-Kh224 isolate has been considered the most potent, this was identified by biochemical, chemotaxonomic, morphological and physiological properties consistent with classification in the genus Streptomyces, with the nearest species being Streptomyces microflavus. Furthermore, a phylogenetic analysis of the 16S rDNA gene sequence and ribosomal database project consistent with conventional taxonomy confirmed that strain KSA-Kh224 was most similar to Streptomyces microflavus (98%). The active metabolite was extracted using Ethyl acetate (1:1, v/v) at pH 7.0. The separation of the active ingredient of the antimicrobialagent and its purification was performed using both thin layer chromatography (TLC) and column chromatography (CC) techniques. The chemical characteristics of the antimicrobial agent(s) viz. elemental analysis and spectroscopic characteristics have been investigated. This analysis indicates a suggested empirical formula of $C_{41}H_{65}NO_{11}$, ultraviolet (UV) absorption spectrum recorded a maximum absorption peaks at 212 and 245 nm. The Infra-red (IR) spectrum showed exhibits characteristic bands at 3398.4 (OH), 2956.3 ~2922.6 (CH, CH_2), 1652.73 (C=O) and 1594.84 (C=C) cm^{-1}. The Mass spectrum showed that the molecular weight at 747.37. The minimum inhibition concentrations "MICs" of the antibiotic were also determined. The collected data emphasized that the antibiotic was characterized as Irumamycin antibiotic.

Key words: *Streptomyces microflavus, Conventional taxonomy, Phylogenetic analysis, Fermentation, Biological activities*

Introduction

Actinomycetales are gram-positive, mycelium-forming, soil bacteria that play an important role in mineralization processes in nature Manteca *et al.* (2005). They are characterized by their tough powdery, pigmented colonies. They are free living, saprophytic bacteria it considered as an intermediate group between bacteria and fungi Jarallah (2014). Hotam *et al.* (2013), reported that, the actinomycetes have proved their ability to produce a variety of bioactive secondary metabolites and of other therapeutically useful compounds. The bioactive secondary metabolites produced by actinomycetes include antibiotics, antitumor agents, immune- suppressive agents and enzymes Kekuda *et al.* (2010). These metabolites are known to possess antibacterial, antifungal, antioxidant, neuritogenic, anti-cancer, anti- algal, anti-helmintic, anti-malarial and anti- inflammatory Ravikumar *et al.* (2011). Among actinomycetes, the genus *Streptomyces* has long been recognized as a rich source of useful secondary metabolites and continues to be a major source of new bioactive molecules Berdy (2005). They are the origin of a good number of marketed antibiotics Pelaez (2006). Metabolites with antibiotic activity were isolated mainly from *Streptomyces* species, representing some 70% to 80% of the all isolated compounds. This genus is the largest, comprised 80% to 90% of the total of actinobacteria population, they are found predominantly in soil and they are noticeable for the odor of tilled soil which is attributed to geosmins, a group of their secondary metabolites Raja and Prabakarana (2011). The resistance of a large number of pathogenic bacteria and fungi to bioactive secondary metabolites in common use is presently an urgent focus of research, and new antifungal and antibacterial molecules are necessary to combat these pathogens Lilia *et al.* (2004). Irumamycin a 20-membered ring macrolide antibiotic active against phytopathogenic fungi and Gram positive bacteria produced by *Streptomyces* sp. AM-3603, which was isolated from a soil sample collected in Iruma-shi, Saitama, Japan Omura *et al.* (1982a). Irumamycin is a neutral lipophylic compound with a melting point of 95~97°C; It does not have a characteristic UV absorption. The IR spectrum exhibits characteristic bands at 3400 (OH), 2956.3~2922.6 (CH, CH_2), 1700 (C=O) and 1590 (C=C) cm^{-1}. The molecular formula $C_{41}H_{63\sim65}NO_{11\sim12}$ (mol. wt. 745~763) was deduced for irumamycin on the basis of the elementary analysis values and Mass spectroscopic data. It is soluble in such organic solvents as methanol, ethanol, acetone, ethyl acetate, diethyl ether, pyridine, chloroform, carbon tetrachloride, benzene, toluene and dimethyl sulfoxide, but it is insoluble in n-hexane, petroleum ether or water Omura *et al.* (1982b&c). In the present study were describe the isolation of an actinomycete strain KSA-Kh224 from Al-Khurmah governorate, KSA, which generates a production the bioactive substances that demonstrated inhibitory affects against microbial pathogenic. The identification of this strain based on the cultural, morphology, physiology and biochemical characteristics,

as well as 16s rDNA methodology. The primary bioactive substances were tested against Gram positive and Gram negative bacteria and unicellular & filamentous fungi. One major active compound was extracted from the purified fermented broth and chemically characterized as Irumamycin antibiotic, based on the elemental analysis and spectroscopic data obtained from the application of UV, FT-IR and Mass Spectrum and by comparison with published data.

Materials and Methods

Actinomycete isolate

The actinomycete isolate KSA-Kh224 was isolated from soil sample collected from Al-Khurmah governorate, Saudi Arabia kingdom. It was purified using the soil dilution plate technique described by Williams and Davis (1965).

Test organisms

The test strains *Staphylococcus aureus*, NCTC 7447; *Bacillus subtilis*, NCTC 1040 ; *Bacillus pumilus*, NCTC 8214 ; *Micrococcus luteus*, ATCC 9341; *Escherichia coli*, NCTC 10416; *Klebsiella pneumonia*, NCIMB 9111; *Pseudomonas aeruginosa*, ATCC 10145; *Candida albicans*, IMRU 3669; *Saccharomyces cervisiae* ATCC 9763; *Aspergillus flavus*, IMI 111023, *Aspergillus fumigatous*, ATCC 16424; *Aspergillus niger* IMI 31276; *Fusarium oxysporum* and *Penicillium chrysogenium* was collection, National Research Centre, Dokki-Giza, Egypt.

Culture media

The inoculum medium used in the cultivations, had the following composition (in g/L distilled water): soluble starch, 20; KNO_3, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 and CaCO3, 0.1. pH 7.2 for investigating its potency to produce antimicrobial agents.

Screening for antimicrobial activity

The anti- microbial activity was determined according to Kavanagh (1972) and Zamanian *et al*. (2005).

Conventional Taxonomy

The cultural, morphological, physiological and biochemical characteristics of strain KSA-Kh224 were assessed following the guidelines adopted by the International *Streptomyces* Project (ISP) Shrilling and Gottlieb (1966). The diaminopimelic acid (LL-DAP) isomers (chemotaxonomy character) in the cell wall were analyzed as described by Lechevalier and Lechevalier (1980). The media composition and the cultivation conditions were implemented as described by Shrilling and Gottlieb (1966). Colors characteristics were assessed on the scale developed by Kenneth and Deane (1955).

DNA Isolation and Manipulation

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

Amplification and Sequencing of the 16S rDNA Gene

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

Sequence Similarities and Phylogenetic Analysis

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

Fermentation

The actinomycete isolate KSA-Kh224 inoculum was introduced aseptically into each sterile flask containing the following ingredients (g/l): soluble starch, 20; KNO_3, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 and CaCO3, 0.1. The pH was adjusted at 7.2 before sterilization. The flasks were incubated on a rotary shaker (200 rpm) at 30 ^{0}C for 5 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 the test organisms as described by Neto *et al.* (2005).

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. (Atta *et al.* (2013).

Precipitation

The precipitation process of the crude compound dissolved in the least amount of the solvent 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. (Atta (2010).

Separation

Separation of the antimicrobial agent(s) into its individual components was conducted by thin layer chromatography using n-Butanol: acetic acid: water (3:1:1 v/v), Benzene: acetone (1:1 v/v), chloroform: methanol (9:1 v/v), Ethanol: water (2:1 v/v), Ethyl acetate: pyridine: water (1:1:4 v/v), Benzene: ethyl acetate (1:1 v/v). as a solvent systems (Atta *et al.* (2009).

Purification

The purification of the antimicrobial agent(s) was carried out using silica gel column (2 X 25) chromatography. Chloroform and Methanol (10:2 v/v), was used as an eluting solvent. The column was left for overnight until the silica gel (Prolabo) was completely settled. One-ml crude precipitate to be fractionated was added on the silica gel column surface and the extract was adsorbed on top of silica gel. Fifty fractions were collected (each of 5 ml) and tested for their antimicrobial activities (Learn-Han *et al.* (2012) and Atta *et al.* (2014).

Elemental and Spectroscopic analysis

The elemental analysis C, H, O, and N and Spectroscopic analysis IR, UV and Mass spectrum were determined at the micro-analytical center of Cairo University, Egypt.

Determination of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) could be determined by the cup assay method. (Kavanagh (1972).

Characterization of the antibiotic

The antibiotic produced by *Streptomyces* sp. was identified according to the recommended international references of Umezawa (1977); Berdy (1974); Berdy (1980a b & c) and Omura *et al.* (1982a, b&c).

Results

Screening for the antimicrobial activities

The metabolites of the *Streptomyces* sp. exhibited various degrees of activities against Gram positive bacteria and filamentous fungi viz: *Staphylococcus aureus*, NCTC 7447; *Bacillus subtilis*, NCTC 1040; *Bacillus pumilus*, NCTC 8214; *Micrococcus luteus*, ATCC 9341; *Aspergillus flavus*, IMI 111023, *Aspergillus fumigatous*, ATCC 16424 and *Penicillium chrysogenium* (Table 1).

Identification of the Most Potent Actinomycete Isolate

Morphological Characteristics

The vegetative mycelia grew abundantly on both synthetic and complex media. The aerial mycelia grew abundantly on Starch- nitrate agar medium Oat-meal agar medium (ISP-3) and Inorganic salts-starch agar medium (ISP-4). The Spore chains were spirals, and had a smooth surface (Plate 1). Neither both sclerotic granules and sporangia nor flagellated spores were observed (Table 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 KSA-Kh224 could hydrolyzes starch, protein, pectin and lecithin, whereas both lipid hydrolysis and catalase test are negative, melanin pigment is positive, degradation of esculin & xanthin was positive, H_2S production, citrate utilization, urea and KCN utilization were positive, whereas nitrate reduction is negative (Table 2). The isolate KSA-Kh224 utilizes D-mannose, D-mannitol, D-glucose, D-fructose, D-galactose, L-rhamnose, sucrose, raffinose, starch, phenylalanine, arginine, tyrosine and cycteine, but do not utilize D-xylose, maltose, lactose, L-arabinose, *meso*-inositol and histidine. Growth was detected in presence of up to (5%) NaCl. The isolate KSA-Kh224 utilizes sodium azid (0.01%), phenol (0.01%); but do not utilize in thallous acetate (0.001). Good growth could be detected within a temperature range of 30 to 50 ºC. Good growth could be detected within a pH value range of 5 to 9. Moreover, the actinomycete isolate KSA-Kh224 are active against *Staphylococcus aureus*, NCTC 7447; *Bacillus subtilis*, NCTC 1040; *Bacillus pumilus*, NCTC 8214; *Micrococcus luteus*, ATCC 9341*; Aspergillus flavus*, IMI 111023, *Aspergillus fumigatous*, ATCC 16424 and *Penicillium chrysogenium* (Table 2).

Color and Culture Characteristics

The actinomycete isolate KSA-Kh224 shows the aerial mycelium is gray; substrate mycelium is light yellowish brown, and the diffusible pigment moderate brown to deep brown for ISP-3, 4 , 6 & 7 (Table 3).

Taxonomy of Actinomycete Isolate

This was performed basically according to the recommended international Key's viz. Buchanan and Gibsons (1974); Williams (1989); and Hensyl (1994) and Numerical taxonomy of *Streptomyces* species program (PIB WIN). On the basis of the previously collected data and in view of the comparative study of the recorded properties of actinomycete isolate in relation to the closest reference strain, viz. *Streptomyces microflavus* it could be stated that the actinomycetes isolate KSA-Kh224 is suggestive of being likely belonging to *Streptomyces microflavus*, KSA-Kh224 (Table 4).

Molecular phylogeny of the selected isolate

The 16_S rDNA sequence of the local isolate was compared to the sequences of *Streptomyces* spp. In order to determine the relatedness of the local isolate to these *Streptomyces* strains. The phylogenetic tree (as displayed by the Tree View program) revealed that the locally isolated strain is closely related to *Streptomyces* sp., the most potent strain evidenced an 98% similarity with *Streptomyces microflavus* (Fig. 1).

Fermentation, Extraction and Purification

The fermentation process was carried out for five days at 30°C. After incubation period, the filtration was conducted followed by centrifugation at 4000 r.p.m. for 15 minutes. The entire culture broth (20 liters) was centrifuged (4000 rpm, 15 minutes) to separate the mycelium and the supernatant. The supernatant was extracted with ethyl acetate (1:1, v/v) and the organic layer was evaporated to give an oily brownish material. The oily material was then dissolved in 15% aqueous methanol and defatted by partitioning with petroleum ether (b.p. 60-80°C) to give a solid extract. Separation of antimicrobial agent into individual components was carried out by thin-layer chromatography using a solvent system composed of n-Butanol: acetic acid: water (3:1:1 v/v), Benzene: acetone (1:1 v/v), chloroform: methanol (9:1 v/v), Ethanol: water (2:1 v/v), Ethyl acetate: pyridine: water (1:1:4 v/v), Benzene: ethyl acetate (1:1 v/v). Only one band of antimicrobial agent at R_f = 0.6, 0.5, 0.6, 0.7, 0.4 and 0.2 respectively. The purification process through column chromatography packed with silica gel, and eluting solvent was composed of chloroform and methanol (10:2 v/v), revealed that the most active fractions against the tested organisms ranged between, 18 to 25.

Physicochemical characteristics

The purified antimicrobial agent produced by *Streptomyces microflavus,* KSA-Kh224 are produces characteristic odour, their melting points are 95 °C. The compound is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10 % isopropyl alcohol, diethyl ether, carbon tetrachloride but insoluble in water, petroleum ether, n-hexane and benzene.

Elemental analysis

The elemental analytical data of antimicrobial agent produced by *Streptomyces microflavus,* showed the following: The elemental analytical data of the antibiotic indicated that: C= 65.7%, H= 8.56%, N= 1.85; O= 23.89 and S= 0.0. This analysis indicates a suggested empirical formula of $C_{41}H_{65}NO_{11}$.

Spectroscopic Characteristics

The spectroscopic analysis of the purified of antimicrobial agent produced by *Streptomyces microflavus,* the ultraviolet (UV) absorption spectrum recorded a maximum absorption peaks at 212 and 245 nm (Fig. 2). The Infra-red (IR) spectrum showed exhibits characteristic bands at 3398.4 (OH), 2956.3~2922.6 (CH, CH_2), 1652.73 (C=O) and 1594.84 (C=C) cm^{-1}, (Fig.3). The Mass spectrum showed that the molecular weight at 747.37 (Fig.4).

MIC of antimicrobial agent

The MIC of antibiotic produced by *Streptomyces microflavus,* KSA-Kh224 for *Bacillus subtilis*, NCTC 1040 and *Bacillus pumilus*, NCTC 8214 *was* 7.8 µg / ml*,* whereas, *Staphylococcus aureus*, NCTC 7447 and *Micrococcus luteus*, ATCC 9341 was 15.6 µg / ml. *Aspergillus flavus*, IMI 111023 was 31.25 µg / ml. Moreover, *Aspergillus fumigatous*, ATCC 16424 and *Penicillium chrysogenium* was 46.87 µg / ml.

Identification of the antimicrobial agent

On the basis of the recommended keys for the identification of antibiotic, it could be stated that the antibiotic suggestive of being belonging to Irumamycin antibiotic (Omura *et al.* (1982a, b&c) and Sigrid *et al.* (2008)

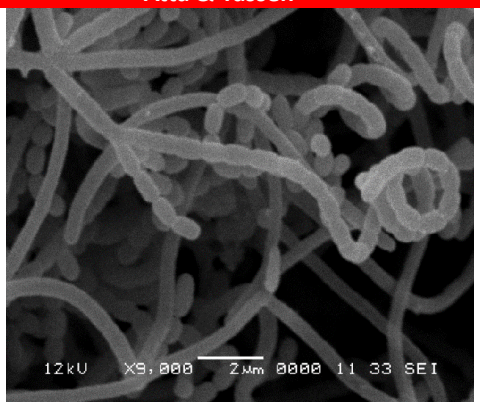

Plate 1. Scanning electron micrograph of the actinomycete isolate KSA-Kh224 growing on starch nitrate agar medium showing spore chain Spiral shape and spore surfaces smooth (X9,000).

Discussion

The *Streptomyces microflavus* was isolated from Al-Khurmah governorate, KSA. The isolate was growing on production medium had the following composition (in g/L distilled water): Starch, 20; KNO_3, 2.0; K_2HPO_4, 1.0; $MgSO_4.7H_2O$, 0.5; KCl, 0.5 and CaCO3, 0.1. pH 7.2 for investigating its potency to produce antimicrobial agents. The actinomycete isolate, exhibited a wide spectrum antimicrobial agents against gram positive bacteria and filamentous fungi Atta *et al.* (2013) and Hosny *et al.* (2015). Due to the selective isolation of soil actinomycetes for finding novel strains which can produce useful bioactive compounds, thus various culture media and techniques have been developed Hozzein *et al*. (2008) and Dhananjeyan *et al*. (2010). Identification process had been performed Williams (1989); Hensyl (1994) and Holt *et al*. (2000). The morphological characteristics and microscopic examination emphasized that the spore chain is spiral. Spore mass is gray, while spore surface is smooth, substrate mycelium is light yellowish-brown and diffusible pigment moderate brown. The results of physiological, biochemical characteristics (Table 2) and cell wall hydrolysate of actinomycete isolate, exhibited that the cell wall containing LL-diaminopimelic acid (DAP). These results emphasized that the actinomycetes isolate related to a group of *Streptomyces* as previously studied Reddy *et al*. (2011); Afifi *et al*. (2012) and Muharram *et al*. (2013). The phylogenetic tree (diagram) revealed that the local isolate is closely related *Streptomyces microflavus*, similarity matrix is 98% as identified strain of *Streptomyces microflavus* based on polyphasic taxonomical analysis. In view of all the previously recorded data, the identification of actinomycete isolate KSA-Kh224 was suggestive of being belonging to *Streptomyces microflavus*, KSA-Kh224, as previously reported Sigrid *et al*. (2008) and Fen *et al.* (2014).

The active metabolites were extracted by ethyl acetate at pH 7. Similar results were obtained by Saurav and Kannabiran (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 brownish ppt. and then tested for their antimicrobial activity. Separation of antimicrobial agents into individual components has been tried by thin-layer chromatography using a solvent system composed of n-Butanol: acetic acid: water (3:1:1 v/v), Benzene: acetone (1:1 v/v), chloroform: methanol (9:1 v/v), Ethanol: water (2:1 v/v), Ethyl acetate: pyridine: water (1:1:4 v/v), Benzene: ethyl acetate (1:1 v/v). Only one band of antimicrobial agent at R_f = 0.6, 0.5, 0.6, 0.7, 0.4 and 0.2 respectively. Similar results were obtained by Atta *et al.* (2009) and Oskay (2011). For the purpose of purification process, the antibiotic were allowed to pass through a column chromatography packed with silica gel and eluting solvent was composed of chloroform and methanol (10:2 v/v), fifty fractions were collected and tested for their activities. The most active fractions against the tested organisms ranged between, 18 to 25. Similarly, many workers used a column chromatography packed with silica gel and an eluting solvent composed of various ratios of chloroform and methanol Narayana and Vijayalakshmi (2008) and Saurav and Kannabiran (2010).

The elemental analytical data of antimicrobial agent produced by *Streptomyces microflavus,* showed the following: The elemental analytical data of the antibiotic indicated that: C= 65.7%, H= 8.56%, N= 1.85; O= 23.89 and S= 0.0. This analysis indicates a suggested empirical formula of $C_{41}H_{65}NO_{11}$. The spectroscopic analysis of the purified of antimicrobial agent produced by *Streptomyces microflavus,* the ultraviolet (UV) absorption spectrum recorded a maximum absorption peaks at 212 and 245 nm (Fig. 3). The Infra-red (IR) spectrum showed exhibits characteristic bands at 3398.4 (OH), 2956.3~2922.6 (CH, CH_2), 1652.73 (C=O) and 1594.84 (C=C) cm^{-1}, (Fig.4). The Mass spectrum showed that the molecular weight at 747.37. Similar investigations and results were attained by Awad and El-Shahed (2013) and Atta *et al.* (2013). The MIC of antibiotic produced by *Streptomyces microflavus,* KSA-Kh224 for *Bacillus subtilis*, NCTC 1040 and *Bacillus pumilus*, NCTC 8214 *was* 7.8 µg / ml, whereas, *Staphylococcus aureus*, NCTC 7447 and *Micrococcus luteus*, ATCC 9341 was 15.6 µg / ml. *Aspergillus flavus*, IMI 111023 was 31.25 µg / ml. Moreover, *Aspergillus fumigatous*, ATCC 16424 and *Penicillium chrysogenium* was 46.87 µg / ml. Similar investigations and results were attained by Duraipandiyan *et al.* (2010); Learn-Han *et al.* (2012) and Singh *et al.* (2014). Identification purified antibiotic according to recommended international keys indicated that the antibiotic is suggestive of being belonging to Irumamycin antibiotic Omura *et al.* (1982a, b&c) and Sigrid *et al.* (2008).

Table 1. Mean diameters of inhibition zones (mm) caused by 100µl of the antimicrobial activities produced by KSA-Kh224 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	30.0
Bacillus pumilus, NCTC 8214	31.0
Micrococcus luteus, ATCC 9341	30.0
Bacillus subtilis, NCTC 1040	32.0
2. Gram Negative	
Escherichia coli, NCTC 10416	0.0
Klebsiella pneumonia, NCIMB 9111	0.0
Pseudomonas aeruginosa, ATCC 10145	0.0
B- Fungi	
1-Unicellular fungi	
Candida albicans, IMRU 3669	0.0
Saccharomyces cervisiae ATCC 9763	0.0
2-Filamentous fungi	
Aspergillus niger IMI 31276	0.0
Aspergillus fumigatous, ATCC 16424	23.0
Aspergillus flavus, IMI 111023	25.0
Fusarium oxysporum	0.0
Penicillium chrysogenum	22.0

Table 2. The morphological, physiological and biochemical characteristics of the actinomycete isolate KSA-Kh224

Characteristic	Result	Characteristic	Result
Morphological characteristics:		Mannitol	++
Spore chains	Spirals	L- Arabinose	-
Spore mass	gray	*meso*-Inositol	-
Spore surface	smooth	Lactose	-
Color of substrate mycelium	Light yellowish brown	Maltose	-
Motility	Non-motile	D-fructose	+
Cell wall hydrolysate		**Utilization of amino acids:**	
Diaminopimelic acid (DAP)	LL-DAP	L-Cycteine	+
Sugar Pattern	Not-detected	L-Valine	-
Physiological and biochemical properties:		L-Histidine	-
Hydrolysis of:-		L-Phenylalanine	+
Starch	+	L-Arginine	+
Protein	+	L-Tyrosine	+
Lipid	-	**Growth with (% w/v)**	
Pectin	+	Sodium azide (0.01)	+
Lecithin	+	Phenol (0.1)	+
Catalase test	-	Thallous acetate (0.001)	-
Production of melanin pigment on:		**Growth at different temperatures (˚C):**	
Peptone yeast- extract iron agar	+	20	-
Tyrosine agar medium	+	25	±
Tryptone– yeast extract broth	-	30-50	+
Degradation of:		55	-
Xanthin	+	**Growth at different pH values:**	
Esculin	+	4	-
H_2S Production	+	5-9	+
Nitrate reduction	-	10	-
Citrate utilization	+	**Growth at different concentration of NaCl (%)**	
Urea test	+	1-5	+
KCN test	+	7	-
Utilization ofcarbon sources		**Antagonistic Effect:**	
D-Xylose	-	*Staphylococcus aureus*, NCTC 7447; *Bacillus subtilis*, NCTC 1040 ; *Bacillus pumilus*, NCTC 8214 ; *Micrococcus luteus*, ATCC 9341; *A. flavus*, IMI 111023, *A. fumigatous*, ATCC 16424 and *Penicillium chrysogenium*	+
D- Mannose	+		
D- Glucose	+		
D- Galactose	+		
Sucrose	+		
L-Rhamnose	++		
Raffinose	+++		
Starch	+++		

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

Table 3. Cultural characteristics of the actinomycete isolate KSA-Kh224.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
1- Starch-nitrate agar medium	Good	264-L .Gray Light gray	57-1.br light brown	58 m-br moderate brown
2- Yeast extract - Malt extract agar medium (ISP-2)	No growth	-	-	-
3- Oat-meal agar medium (ISP-3)	Good	264-L .Gray Light gray	76.1.y Br light yellowish brown	58 m-br moderate brown
4- Inorganic salts-starch agar medium (ISP-4)	Good	264-L .Gray Light gray	76.1.y Br light yellowish brown	-
5- Glycerol-Asparagine agar medium (ISP-5)	Moderate	264-L .Gray Light gray	76.1.y Br light yellowish brown	-
6- Melanin test: a- Tryptone-yeast extract broth (ISP-1)	No growth	-	-	-
b- Peptone yeast extract-iron agar medium (ISP-6)	Good	264-L .Gray Light gray	57-1.br light brown	59-d.Br Deep brown
c- Tyrosine agar (ISP-7)	Good	264-L .Gray Light gray	57-1.br light brown	59-d.Br Deep brown

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

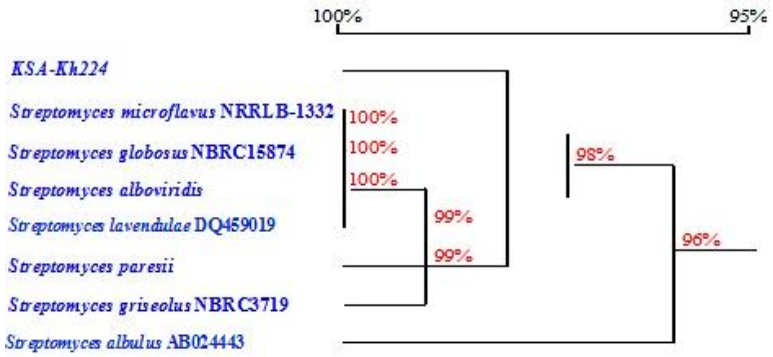

Fig. 1. The phylogenetic position of the local *Streptomyces* sp. strain among neighboring species. The phylogenetic tree was based on the multiple alignments options of 16_SrDNA sequences.

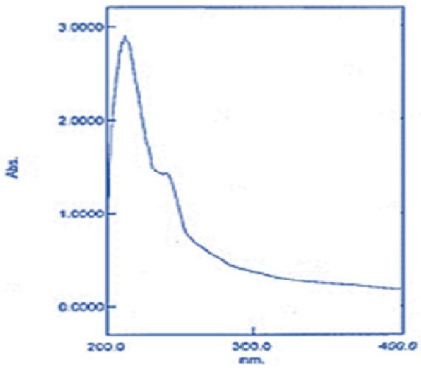

Fig. 2. Ultraviolet absorbance of antibiotic produced by *Streptomyces microflavus,* KSA-Kh224

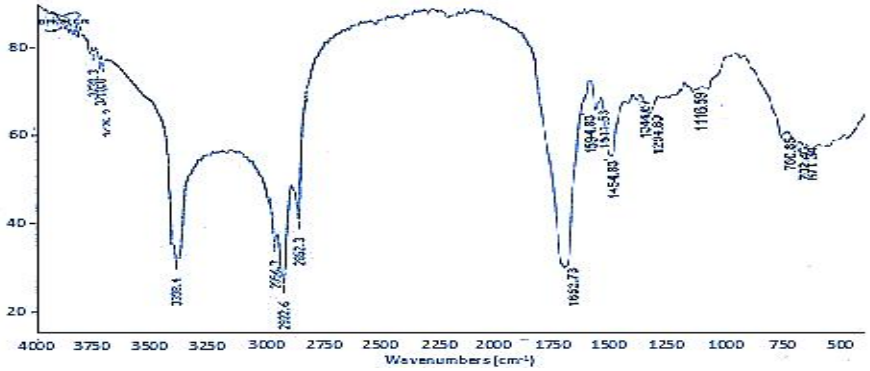

Fig. 3. FTIR spectrum of antibiotic produced by *Streptomyces microflavus,* KSA-Kh224

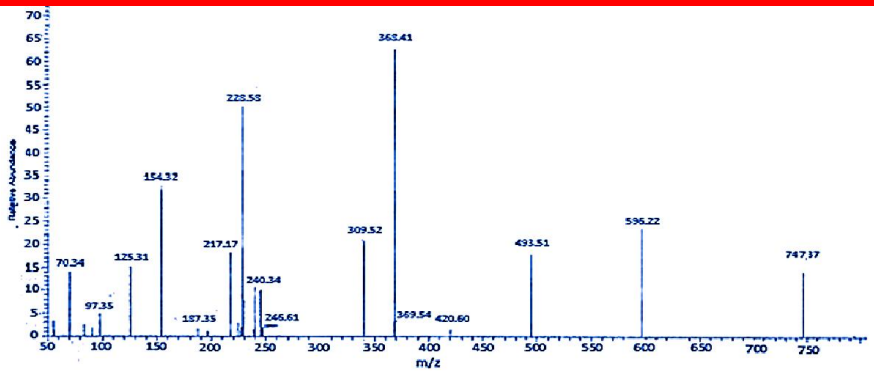

Fig. 4. Mass spectrum of antibiotic produced by *Streptomyces microflavus,* KSA-Kh224

Conclusion

The present study mainly involved in the isolation of Actinomycetes based on the cultural, morphology, physiology and biochemical characteristics, as well as 16s rDNA methodology. Further work should be focused in most potent *Streptomyces microflavus* for production the antimicrobial agent(s), against Gram positive and filamentous Fungi. The physico-chemical characteristics of the purified antibiotic viz. color, melting point, solubility, elemental analysis and spectroscopic characteristics have been investigated. The Irumamycin antibiotic produced by *Streptomyces microflavus*, KSA-Kh224 demonstrated obvious inhibitory affects against *Staphylococcus aureus, NCTC 7447; Bacillus subtilis, NCTC 1040; Bacillus pumilus, NCTC 8214; Micrococcus luteus, ATCC 9341; Aspergillus flavus, IMI 111023; Aspergillus fumigatous, ATCC* 16424 and *Penicillium chrysogenium*.

References

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

Anderson, A.S., and Wellington, E.M.H. (2001). The taxonomy of *Streptomyces* and related genera. Int. J. Syst. Evol. Microbiol., 51: 797-814.

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.

Atta, H. M.; Abul-hamd, A. T. and Radwan, H. G. (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.

Atta, H. M. 2014. Bioactive Secondary Metabolites from *Streptomyces* sp: Taxonomy, Fermentation, Purification and Biological Activities. New York Science Journal, 2013;6(6):99-110

Atta, H.M.; Bayoumi, R.; El-Sehrawi, M. and Selim Sh. M. (2013). Application Biotechnology of Recycling Agricultural Waste In Al-Khurmah Governorate For Production Antimicrobial Agent(S) By Actinomycetes Isolates Under Solid State Fermentation Condition. Life Science Journal 2013 10 (4):1749-1761.

Awad, H.M. and El-Shahed, K.Y.I. (2013). A Novel *Actinomycete* sp. Isolated from Egyptian Soil has β-Lactamase Inhibitor Activity and Belongs to the *Streptomyces rochei* Phylogenetic Cluster. World Applied Sciences Journal 21 (3): 360-370, 2013

Berdy J. (2005). Bioactive microbial metabolites. J Antibiot. 2005; 58; 1-26.

Berdy, J. (1974). Recent development of antibiotic research and classification of antibiotic according to chemical structure. Adv. App. Microbiol., 14: 309-406.

Berdy, J. (1980a). Recent advances in and prospects of antibiotics research. Proc. Biochem., 15: 28-35.

Berdy, J. (1980b). CRC Handbook of antibiotic compounds. Vol I. CRC Press, Boca Raton, Florida.

Berdy, J. (1980c). CRC Handbook of antibiotic compounds. Vol II. CRC Press, Boca Raton, Florida.

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

Dhananjeyan, K.J.; Paramasivan, R.; Tewari, S.C.; Rajendran, R.; Thenmozhi, V.; Leo, S.V.J.; Venkatesh, A. and Tyagi, B.K. (2010). Molecular identification of mosquito vectors using genomic DNA isolated from eggshells, larval and pupal exuvium. Trop. Biomed. ; 27:47–53.

Duraipandiyan, V.; Sasi, A.H.; Islam, V.I.; Valanarasu, M. and Ignacimuthu, S. (2010). Antimicrobial properties of actinomycetes from the soil of Himalaya. J Med Mycol. 2010;20(1):15-20.

Edwards, U.; Rogall, T.; Blocker, H.; Emde, M. and Bottger, E.C. (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.

Fen, G.; Yuanhua, W. and Mengliang W. (2014). Identification and antifungal activity of an actinomycete strain against *Alternaria* spp. Spanish Journal of Agricultural Research 2014 12(4): 1158-1165

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.

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

Holt, J.G.; Krieg, N.R.; Sneath, P.H.A; Staley J.T. and Williams S.T. (2000). Bergey's Manual of Determinative Bacteriology. 9th Ed.

Baltimore: Williams and Wilkins, London.
Hosny, A.; Sheir, D.; El-Diwany, A.; Abdelwahed, N. A, Fallarero, A. and Vuorela, P. M 2015. Production and characterization of antimicrobial compound produced by *Streptomyces atrovirens* H33. Biolife 3(2):476-482.
Hotam, S. C.; Bhavana, S.; Anju, R. S.; Saurabh, S. (2013). Diversity and Versatility of Actinomycetes and its Role in Antibiotic Production. Journal of Applied Pharmaceutical Science Vol. 3 (8 Suppl 1), pp. S83-S94, September, 2013.
Hozzein, W.N.; Ali, M.I.A. and Rabie, W. (2008). A new preferential medium for enumeration and isolation of desert actinomycetes. World J. Microbiol. Biotechnol. 24: 1547-1552.
Jarallah, E.M. (2014). Screening for Antimicrobial Agents Producing Actinomycetes Isolated from Agricultural Soils in Hilla/Iraq. *IOSR Journal of Pharmacy and Biological Sciences 9 (2): PP 47-52.*
Kang, S.G.; Park, H.U.; Lee, H.S.; Kim, H.T. and Lee, KJ. (2000). New beta-lactamase inhibitory protein (BLIP-I) from *Streptomyces exfoliatus* SMF19 and its roles on the morphological differentiation. J. Biol. Chem. 275:16851–16856.
Kavanagh, F. (1972). Analytical Microbiology. Vol. 2, Acad. Press, New York.
Kekuda, T.R.P.; Shobha, K.S. and Onkarappa, R. (2010). Studies on antioxidant and anthelmintic activity of two *Streptomyces species* isolated from Western Ghat soils of Agumbe, Karnataka. J Pharm Res. 2010;3:26-9.
Kenneth, L.K. and Deane, B.J. (1955). Color universal language and dictionary of names. United States Department of Commerce. National Bureau of standards. Washington, D.C., 20234.
Learn-Han, L.; Yoke-Kqueen, C.; Shiran, M. S.; Nurul-Syakima, A.M.; Yi-Li, T.; Hai-Peng, L. and KuiHong, L. (2012). Molecular characterization of Antarctic actinobacteria and screening for antimicrobial metabolite production. *MIRCEN Journal of Applied Microbiology and Biotechnology,* 28 (5): 2125–2137.
Lechevalier, M.P. and Lechevalier, H.A. (1980). The chemotaxonomy of actinomycetes. In: Actinomycete Taxonomy. A. Dietz and D.W. Thayer, (Eds.), Special publication. Arlington S I M, USA, 6: 227-291.
Lilia F.; Serge F.; Raoudha M.; Lotfi M.and Hartmut, L. (2004). Purification and structure elucidation of antifungal and antibacterial activities of a newly isolated *Streptomyces* sp. strain US80. Online http://wwwuser.gwdg.de/~hlaatsc/156_US80.pdf.
Manteca, A.; Fernandez. M. and Sanchez, J. (2005). A death round affecting a young compartmentalized mycelium precedes aerial mycelium dismantling in confluent surface cultures of *Streptomyces antibioticus*. *Microbiology*. 2005; 151:3689-3697.
Muharram, M.M.; Abdelkader M.S. and Alqasoumi, S.I. (2013). Antimicrobial activity of soil actinomycetes isolated from Alkharj, KSA. Int. Res. J. Microbiol., 4: 12-20.
Narayana, K.J.P. and Vijayalakshmi, M. (2008). Optimization of antimicrobial metabolites production by *Streptomyces albidoflavus*. Res J Pharmacol. 2008;2(1):4-7.
Neto, A.B.; Hirata, D.B.; Cassiano Filho, L.C.M.; Bellao, C.; Badino Junior A.C. and. Hokka, C.O (2005). A study on clavulanic acid production by *Streptomyces clavuligerus* in batch, fed-batch and continuous processes Brazilian Journal of Chemical Engineering, 22(4): 557-563.
Numerical taxonomy program (1989). Numerical taxonomy of *Streptomyces* species program (PIB WIN) (*Streptomyces* species J. Gen Microbiol. 1989 13512-133.
Omura, S.; Nakagawa, A. and Tanaka, Y. (1982b). New macrocyclic antibiotics, irumamycin and hitachimycin (stubomycin). In Trends in Antibiotic Research. Eds. H. Umezawa , A. L. Demain, T. Hata & C. R. Hutchinson, pp. 135-145, Japan Antibiotics Res. Assoc., Tokyo, 1982
Omura, S.; Nakagawa A. and Tanaka, Y. (1982c). Structure of a new antifungal antibiotic, irumamycin. J. Org. Chem. 47: 5413 - 5415, 1982.
Omura, S.; Tanaka, Y. Nakagawa, A. Iwai, Y. Inoue M. and Tanaka, H. (1982a). Irumamycin, a new antibiotic active against phytopathogenic fungi. J. Antibiotics 35: 256257, 1982
Oskay, M. (2011). Effects of some Environmental Conditions on Biomass and Antimicrobial Metabolite Production by *Streptomyces* sp., KGG32. Int J Agric Biol. 2011;13(3):317-324.
Pelaez, F.T. (2006). He historical delivery of antibiotics from microbial natural products-Can history repeat? Biochem Pharmacol. 2006; 71: 981– 990.
Raja, A. and Prabakarana, P. (2011). Actinomycetes and Drug-An Overview. Amer. J. Drug Disc. Develop. 1: 75–84.
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; 4:294-6.
Reddy, N.G.; Ramakrishna, D.P.N and Gopal, S.V.R. (2011). A morphological, physiological and biochemical studies of marine *Streptomyces rochei* (MTCC 10109) showing antagonistic activity against selective human pathogenic microorganisms. Asian Journal of Biological Science, 4(1): 1-14.
Sambrook, J.; Fritsch E.F. and Maniatis, T.A. (1989). Molecular Cloning: A Laboratory Manual. 2nd Edn., Cold Spring Harbor Laboratory Press, New York, USA., ISBN-13: 9780879695774, Pages: 397.
Sanger, F.; Nicklen, S. and Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci., 74: 5463-5467.
Saurav, K. and Kannabiran, K. (2010). Diversity and optimization of process parameters for the growth of *Streptomyces VITSVK* 9 sp. isolated from Bay Of Bengal, India. J Nat Env Sci. 2010;1(2):56-65.
Shrilling, E.B. and Gottlieb, D. (1966). Methods for characterization of *Streptomyces* species. International Journal of Systematic Bacteriology, 16(3): 313-340.
Sigrid H.; Espen F.; Kjell, D.; Elena I.; Trond E. and Sergey B. Z. (2008). Characterization of *Streptomyces* spp. Isolated from the Sea

Surface Microlayer in the Trondheim Fjord, Norway. *Marine Drugs* 2008, *6*, 620-635.
Singh, R.; Pandey, B. and Mathew, C.M. (2014). Production, purification and optimization of Streptomycin from isolated strain of *Streptomyces griseus* and analysis by HPLC. IndianJ.Sci.Res.4 (1):149-154, 2014
Umezawa, H. (1977). Recent advances in bio-active microbial secondary metabolites. Jap. J. Antibiotic. Suppl., 30: 138-163.
Williams, S.T. (1989). Bergey's Manual of Systematic Bacteriology. Vol. 4, Williams and Williams, Baltimore, MD., USA.
Williams, S.T. and Davies, F. L. 1965. Use of antibiotics for selective isolation and enumeration of actinomycetes in soil. J. Gen. Microbiol., 38:251-262.
Zamanian, S.; Shahidi Bonjar, G. H. and Saadoun, I. (2005). First report of antibacterial properties of a new strain of *Streptomyces plicatus* (strain 101) against *Erwinia carotovora* from Iran. Biotechnology, 4: 114-120.