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Original Research Article

Phylogenetic characterization, fermentation and biological activities of an antibiotic producing *Streptomyces clavuligerus* isolated from KSA

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Keywords

Actinomycetes, Conventional taxonomy, Phylogenetic analysis, Fermentation, Biological activities and clavulanic acid

A B S T R A C T

This work was carried out for the biosynthesis of antimicrobial substance that demonstrated inhibitory effects against pathogenic microorganisms from *Streptomyces* sp. The KSA-T180 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 clavuligerus*. Furthermore, a phylogenetic analysis of the 16S rDNA gene sequence and ribosomal database project consistent with conventional taxonomy confirmed that strain KSA-T180 was most similar to *Streptomyces clavuligerus* (98%). The active metabolite was extracted using n-Butanol (1:1, v/v) at pH 7.0. The separation of the active ingredient of the antibacterial agent and its purification was performed using both thin layer chromatography (TLC) and column chromatography (CC) techniques. The chemical characteristics of the antibacterial agent(s) viz. elemental analysis and spectroscopic characteristics have been investigated. This analysis indicates a suggested empirical formula of $C_8H_8NO_5$, ultraviolet (UV) absorption spectrum recorded a maximum absorption peak at 285 nm, Infra-red (IR) spectrum showed characteristic twenty-three bands and Mass spectrum showed that the molecular weight at 200.0. The minimum inhibition concentrations "MICs" of the antibiotic were also determined. The collected data emphasized that the antibiotic was characterized as clavulanic acid

Introduction

Antibiotics are complex chemical secondary metabolites, which are produced by microorganisms and acts against other microorganisms (Singh *et al.*, 2014).

Antibiotics are used to prevent infections after surgery or at open wounded areas (Cimochowski *et al.*, 2001). Actinomycetes are a major source of bioactive natural products. More than 10,000 substances with bioactivity have been isolated so far from terrestrial and marine actinomycetes (Berdy 2005, 2012) and many are clinically used as antitumor agents, antibiotics, or immunosuppressants. Members of Actinomycete genera are gram positive bacteria with high GC content in their DNA (Kieser *et al*., 2000). They are well known for production of a wide range of secondary metabolites like antibiotics, antitumor compounds, immunosuppressants, herbicides, antiviral and antiparasitic agents. There are 23,000 biologically active secondary metabolites produced by microorganisms has been identified up to now, and 10,000 of them are produced by the order of Actinomycetales. *Streptomyces* spp. produce 7,600 of these 10,000 secondary metabolites (Sacramento *et al*., 2004 and Olano *et al*., 2008). *Streptomyces* spp. are filamentous, spore forming and strictly aerobic bacteria which belong to Actinomycetes order (Paradkar *et al*., 2003). High GC content genome (more than 70 mole %) and large linear plasmids (10-600 kb) are distinctive features of *Streptomyces* species (Kieser *et al*., 2000). Mona-Ibrahim (2012) reported that, the Streptom*yces* generally synthesis a sizeable number of diverse natural secondary metabolites (Onaka *et al.*, 2001), such as antibiotics, insecticides, herbicides, immunosuppressive actions (Mao *et al*., 2007), vitamins, alkaloids, plant growth factor, enzymes and enzyme inhibitors (Augustine *et al*., 2005). Secondary metabolite production of *Streptomyces* is strictly related and regulated with morphological changes (Paradkar *et al*., 2003). *Streptomyces clavuligerus* has been the subject of extensive research in the last 30 years because of its ability to produce β-lactam metabolites with antibiotic, antifungal and β-lactamase-inhibitory activities (Thai *et al.*, 2001 and Bibb, 2005). *Streptomyces clavuligerus* is known to produce 21 secondary metabolites (Gouveia *et al.*, 2001; Ortiz *et al*., 2007 and Rodríguez *et al.*, 2008) including holomycin; a member of the pyrrothine class antibiotics, an antibiotic related to tunicamycin; a glucosamine-containing antibiotic (Kenig and Reading, 1979), and β-lactam metabolites with antibiotic, antifungal activities, which is why it has been studied over three decades (Thai *et al*., 2001). In the present study were describe the isolation of an actinomycete strain KSA-T180 from Taif city, 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 and filamentous fungi. One major active compound was extracted from the purified fermented broth and chemically characterized as clavulanic acid, 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-T180 was isolated from soil sample collected from Taif city, 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 ; *Fusarium oxysporum* and *Penicillium chrysogenum* was collection, National Research Centre, Dokki-Giza, Egypt.

Culture media: The seed medium had the following composition (in g/L distilled water): glycerol, 15; bacto-peptone, 10; malt extract, 10; yeast extract, 1.0; K_2HPO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 0.75; $MnCl_2 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; and $ZnSO_4 \cdot 7H_2O$, 0.001. The pH of the medium was adjusted to 6.8 with NaOH 5.0 M before autoclaving at 121 °C for 15 min. The inoculum medium used in the cultivations, based on that proposed by (Maranesi *et al*., 2005), had the following composition (in g/L distilled water): glycerol, 10; soybean, 20; K_2HPO_4, 1.2; $MnCl_2 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001, pH 6.8. The composition of the production medium was similar to that used for the inoculum, except for the concentration of glycerol (5.0 g/L).

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

Conventional Taxonomy: The cultural, morphological, physiological and biochemical characteristics of strain KSA-T180 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 analysed 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 seven 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.ACAAGCCCTGGAAACGGG GT-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: A loopful of the, *Streptomyces* sp. from the 5-day culture age was inoculated into 250 ml Erlenmeyer flasks containing 75 ml of antibiotic production medium had the following composition (in g/L distilled water): glycerol, 5; soybean flour, 20; K_2HPO_4, 1.2; $MnCl_2 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001, pH 6.8. 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 (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, 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 *et al.,* 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). as a solvent system (Atta *et al.,* 2009).

Purification: The purification of the antimicrobial agent(s) was carried out using silica gel column (2 X 25) chromatography. Chloroform-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 (Lu *et al.,* 2008).

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 Eric, 1999).

Result and Discussion

Screening for the antimicrobial activities

The metabolites of the *Streptomyces* sp. exhibited various degrees of activities against Gram positive and Gram negative bacteria viz: *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 (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 rectiflexibiles, 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-T180 could hydrolyzes starch, protein, lipid and lecithin, whereas pectin hydrolysis and catalase test are negative, melanin pigment is negative, degradation of esculin & xanthin was positive, citrate utilization, urea and KCN utilization were positive, whereas nitrate reduction, H_2S production is negative (Table 2).

The isolate KSA-T180 utilizes *meso*-inositol, starch, L-phenylalanine, L-valine, L-arginine, L-tyrosine and L-histidine, but do not utilize D-mannose, D-mannitol, D-glucose, D-fructose, D-xylose, D-galactose, maltose, lactose, L-rhamnose, sucrose, raffinose, L-arabinose, and cycteine. Growth was detected in presence of up to (7%) NaCl. The isolate KSA-T180 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 45ºC. Good growth could be detected within a pH value range of 5 to 9. Moreover, the actinomycete isolate KSA-T180 are active against *Bacillus subtilis*, NCTC 1040*; Micrococcus luteus*, ATCC 9341, but not active against *Saccharomyces cerevisiae* ATCC 9763 and *Aspergillus niger* IMI 31276 (Table 2).

Color and Culture Characteristics

The actinomycete isolate shows the aerial mycelium is grayish yellow; substrate mycelium is light yellowish brown, and the diffusible pigment moderate yellowish brown for ISP-2, 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 clavuligerus* it could be stated that the actinomycetes isolate KSA-T180 is suggestive of being likely belonging to *Streptomyces clavuligerus*, KSA-T180.

Molecular phylogeny of the selected isolate

The 16S rDNA sequence of the local isolate was compared to the sequences of *Streptomyces* spp. In order to determine the relatedness of the local isolate to 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 clavuligerus* (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 n-butanol (1:1, v/v) and the organic layer was evaporated to give an oily material. The oily material was then dissolved in 15% aqueous methanol and defatted by partitioning with petroleum ether (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). Only one band at $R_f = 0.6$ showed antibacterial activity. The purification process through column chromatography packed with silica gel, revealed that the most active fractions against the tested organisms ranged between, 23 to 30.

Physicochemical characteristics

The purified antibacterial agent produced by *Streptomyces clavuligerus*, KSA-T180 are produces characteristic odour, their melting points are 118°C. The compound is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10 % isopropyl alcohol, but insoluble in petroleum ether, hexan and benzene.

Elemental analysis

The elemental analytical data of β-lactamase inhibitor compound produced by *Streptomyces clavuligerus,* showed the following: The elemental analytical data of the antibiotic indicated that: C=45.65; H=3.8; N= 7.1; O= 43.45 and S= 0.0. This analysis indicates a suggested empirical formula of: $C_8H_8NO_5$.

Spectroscopic Characteristics

The spectroscopic analysis of the purified of β-lactamase inhibitor compound produced by *Streptomyces clavuligerus*, the ultraviolet (UV) absorption spectrum recorded a maximum absorption peaks at 285 nm (Fig. 3). The Infra-red (IR) spectrum showed characteristic bands 589, 600, 650, 708, 734, 800, 850, 880, 900, 950, 976, 1062, 1101, 1224, 1300, 1338, 1550, 1618, 1700, 2800, 2886, 3012 and 3420 (Fig.4). The Mass spectrum showed that the molecular weight at 200.0 (Fig.5).

MIC of β-lactamase Inhibitory Protein

The MIC of antibiotic produced by *Streptomyces clavuligerus*, KSA-T180 for *Staphylococcus aureus*, NCTC 7447 and *Bacillus subtilis*, NCTC 1040 *was* 7.8 µg / ml, whereas, *Bacillus pumilus*, NCTC 8214 and *Micrococcus luteus*, ATCC 9341 was 15.6 µg / ml. *Escherichia coli*, NCTC 10416 and *Klebsiella pneumonia*, NCIMB 9111 was 31.25 µg / ml. Moreover, *Pseudomonas aeruginosa* was 46.87 µg / ml.

Identification of the β-lactamase Inhibitor

On the basis of the recommended keys for the identification of antibiotic, it could be stated that the antibiotic suggestive of being belonging to clavulanic acid (Chen, *et al.*, 2003; Parag *et al.*, 2006 and Awad & El-Shahed, 2013)

The *Streptomyces clavuligerus* was isolated from Taif city, KSA. The isolate was growing on production medium had the following composition (in g/L distilled water): glycerol, 5; soybean flour (SF), 20; K_2HPO_4, 1.2; $MnCl_2 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.001; $ZnSO_4 \cdot 7H_2O$, 0.001, pH 6.8. for investigating its potency to produce antibacterial agents. The actinomycete isolate, exhibited a wide spectrum antibacterial agent (Kavanagh, 1972). 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 grayish yellow, while spore surface is smooth, substrate mycelium is light yellowish brown and diffusible pigment moderate yellowish 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 KSA-T180 is closely related *Streptomyces clavuligerus*, similarity matrix is 98% as identified strain of *Streptomyces clavuligerus.* Similar result for identified strain of *Streptomyces plicatus* (strain 101) by (Kang *et al.*, 2000; Anderson & Wellington, 2001and Zamanian *et al.*, 2005) *Streptoverticillium* sp. and two *Streptomyces* sp. by (Raja *et al*. 2010). In view of all the previously recorded data, the identification of actinomycete isolate KSA-T180 was suggestive of being belonging to *Streptomyces clavuligerus*, KSA-T180, as previously reported (Ghadin *et al*., 2008 and Ubukata *et al*., 2007).

The active metabolites were extracted by n-butanol at pH 7. Similar results were obtained by (Sekiguchi, *et al.*, 2007). 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 antibacterial activity. Separation of antibiotic into individual components has been tried by thin-layer chromatography using a solvent system composed n-butanol-acetic acid -water (3:1:1, v/v) as developing solvent (Zhang *et al*, 2007 and Atta *et al.*, 2009). 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, 23 to 30. Similarly, many workers used a column chromatography packed with silica gel and an eluting solvent composed of various ratios of chloroform and methanol (Criswell *et al.* 2006; El-Naggar *et al*., 2006 and Sekiguchi, *et al.,* 2007).

The elemental analytical data of antibacterial agent produced by *Streptomyces clavuligerus,* showed the following: The elemental analytical data of the antibiotic indicated that: C=45.65; H=3.8; N= 7.1; O= 43.45 and S= 0.0. This analysis indicates a suggested empirical formula of: $C_8H_8NO_5$. The spectroscopic analysis of the purified of antibacterial agent produced by *Streptomyces clavuligerus,* the ultraviolet (UV) absorption spectrum recorded a maximum absorption peak at 285 nm (Fig. 2).

Table.1 Mean diameters of inhibition zones (mm) caused by 100µl of the antimicrobial activities produced by KSA-T180 in the agar plate diffusion assay (The diameter of the used cup assay was 10 mm).

Test organism	Mean diameters of inhibition zone (mm)
***Staphylococcus aureus*, NCTC 7447**	33.0
***Bacillus subtilis*, NCTC 1040**	31.0
***Bacillus pumilus*, NCTC 8214**	30.0
***Micrococcus luteus*, ATCC 9341**	29.0
***Escherichia coli*, NCTC 10416**	29.0
***Klebsiella pneumonia*, NCIMB 9111**	27.0
***Pseudomonas aeruginosa*, ATCC 10145**	24.0
***Candida albicans*, IMRU 3669**	0.0
***Saccharomyces cervisiae* ATCC 9763**	0.0
Aspergillus flavus, IMI 111023	0.0
Aspergillus fumigatous, ATCC 16424	0.0
Fusarium oxysporum	0.0
Penicillium chrysogenum	0.0

Plate.1 Scanning electron micrograph of the actinomycete isolate KSA-T180 growing on starch nitrate agar medium showing spore chain Spiral shape and spore surfaces smooth (X7,500)

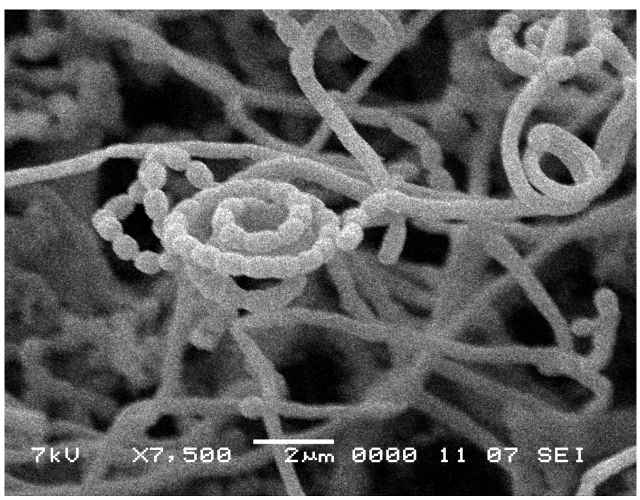


Table.2 The morphological, physiological and biochemical characteristics of the actinomycete isolate KSA-T180

Characteristic	Result	Characteristic	Result
Morphological characteristics:		**Mannitol**	-
Spore chains	**Rectiflexibiles**	L- Arabinose	-
Spore mass	**grayish yellow**	*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 inhibitors	
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	-	**30-45**	+
Degradation of:		**50**	-
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	+	**1-7**	+
KCN test	+	**10**	-
Utilization of carbon sources		Antagonistic Effect:	
D-Xylose	-	*Bacillus subtilis*	+
D- Mannose	-	*Micrococcus luteus*	+
D- Glucose	-	*Saccharomyces cerevisiae*	-
D- Galactose	-	***Aspergillus niger***	-
Sucrose	-		
L-Rhamnose	-		
Raffinose	-		
Starch	+++		

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

Table.3 Cultural characteristics of the actinomycete isolate KSA-T180

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
1- Starch-nitrate agar medium	Good	**90-gy-y** grayish yellow	**76.1.y Br** light yellowish brown	**77 m-y Br** moderate yellowish brown
2- Yeast extract - Malt extract agar medium (ISP-2)	No growth	-	-	-
3- Oat-meal agar medium (ISP-3)	Good	**90-gy-y** grayish yellow	**76.1.y Br** light yellowish brown	-
4- Inorganic salts-starch agar medium (ISP-4)	Good	**90-gy-y** grayish yellow	**76.1.y Br** light yellowish brown	-
5- Glycerol-Asparagine agar medium (ISP-5)	Moderate	**90-gy-y** grayish yellow	93-y-Gray yellowish gray	-
6- Melanin test: a- Tryptone-yeast extract broth (ISP-1)	No growth	-	-	-
b- Peptone yeast extract-iron agar medium (ISP-6)	Good	**90-gy-y** grayish yellow	**76.1.y Br** light yellowish brown	**77 m-y Br** moderate yellowish brown
c- Tyrosine agar (ISP-7)	Good	**90-gy-y** grayish yellow	**76.1.y Br** light yellowish brown	**77 m-y Br** moderate yellowish 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 16S rDNA sequences

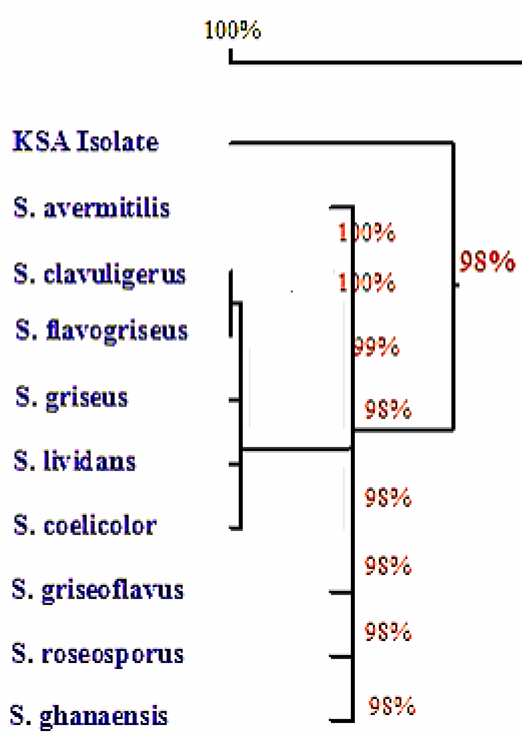


Fig.2 Ultraviolet absorbance of antibiotic produced by Streptomyces clavuligerus, KSA-T180

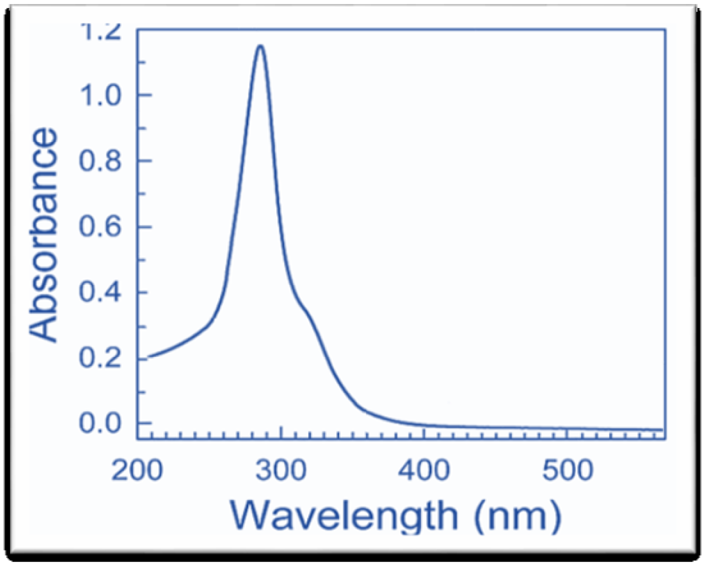


Fig.3 FTIR spectrum of antibiotic produced by *Streptomyces clavuligerus,* KSA-T180

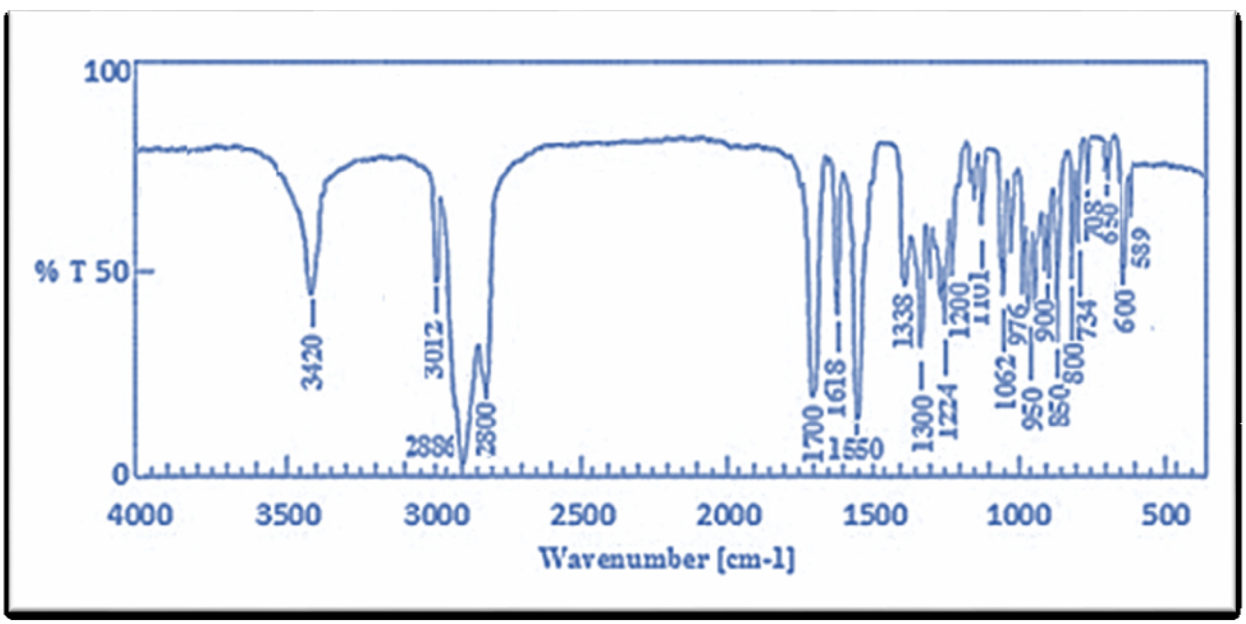
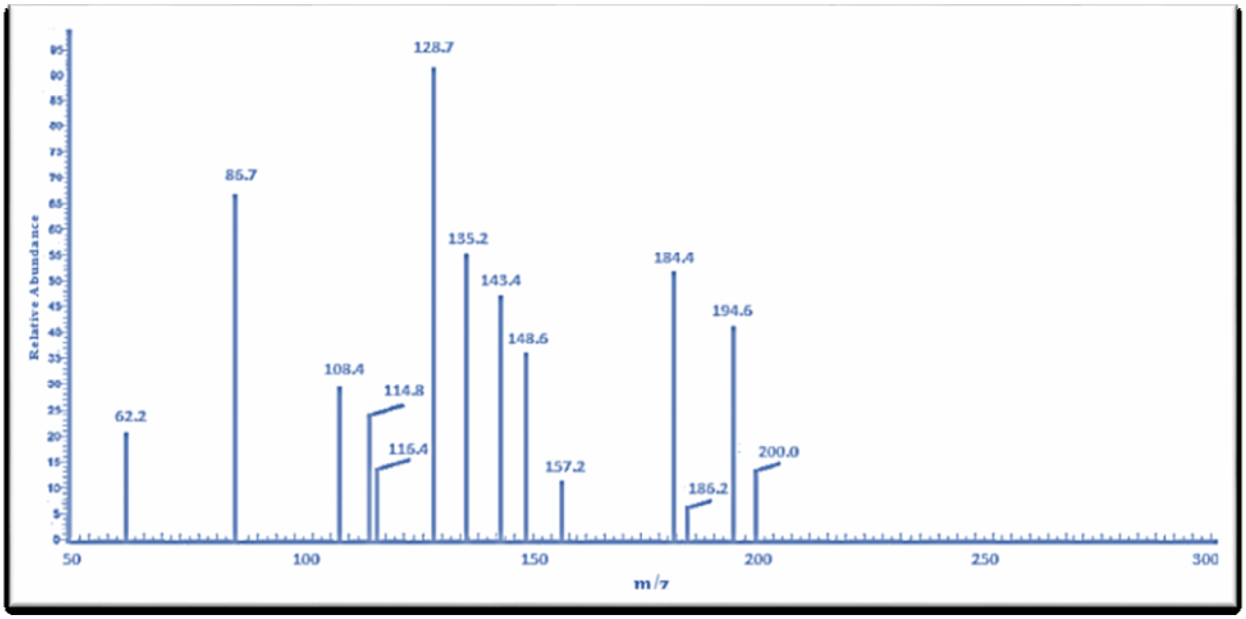


Fig.4 Mass spectrum of antibiotic produced by *Streptomyces clavuligerus*, KSA-T180



The Infra-red (IR) spectrum showed characteristic bands 589, 600, 650, 708, 734, 800, 850, 880, 900, 950, 976, 1062, 1101, 1224, 1300, 1338, 1550, 1618, 1700, 2800, 2886, 3012 and 3420 (Fig.3). The Mass spectrum showed that the molecular weight at 200.0. Similar investigations and results were attained by (Baptista-Neto *et al.*, 2000; Parag *et al.*, 2006; Awad and El-Shahed, 2013 and Atta *et al.*, 2013). The MIC of antibiotic produced by *Streptomyces clavuligerus*, KSA-T180 for *Staphylococcus aureus*, NCTC 7447 and *Bacillus subtilis*, NCTC 1040 *was* 7.8 µg / ml, whereas, *Bacillus pumilus*, NCTC 8214 and *Micrococcus luteus*, ATCC 9341 was 15.6 µg / ml. *Escherichia coli*, NCTC 10416 and *Klebsiella pneumonia*, NCIMB 9111 was 31.25 µg / ml. Moreover, *Pseudomonas aeruginosa* was 46.87 µg / ml. Similar investigations and results were attained by (Parag *et al.*, 2006 and Awad *et al.*, 2009). Identification purified antibiotic according to recommended international keys indicated that the antibiotic is suggestive of being belonging to clavulanic acid antibiotic (Chen, *et al.*, 2003; Parag *et al.*, 2006 and Awad & El-Shahed, 2013).

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