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ORIGINAL ARTICLE

Biochemical studies on antibiotic production from *Streptomyces* sp.: Taxonomy, fermentation, isolation and biological properties

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Abstract Tunicamycin is a nucleotide antibiotic which was isolated from the fermentation broth of a *Streptomyces* strain No. T-4. According to the morphological, cultural, physiological and biochemical characteristics, and 16S rDNA sequence analysis, strain T-4 was identified as *Streptomyces torulosus*. It is active *in vitro* against some microbial pathogenic viz: *Staphylococcus aureus*, NCTC 7447; *Micrococcus lutea*, ATCC 9341; *Bacillus subtilis*, NCTC 10400; *B. pumilus*, NCTC; *Klebsiella pneumonia*, NCIMB 9111; *Escherichia coli*, NCTC 10416; *Pseudomonas aeruginosa*, ATCC 10145; *Saccharomyces cerevisiae* ATCC 9763; *Candida albicans*, IMRU 3669; *Aspergillus flavus*, IMI 111023; *Aspergillus niger* IMI 31276; *Aspergillus fumigatus* ATCC 16424; *Fusarium oxysporum*; *Rhizoctonia solani*; *Alternaria alternata*; *Botrytis fabae* and *Penicillium chrysogenium*. The production media were optimized for maximum yield of secondary metabolites. The metabolites were extracted using *n*-butanol (1:1, v/v) at pH 7.0. The chemical structural analysis with UV, IR, and MS spectral analyses confirmed that the compound produced by *Streptomyces torulosus*, T-4 is tunicamycin antibiotic.

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1. Introduction

Actinomycetes have provided important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances (Olano et al., 2009). *Streptomyces* is the largest genus of Actinobacteria and the type genus of the family *Streptomycetaceae* (Kampfer, 2006). Over 500 species of *Streptomyces* have been described by Euzeby (2008). As with the other Actinobacteria, Streptomycetes are Gram-positive and have genomes with high GC-content (Madigan and Martinko, 2005). *Streptomyces* sp. are widely recognized as industrially important organisms for their ability to elaborate different kinds of novel secondary metabolites (Bibb, 2005). Tunicamycins are nucleotide antibiotics produced by several *Streptomyces* species. They are potent inhibitors of the UDP-GlcNAc:polyprenol phosphate GlcNAc-1-P translocase family and are often used to block protein *N*-glycosylation. The structures are highly unusual but well characterized (Tamura, 1982; Tkacz, 1983; Eckardt, 1983) and are composed of uracil, *N*-acetylglucosamine (GlcNAc), a unique 11-carbon 2-aminodialdose sugar

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called tunicamine, and an amide-linked fatty acid. The αβ-1,1′-glycosidic linkage between tunicamine and the GlcNAc substituent is also unique to the tunicamycin family of compounds. Tunicamycin structural variants occur that differ only in the nature of the *N*-linked acyl chain. We have recently introduced a structure-based naming system that identifies each tunicamycin by its signature fatty acid, i.e. Tun 13:1–Tun 18:1 (Tsvetanova and Price, 2001). Although a great deal is known about tunicamycin structure and function, no previous analysis of tunicamycin biosynthesis has been reported. The key to understanding the biosynthesis of tunicamycin is the origin of the 11-carbon tunicamine dialdose sugar and the kinetics for the formation of the, β-1″, 11′-glycosidic bond. A large number of natural products of *Streptomyces* origin are synthesized from 2-carbon units via a polyketide-type reaction sequence (Khosla, 2000). However, other long chain sugars such as sialic acids, ketodeoxyoctulosonate (KDO) and ketodeoxyheptulosonate are synthesized from aldol condensation of lower sugars with phosphoenolpyruvate (PEP) (Subramaniam et al., 1998). In addition, the biosynthesis of similar nucleoside antibiotics, polyoxins and nikkomycins, occurs by ligation of PEP and uridine-5-aldehyde, generating 8-carbon octofuranuloseuronic acid nucleoside as an intermediate (Isono and Suhadolnik, 1976; Isono et al., 1978; Schuz et al., 1992). Here, metabolic radiolabeling experiments and stable isotope incorporations have been applied to unravel the metabolic origin of the 11-carbon dialdose sugar, tunicamine. The [2-^{14}C] uridine and [1-^{14}C] glucosamine are efficiently incorporated into tunicamycin by resting cells of *Streptomyces chartreusis* and that the [1-^{14}C] glucosamine feeds into both the 11-carbon tunicamine and the attached α-1″-GlcNAc residue. Stable isotope incorporations using ^{2}H- or ^{13}C-labeled glucose and competitive metabolic experiments were monitored by LC-ESI-CID-MS and NMR (H-1, C-13, and HSQC) spectroscopy. The isotopic labeling patterns were consistent with carbon–carbon bond formation between a 5-carbon precursor derived from uridine and a 6-carbon hexose intermediate, the latter most probably derived from UDP-GlcNAc. Heteronuclear C-13/H-1 NMR correlations showed an equal incorporation of ^{13}C label from [1-^{13}C] glucose into both the β-11′ and α-1″ anomeric carbons, indicating that both arise from a common precursor pool. Hence, both the pseudo-aminogalactopyranosyl (pseudoGalN) rings of tunicamine and the α-1″-linked GlcNAc residue are initially derived from the sugar nucleotide UDP-GlcNAc. Based on the results of these experiments a biosynthetic pathway is proposed for tunicamycin for the first time (Schuz et al., 1992).

In the present work we describe the isolation of an actinomycete strain from Taif City, KSA soil, with high potential of antimicrobial activity. The identification of this strain, based on the cultural, morphology, physiology and biochemical charateristics, as well as 16s rDNA analysis, is also reported. The primary bioactive substance was isolated, purified and its biological activities were determined.

2. Materials and methods

2.1. Actinomycete strain

Strain T-4 was isolated from a suspension of a soil sample (Williams and Davies, 1965) collected from Taif City, Kingdom of Saudi Arabia, and inoculated onto a starch–nitrate agar. Plates were incubated at 35 °C for seven days. The isolates were individually maintained on starch–nitrate agar at 4 °C and stored as a mixture of hyphae and spores in 20% glycerol at 80 °C the selected isolate was allowed to grow in a starch nitrate broth in a purpose to get a clear supernatant for antimicrobial activity.

2.2. Test organisms

2.2.1. Bacteria

i. Gram-positive bacteria: *Staphylococcus aureus*, NCTC 7447; *Bacillus subtilis*, NCTC 1040; *B. pumilus*, NCTC 8214 and *Micrococcus luteus*, ATCC 9341.
ii. Gram-negative bacteria: *Escherichia coli*, NCTC 10416; *Klebsiella pneumonia*, NCIMB 9111 and *Pseudomonas aeruginosa*, ATCC 10145.

2.2.2. Fungi

i. Unicellular fungi: *Candida albicans*, IMRU 3669 and *Saccharomyces cervisiae*, ATCC 9763.
ii. Filamentous fungi:*Aspergillus niger*, IMI 31276; *A. flavus*, IMI111023; *A. fumigatous*, ATCC 16424; *Fusarium oxysporum; Rhizoctonia solani; Alternaria alternate; Botrytis fabae* and *Penicillium chrysogenium*.

2.3. Screening for antimicrobial activity

The antimicrobial activity was determined by cup method assay according to Kavanagh (1972).

2.4. Taxonomic studies of actinomycete isolate

Morphological characteristics of the most potent produce strain T-4 grown on starch nitrate agar medium at 35 °C for 5 days was examined under scanning electron microscopy (JEOL Technics Ltd.).

2.5. Physiological and biochemical characteristics

The ability of the strain to produce different enzymes was examined by using standard methods. Lecithinase was conducted on egg-yolk medium according to the method of Nitsh and Kutzner (1969); lipase (Elwan et al., 1977); protease (Chapman, 1952); pectinase according to the method of Hankin et al. (1971); α-amylase according to the method of Cowan (1974) and catalase test according to the method of Jones (1949). Melanin pigment according to the method of Pridham et al. (1957). Degradation of esculin and xanthine according to the method of Gordon et al. (1974). Nitrate reduction according to the method of Gordon (1966). Hydrogen sulfide production and oxidase test according to the method of Cowan (1974). The utilization of different carbon and nitrogen sources was according to the methods of Pridham and Gottlieb (1948). Cell wall was performed by the method of Becker et al. (1964) and Lechevalier and Lechevalier (1970). Cultural characteristics such as color of aerial mycelium, color of substrate mycelium and pigmentation of the selected actinomycete were recorded on ISP agar medium (Shirling and Gottlieb, 1966). Colors characteristics were assessed on the scale developed by Kenneth

and Deane (1955). In addition, the sensitivity of the strains to different antibiotics was determined by paper disk method (Cappuccino and Sherman, 2004).

2.6. DNA isolation and manipulation

The locally isolated actinomycete strain was grown for 5 days on a starch agar slant at 35 °C. Two milliliters of a spore suspension were inoculated into the starch–nitrate broth and incubated for 3 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).

2.7. 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′-ACAA GCCCTGGAAACGGGGT-3′, in accordance with the me thod 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). The 16S rDNA 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).

2.8. 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 evaluated using BioEdit software (Hall, 1999). The phylogenetic tree was displayed using the TREE VIEW program.

2.9. Factors effecting on the biosynthesis of the antimicrobial agent

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

2.9.1. Fermentation

The *Streptomyces torulosus*, T-4 inoculum was introduced aseptically into each sterile flask containing the following ingredients (g/l): glucose, 20; KNO_3, 2.0; K_2HPO_4, 0.8; $MgSO_4{\cdot}7H_2O$, 0.7 and KCl, 0.5. The pH was adjusted at 7.0 before sterilization. After 5 days of incubation at 35 °C. Filtration was carried out through cotton wool and followed by centrifugation at 5000 rpm for 15 min.

2.9.2. Extraction

The culture filtrates were extracted twice with n-Butanol and the pooled solvent extracts were evaporated to dryness under vacuum to yield a crude residue.

2.9.3. Precipitation

The precipitation process of the crude compound was carried out using petroleum ether (b.p. 60–80 °C) followed by centrifugation at 5000 rpm for 15 min.

2.9.4. Purification by TLC

Separation of the antimicrobial compound into its individual components was conducted by thin-layer chromatography using chloroform and methanol (24:1, v/v) as a solvent system.

2.10. Purification by column chromatography

The purification of the antimicrobial compound was carried out using silica gel column (2.5 × 50) chromatography, chloroform and methanol 10:2 (v/v) was used as an eluting solvent. The column was left overnight until the silica gel (Prolabo) was completely settled. One milliliter of crude extract to be fractionated was added on the silica gel column surface and the extract was adsorbed on top of silica gel. Fifty fractions were collected (each of 5 ml) and tested for their antimicrobial activities.

2.11. Physico-chemical properties

2.11.1. Elemental analysis

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

2.11.2. Spectroscopic analysis

The IR, UV and mass spectra were determined at the microanalytical center of Cairo University, Egypt.

2.11.3. Biological activity

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

2.11.4. Characterization of the antimicrobial agent

The antibiotic produced by *Streptomyces torulosus*, T-4 was identified according to the recommended international references of (Umezawa, 1977; Berdy, 1974, 1980a,b,c).

3. Results

3.1. Screening for the antimicrobial activities

Ninety-seven actinomycete strains were isolated from fifty soil samples collected from the Taif City, Kingdom of Saudi Arabia. Only one actinomycete culture T-4 was found exhibited to produce wide spectrum of antimicrobial activities (Gram-positive and Gram-negative bacteria and unicellular and filamentous fungi) (Table 1).

Table 1 Antimicrobial potentialities of the antibiotic-producing microorganisms isolated from various localities in Taif governorate.

Organism number	Mean values of inhibition zones (in mm) against												
	Bacteria						Fungi						
	Staphylococcus aureus, NCTC 7447	*Bacillus subtilis*, NCTC 1040	*Bacillus pumilus*, NCTC 8214	*Micrococcus luteus*, ATCC 9341	*E. coli* NCTC 10416	*Klebsiella pneumonia*, NCIMB 9111	*Pseudomonas aeruginosa*, ATCC 10145	*Candida albicans*, IMRU 3669	*S. cervicea* ATCC 9763	*A. niger*, IMI 31276	*A. fumigatus*	*A. flavus*, IMI 111023	*Fusarium oxysporum*	*P. chrysogenum*
T-4	24.0	22.0	22.5	22.0	22.0	21.0	20.0	22	23.0	30.0	28.0	29.0	27.0	25.0
T-5	25.0	24.0	23.0	25.5	0.0	0.0	0.0	–	0.0	0.0	0.0	0.0	0.0	0.0
T-6	20.0	18.0	18.0	18.0	17.0	15.0	12.0	18	18.0	30.0	27.0	26.0	25.0	23.0
T-7	13.0	12.0	12.0	0.0	0.0	0.0	0.0	–	0.0	0.0	0.0	0.0	0.0	0.0
T-16	21.0	20.0	20.0	22.0	20.0	19.0	16.0	15	15.0	0.0	0.0	0.0	0.0	0.0
T-18	0.0	0.0	0.0	–	0.0	0.0	0.0	–	0.0	30.0	27.0	26.0	25.0	23.0
T-19	19.0	22.0	21.0	22.0	22.0	17.0	0.0	18	19.0	25.0	22.0	23.0	21.0	19.0
T-27	23.0	21.0	20.0	20.0	21.0	18.0	16.0	19	20.0	30.0	27.0	28.0	26.0	25.0
T-30	30.0	30.0	29.5	27.0	–	0.0	0.0	0.0	–	0.0	0.0	0.0	–	0.0
T-32	20.0	21.0	21.0	20.0	18.0	15.0	0.0	–	0.0	0.0	0.0	0.0	0.0	0.0
T-35	18.0	17.0	17.0	17.0	13.0	0.0	0.0	–	0.0	0.0	0.0	0.0	0.0	0.0
T-72	14. 0	13.0	12.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
T-88	15.0	14.0	14.0	12.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

Plate 1 Scanning electron micrograph of the actinomycete isolate T-4 growing on starch–nitrate agar medium showing spore chain spiral shape and spore surfaces warty (25,000×).

3.2. Identification of the 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 and oatmeal agar medium (ISP-3). The spore chains were spiral, and had a warty surface (Plate 1). Neither both sclerotic granules and sporangia nor flagellated spores were observed.

3.3. Cell wall hydrolysate

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

3.4. Physiological and biochemical characteristics

The actinomycete isolate T-4 could hydrolyze starch, protein, and cellulose, whereas lipid, pectin, lecithin and catalase are negative. Melanin pigment is positive, degradation of xanthine, esculine, production of H_2S, nitrate reduction, decomposition of urea and utilization of citrate and KCN are positive. The isolate under study utilizes D-xylose, D-mannose, D-glucose, D-fructose, D-galactose, mannitol, *meso*-inositol, sucrose, rhamnose, L-arabinose, raffinose, starch and trehalose, but do not utilize lactose, maltose, and ribose. Good growth on L-glycine, L-asparagines, L-leucine L-histidine, L-phenyl alanine and L-lysine. No growth on L-valine, and L-methionine. On the other hand, the isolate T-4 decreased at high NaCl concentration above (5% w/v). The growth is not inhibited in the presence of phenol and 45 °C. The actinomycete isolate T-4 is not sensitive to Ampicillin (25 µg/ml), Nalidixic acid (30 µg/ml), Cefoperazone (75 µg/ml), and Fusidic acid (10 µg/ml), Gentamicin (10 µg/ml) and Kanamycin (30 µg/ml) (Table 2).

Table 2 The morphological, physiological and biochemical characteristics of the actinomycete isolate T-4.

Characteristic	Result	Characteristic	Result
Morphological characteristics		Mannitol	+ +
Spore chains	Spiral	L-Arabinose	+
Spore mass	Gray	*meso*-Insitol	+ +
Spore surface	Warty	Lactose	–
Color of substrate mycelium	Light brown–deep brown	Maltose	–
Diffusible pigment	Yellowish brown	Trehalose	+ +
Motility	Non-motile	D-Ribose	–
Cell wall hydrolysate		D-Fructose	+ +
Diaminopimelic acid (DAP)	LL-DAP	*Utilization of amino acids*	
Sugar pattern	Not detected	L-Glycine	+
Physiological and biochemical properties		L-Leucine	+
Hydrolysis of		L-Histidine	+
Starch	+	L-Phenylalanine	+
Protein	+	L-Asparagine	+
Lipid	–	L-Methionine	–
Pectin and lecithin	–	L-Lysine	+
Cellulose	+	L-Valine	–
Catalase test	–	*Growth with (% w/v)*	
Production of melanin pigment on		Sodium azide (0.01)	–
Peptone yeast- extract iron agar	+	Phenol (0.1)	+
Tyrosine agar medium	+	Thallous acetate (0.001)	–
Tryptone – yeast extract broth	–	*Growth at different temperatures (°C)*	
Degradation of		10	–
Xanthin	+	30–45	+ +
Esculin	+	50	±
H_2S Production	+	55	–
Nitrate reduction	+	*Growth at different pH values*	
Citrate utilization	+	6–8	+
Urea test	+	9	–
KCN test	+	*Growth at different concentration of NaCl (%)*	
Utilization of carbon sources		1–5	+
D-Xylose	+	7	–
D-Mannose	+	*Resistance to*	
D-Glucose	+ + +	Ampicillin (25 μg/ml) and	+
D-Galactose	+	Nalidixic acid (30 μg/ml)	+
Sucrose	+ +	Cefoperazone (75 μg/ml)	+
L-Rhamnose	+ +	Gentamicin (10 μg/ml)	+
Raffinose	+ +	Kanamycin (30 μg/ml)	+
Starch	+ + +	Fusidic acid (10 μg/ml)	+

+ = positive , – = negative , ± = doubtful results, , + + = moderate growth and + + + = good growth.

3.5. Color and culture characteristics

The isolate T-4 shows that the aerial mycelium is light gray; substrate mycelium is light brown, and the diffusible pigment is moderate yellowish brown or not produced diffusible (Table 3).

3.6. Taxonomy of actinomycete isolate, T-4

This was performed basically according to the recommended International Key's viz. Buchanan and Gibbons (1974) and Hensyl (1994) and numerical taxonomy of *Streptomyces* species program. On the basis of the previously collected data and in view of the comparative study of the recorded properties of T-4 in relation to the most closest reference strain, viz. *Streptomyces torulosus*, it could be stated that actinomycetes isolate, T-4 is suggestive of being likely belonging to *Streptomyces torulosus*, T-4 (Table 4).

3.6.1. Amplification of the 16S rDNA gene

The 16S rDNA gene was amplified by polymerase chain reaction (PCR) using the universal primers. The primers that was used to 16S rDNA 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.

3.6.2. 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., rather related to *Streptomyces* sp., rather than to *Streptomyces torulosus* (Fig. 1). Multiple sequence alignment was conducted by the sequences of the 16S rDNA gene of *Streptomyces torulosus*. Computer assisted DNA searches

Table 3 Culture characteristics of the actinomycete isolate T-4.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigments
1. Starch–nitrate agar medium	Good	L.gray264 – light gray	57-l.br – light brown	77-m.ybr – moderate yellowish brown
2. Tryptone yeast extract broth (ISP-1)	No growth	–	–	–
3. Yeast extract malt extract agar medium (ISP-2)	No growth	–	–	–
4. Oatmeal agar medium (ISP-3)	Good	L.gray264 – light gray	57-l.br – light brown	–
5. Glycero asparagine agar medium (ISP-4)	Poor	L.gray264 – light gray	57-l.br – light brown	–
6. Inorganic salts starch agar medium (ISP-5) –		Moderate	L.gray264 – light gray	86-l. yellow – light yellow
7. Peptone yeast extract–iron agar medium (ISP-6) 59-d.br – deep brown		Moderate	L.gray264 – light gray	57-l.br – light brown
8. Tyrosine agar medium (ISP-7) 59-d.br – deep brown		Moderate	L.gray264 – light gray	57-l.br – light 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 characteristic properties of T-4 in relation to reference strain, *Streptomyces torulosus* (Williams et al., 1989, p. 2448 and Table 29-12).

Characteristics	T-4	*Streptomyces torulosus*
Morphological characteristics		
Spore mass	Gray	Gray
Reverse color	Light yellow/light brown	Light yellow
Spore chain	Spiral	Spiral
Spore surface	Warty	Warty and spiny
Motility	Non-motile	Non-motile
Cell wall hydrolysate		
Diaminopimelic acid (DAP)	LL-DAP	LL-DAP
Sugar pattern	Not detected	Not detected
Melanin pigment	+	+
Utilization of carbon sources		
L-Arabinose	+	+
D-Fructose	+	+
D-Galactose	+	+
D-Glucose	+	+
Meso-Inositol	+	+
D-Mannitol	+	+
Raffinose	+	+
Sucrose	+	ND
D-Xylose	+	+

ND = no data.

against bacterial database similarly revealed that the 16S rDNA sequence was 98% identical *Streptomyces torulosus* (Fig. 1).

3.7. Factors effecting on the biosynthesis of the antimicrobial agent

The maximum inhibition zones of produced antibiotic against tested microorganisms reached up to 30.0, 28.0, 27.0, 25.0 and 23.0 in case of *A. niger*, IMI 31276; *Staphylococcus aureus*, NCTC 7447, *Bacillus subtilis*, NCTC 1040, *C. albicans*, IMRU 3669 and *Klepseilla pneumonia*, NCIMB, 9111, respectively, at an inoculum size of four (disks per 100 ml media). The level of antibiotic yield increased gradually with increasing the incubation period up to the end of 5 days, after these maximum values 30.0, 28.0, 27.0, 25.0 and 23.0 in case of *A. niger*, IMI 31276; *Staphylococcus aureus*, NCTC 7447; *B. subtilis*, NCTC 1040, *C. albicans* IMRU 3669 and *K. pneumonia*, NCIMB 9111, respectively. The optimum temperature capable of promoting antimicrobial agents biosynthesis was at 35°C, whereas, the diameter of inhibition zone resulted from antimicrobial agent productivity reached up to 31.0, 29.0, 28.0, 25.5 and 24.0 in case of *A. niger*, IMI 31276; *Staphylococcus aureus*, NCTC 7447; *B. subtilis*, NCTC 1040; *C. albicans*, IMRU 3669 and *K. pneumonia*, NCIMB 9111, respectively. The optimum initial pH value capable of promoting antimicrobial agent was found to be at the value of 7.0 since the diameter of inhibition zone resulted from antimicrobial agents productivity reached up to 31.0, 29.0, 28.0, 25.5 and 24.0 in case of *A. niger*, IMI 31276; *Staphylococcus aureus*, NCTC 7447; *B. subtilis*, NCTC 1040; *C. albicans*, IMRU 3669 and *K. pneumonia*, NCIMB 9111, respectively. The effect of the used carbon sources in the production of antimicrobial agent could be arranged in the following descending manner; for *Streptomyces torulosus*, KH-4, glucose > starch > mannitol > sucrose > fructose > Arabinose > D-mannose > D-galactose > xylose > raffinose > Rhamnose.

3.8. Fermentation, extraction and purification

The fermentation process was carried out for five days at 35 °C. After incubation period, the filtration was conducted followed by centrifugation at 4000 rpm for 15 min. The entire

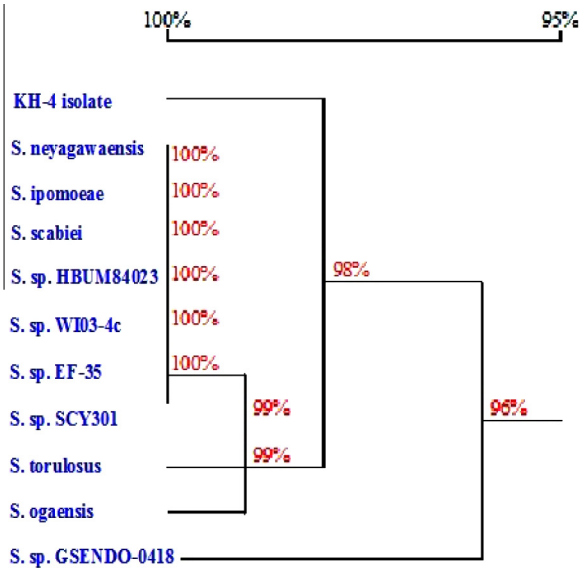

Figure 1 The phylogenetic position of the local *Streptomyces* sp. strain among neighboring species. The phylogenetic tree was based on the pairwise comparisons of 16S rDNA sequences.

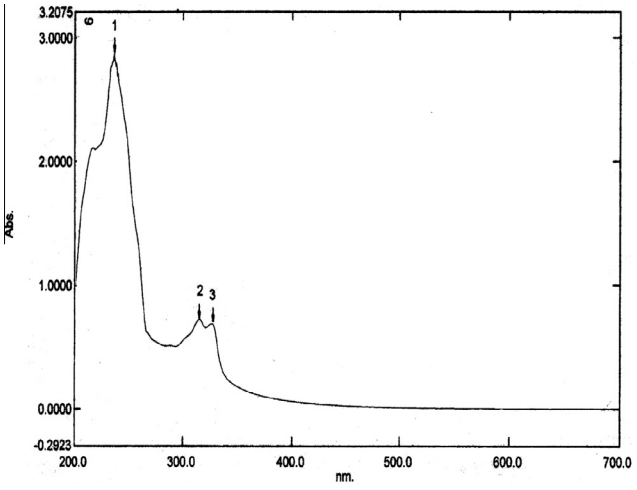

Figure 3 Ultraviolet absorbance of antimicrobial agent produced by *Streptomyces torulosus*, T-4.

culture broth (20 l) was centrifuged (4000 rpm, 15 min) 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. Its color is yellowish. Separation of antimicrobial agent into individual components was carried out by thin-layer chromatography using a solvent system composed of chloroform and methanol (24:1, v/v). Only one band at $R_f = 0.55$ showed antimicrobial activity. The purification process through column chromatography packed with silica gel revealed that the most active fractions against the tested organisms ranged between 14 and 23.

3.9. Physico-chemical characteristics

The purified antimicrobial agent produced by *Streptomyces torulosus*, T-4 produces characteristic odor, their melting points are 235 °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.

3.10. Elemental analysis

The elemental analytical data of the antimicrobial agent produced by *Streptomyces torulosus*, T-4 showed the following:

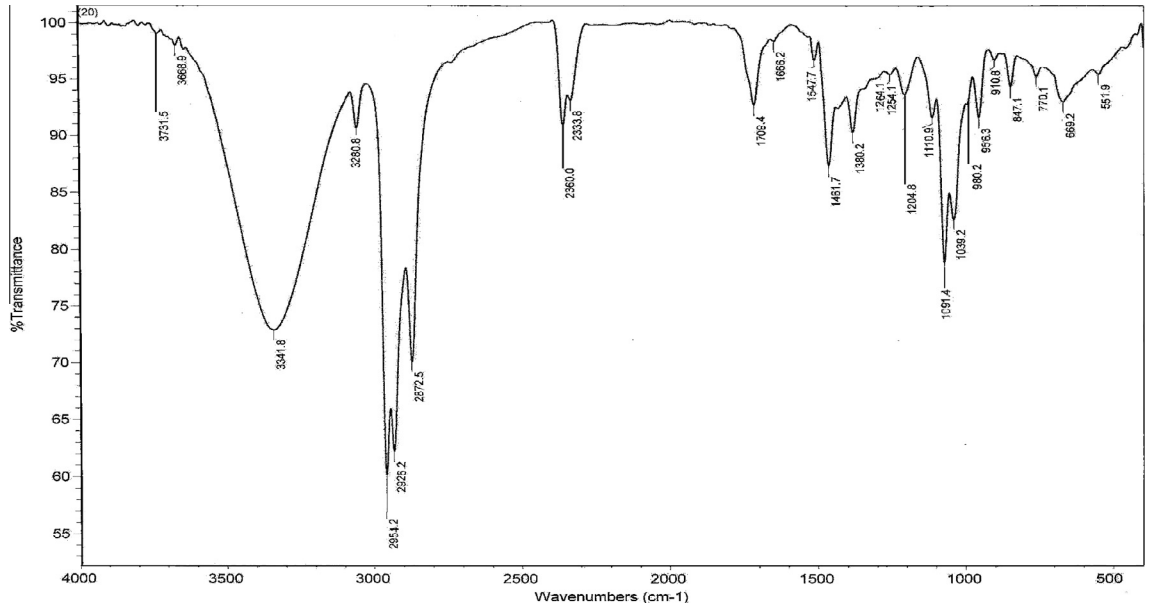

Figure 2 I.R. spectrum of antimicrobial agent produced by *Streptomyces torulosus*, T-4.

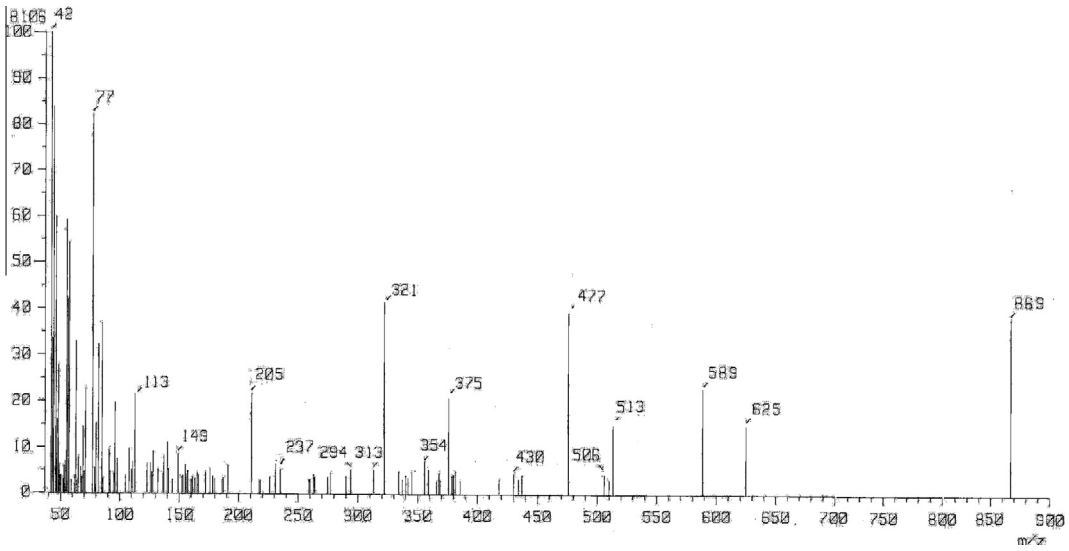

Figure 4 Mass spectrum of antimicrobial agent produced by *Streptomyces torulosus*, T-4.

Table 5 A comparative study of the characteristic properties of the antimicrobial agent produced by *Streptomyces torulosus*, T-4 in relation to reference antibiotic (tunicamycin).

Character	purified antimicrobial agent	Tunicamycin
1. Melting point	235 °C	234–235 °C
2. Molecular weight	865	865.4
Chemical analysis		
C	53.30	53.31
H	6.87	6.86
N	6.61	6.61
O	29.51	29.51
S	0.0	0.0
3. Ultraviolet	260	205 and 260
4. Formula	$C_{38}H_{62}N_4O_{16}$	$C_{38}H_{62}N_4O_{16}$
5. Active against	Active against Gram-positive and Gram-negative bacteria and unicellular and filamentous fungi	Active against Gram-positive and Gram-negative bacteria and unicellular and filamentous fungi

C = 53.30; H = 6.87; N = 6.61; O = 29.51 and S = 0.0. This analysis indicates a suggested empirical formula of $C_{38}H_{62}N_4O_{16}$.

3.11. Spectroscopic characteristics

The spectroscopic analysis of the purified of antimicrobial compound produced by *Streptomyces torulosus*, T-4, the infrared (IR) spectrum showed characteristic band corresponding to 26 peaks 551.9, 669.2, 770.1, 847.1, 910.8, 956.3, 980.2, 1039.2, 1091.4, 1110.9, 1204.8, 1254.1, 1264.1, 1380.2, 1461.7, 1547.7, 1666.2, 1708.4, 2333.8, 2306.0, 2872.5, 2954.2, 3280.8, 3341.8, 3668.9 and 3731.5 (Fig. 2). The ultraviolet (UV) absorption spectrum is recorded a maximum absorption peak at 260 nm (Fig. 3). The Mass spectrum revealed that the molecular weight is 865 (Fig. 4).

3.12. Biological activities of the antimicrobial agent

Data of the antimicrobial agent spectrum indicated that the agent is active against Gram-positive and Gram-negative bacterial and unicellular and filamentous fungi (Table 5).

3.13. Identification of the antimicrobial agent

On the basis of the recommended keys for the identification of antibiotics and in view of the comparative study of the recorded properties of the antimicrobial agent, it could be stated that the antimicrobial compound is suggestive of being belonging to tunicamycin antibiotic (Umezawa, 1977; Berdy, 1974, 1980a,b,c; Tsvetanova et al., 2002) (Table 6).

4. Discussion

The increase in the frequency of multi-resistant pathogenic bacteria has created an urgent demand in the pharmaceutical industry for more rational approaches and strategies to the screening of new antibiotics with a broad spectrum of activity, which resist the inactivation processes exploited by the microbial enzymes (Motta et al., 2004). Ninety-seven actinomycete strains were isolated from fifty soil samples collected from the Taif City, Kingdom of Saudi Arabia. Only one actinomycete culture T-4 was found exhibited to produce wide spectrum antimicrobial activities. Identification process has been carried out according to (Hensyl, 1994; 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 mass is light gray; while spore surface is warty, substrate mycelium is light yellowish brown and no diffusible pigment was produced on ISP-media. The results of physiological, biochemical characteristics and cell wall hydrolysate of actinomycetes isolate, exhibited that the cell wall containing LL-diaminopim-

Table 6 Antimicrobial spectrum of the antimicrobial agent(s) by adding paper disk diffusion method (Kavanagh, 1972).

Test organisms	MIC (μg/ml) concentration of antimicrobial agent produced by *Streptomyces torulosus*, T-4
A. Bacteria	
a. Gram-positive cocci	
Staphylococcus aureus, NCTC 7447	52.7
Micrococcus luteus, ATCC 9341	52.7
b. Gram-positive bacilli	
Bacillus subtilis, NCTC 10400	73.78
Bacillus pumilus, NCTC 8214	73.78
c. Gram-negative bacteria	
Escherichia coli, NCTC 10416	73.78
Klebsiella pneumonia, NCIMB 9111	100 <
Pseudomonas aeruginosa, ATCC 10145	100 <
B. Fungi	
a. Unicellular fungi	
Candida albicans, IMRU 3669	73.78
Saccharomyces cervisiae ATCC 9763	46.9
b. Filamentous fungi	
Aspergillus niger, IMI 31276	15.73
Aspergillus fumigatus, ATCC 16424	31.25
Aspergillus flavus, IMI 111023	22.32
Fusarium oxyspoum	46.9
Rhizoctonia solani	52.7
Alternaria alternata	46.9
Botrytis fabae	46.9
Penicillium chrysogenium	52.7

elic acid (DAP) and sugar pattern of cell wall hydrolysate could not be detected. These results emphasized that the actinomycetes isolate is related to a group of *Streptomyces*. In view of all the previously recorded data, the identification of actinomycete isolate T-4 was suggestive of being belonging to *Streptomyces torulosus*, T-4. The resulted sequence was aligned with available almost complete sequence of type of strains of family streptomycetaeae. The phylogenetic tree (diagram) revealed that the local isolate is closely related to *Streptomyces sp* rather than to *Streptomyces torulosus* by a similarity matrix is 98%.

Maximum antimicrobial activity biosynthesis could be recorded that a different inoculum sizes for four disks; incubation period for five days (Adinarayana et al., 2002); pH 7.0 (Atta, 2010); temperature 35 °C (Kunnari et al., 1997; Atta, 1999); glucose best carbon source (Hoshino et al., 2004); KNO_3 best nitrogen source (Hosokawa et al., 1996; Khalifa, 2008; Atta et al., 2011).

The active metabolites were extracted by n-Butanol at pH 7.0 (Atta, 2010; Atta et al. 2011). 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 antimicrobial activity. Separation of antibiotic 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; Atta et al., 2009). The band with an R_f value at 0.55 which indicated the presence of one compound (Atta, 2010). For the purpose of purification process, the antibiotics 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 14 and 23. 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; Sekiguchi et al., 2007).

The physico-chemical characteristics of the purified antibiotic revealed that, melting point at 235 °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; similar results were recorded by Mellouli et al. (2003), El-Tayeb et al. (2004) and Atta (2010).

A study of the elemental analysis of the antibiotic showed the following C = 53.30; H = 6.87; N = 6.61; O = 29.51 and S = 0.0 lead to an empirical formula of $C_{38}H_{62}N_4O_{16}$. The spectroscopic characteristics of antibiotic revealed the presence of the maximum absorption peak in UV at 260 nm, infrared absorption spectrum showed characteristic band corresponding to 26 peaks 551.9, 669.2, 770.1, 847.1, 910.8, 956.3, 980.2, 1039.2, 1091.4, 1110.9, 1204.8, 1254.1, 1264.1, 1380.2, 1461.7, 1547.7, 1666.2, 1708.4, 2333.8, 2306.0, 2872.5, 2954.2, 3280.8, 3341.8, 3668.9 and 3731.5. Mass spectrum showed that the molecular weight is 865 (Tsvetanova et al., 2002). The MIC of antibiotic under study exhibited fairly active against both Gram-positive and Gram-negative bacteria and unicellular and filamentous fungi. The MIC of antibiotic was determined and the results showed that the minimum inhibitory concentration (MIC) of the antibiotic produced by *Streptomyces torulosus*, T-4 against *Staphylococcus aureus*, NCTC 7447 was 52.7 μg/ml, *Micrococcus lutea*, ATCC 9341 was 52.7 μg/ml, and *B. subtilis*, NCTC 10400 was 73.78 μg/ml, *B. pumilus*, NCTC 8214 was 73.78 μg/ml, *K. pneumonia*, NCIMB 9111, was > 100 μg/ml, *E. coli*, NCTC 10416 was 73.78 μg/ml, and *Pseudomonas aeruginosa*, ATCC 10145 was > 100 μg/ml, for *A. flavus*, IMI 111023 was 31.25 μg/ml and *Saccharomyces cerevisiae*, ATCC 9763 was 46.9 μg/ml, *C. albicans*, IMRU 3669 was 73.78 μg/ml, *A. niger*, IMI 31276 was 15.73 μg/ml, *A. fumigatus*, ATCC 16424 was 31.25 μg/ml, *A. flavus*, IMI 111023 was 22.32 μg/ml, *F. oxysporum* was 46.9 μg/ml, *R. solani* was 52.7 μg/ml, *A. alternata* was 46.9 μg/ml, *B. fabae* was 46.9 μg/ml, *Penicillium chrysogenium* was 52.7 μg/ml. Similar investigations and results were attained by Imnagaki et al. (1998), Sekiguchi et al. (2007) and Atta et al. (2009). Identifi-

cation of antibiotic according to recommended international keys indicated that the antibiotic is suggestive of being belonging to tunicamycin antibiotic (Umezawa, 1977; Berdy, 1974, 1980a,b,c; Tsvetanova et al., 2002).

5. Conclusion

The present study shows the present data focusing on obtaining microbial local isolates which have the ability to produce antimicrobial agent against pathogenic microorganisms (Gram-positive and Gram-negative bacteria and unicellular and filamentous fungi).

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