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Biotechnological Application for Producing Some Antimicrobial Agents by Actinomycetes Isolates from Al-khurmah Governorate.

[1]H.M. Atta, [1]R.. Bayoumi [2]M. El-Sehrawi, [3]A. Aboshady and [3]A. Al-Humiany

[1]Botany and Microbiology Department, Faculty of Science (Boys), Al-Azhar University, Cairo, Egypt
Biotechnology Department. Faculty of Science and Education- Al-Khurmah, Taif University; KSA
[2]Biotechnology Department, Faculty of Science, Taif University,
[3]College of Applied Health Sciences, Tarabah, KSA

Abstract: This work was carried out in the course of a screening program for specifying the bioactive substances that demonstrated inhibitory affects against microbial pathogenic, from actinomycetes strains. Ninety-seven actinomycete strains were isolated from fifty soil samples collected from Al-Khurmah governorate, kingdom of Saudi Arabia. Only one actinomycete culture KH-4 from thirteen cultures was found exhibited to produce wide spectrum antimicrobial activities. The nucleotide sequence of the 16s RNA gene (1.5 Kb) of the most potent strain evidenced an 98% similarity with *Streptomyces torulosus*. From the taxonomic features, the actinomycetes isolate KH-4 matches with *Streptomyces torulosus* in the morphological, physiological and biochemical characters. Thus, it was given the suggested name *Streptomyces torulosus*, KH-4. The parameters controlling the biosynthetic process of antimicrobial agent formation including: different inoculum size, pH values, temperatures, incubation period and different carbon and nitrogen sources were fully investigates.

Key words: Actinomycetes · *Streptomyces torulosus* · 16s RNA · Taxonomy · Physiological activities · Antimicrobial activity

INTRODUCTION

Microbial natural products are the origin of most of the antibiotics in the market today. There is an alarming scarcity of new antibiotics currently under development in the pharmaceutical industry [1]. Still, microbial natural products remain the most promising source of novel antibiotics, although new approaches are required to improve the efficiency of the discovery process [2]. Actinomycetes have provided important bioactive compounds of high commercial value and continue to be routinely screened for new bioactive substances [3]. These searches have been remarkably successful and approximately two-thirds of naturally occurring antibiotics, including many of medical importance, have been isolated from actinomycetes [4]. The actinomycetes are noteworthy as antibiotic producers, making three quarters of all known products; the *Streptomyces* are, especially prolific and can produce a great many antibiotics and other class of biologically active secondary metabolites [5]. They cover around 80% of total antibiotic product, with other genera trailing numerically; *Micromospora* is the runner up with less than one-tenth as many as Streptomyces. If we include secondary metabolites with biological activities other than antimicrobial, actinomycetes are still out in front, over 60%; *Streptomyces* spp. accounting for 80% of these [6].

Streptomyces sp. are widely recognized as industrially important organisms for their ability to elaborate different kinds of novel secondary metabolites [7]. *Streptomyces* sp. are prolific producers of useful bioactive compounds [8], providing 75-80% of the naturally occurring antibiotics discovered till date. They are known to elaborate a wide diversity of natural products including antibiotics, antifungal agents, plant growth factors, enzymes and enzyme inhibitors, antiparasitic, anticancer and immunomodulating agents [9]. *Streptomyces* sp. or strains with novel antibiotics may still exist in nature [10]. They are Gram positive bacteria with distinct features such as high DNA G+C content, presence of LL-Diaminopimelic acid (LL-DAP) and the absence of characteristic sugars in the cell wall [11].

Corresponding Author: Houssam M. Atta, Department of Botany and Microbiology, Faculty of Science (Boys), Al-Azhar University, Cairo, Egypt; and Biotechnology. Faculty of Science and Education- Al-Khurmah, Taif University, KSA. E-mail: houssamatta@yahoo.com

They also produce extensively branched substrate and aerial mycelia [12]. They produce a wide variety of various bioactive compounds, such as antibiotics, enzymes, anticancer and agroactive compounds [13].

The present study described the isolation of an actinomycete strain from Al-Khurmah governorate soil, The identification of this strain, b ased on the cultural, morphology, physiology and biochemical characteristics, as well as 16s rRNA methodology. The primary bioactive substances were tested against Gram positive and Gram negative bacteria and unicellular and filamentous fungi.

MATERIALS AND METHODS

Actinomycete Isolate: The actinomycete KH-4 was isolated from soil sample collected from Al-Khurmah governorate. It was purified using the soil dilution plate technique [14].

Screening for Antimicrobial Activity: The antimicrobial activity was determined by Agar well method assay [15].

Test Organisms:

Bacteria: *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.

Fungi: *Candida albicans*, IMRU 3669; *Saccharomyces cervisiae* ATCC 9763; *Aspergillus flavus*, IMI 111023, *Aspergillus fumigatous*, ATCC 16424; *Fusarium oxysporum*.

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

Physiological and biochemical characteristics: Lecithinase [16]; Lipase[17]; Protease [18]; Pectinase [19]; á-amylase [20] and Catalase test [21]. Melanin pigment [22] were carried out using standard methods. Degradation of both esculin and xanthine [23]. Nitrate reduction [24]. Hydrogen sulphide production and oxidase test [20] were also investigated. The utilization of different carbon and nitrogen sources [25]. Cell wall hydrolysate was performed [26, 27]. The cultural characteristics were studied in accordance with the guidelines established by the International *Streptomyces* Project [28]. Colors characteristics were assessed on the scale developed [29].

DNA Isolation and Manipulation: The locally isolated actinomycete strain was grown for 5 days on a starch agar slant at 35°C. Two ml 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 as previously described [30].

Amplification and Sequencing of the 16s RRNA Gene: PCR amplification of the 16S rRNA gene of the local actinomycete strain was conducted using two primers, StrepF; 5.-ACGTGTGCAGCCCAAGACA-3. and Strep R; 5.ACAAGCCCTGGAAACGGGGT-3 [31]. 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 electrophoresis and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The 16S rRNA gene was sequenced on both strands via the dideoxy chain termination method [32]. The 16S rRNA gene (1.5 kb) sequence of the PCR product was acquired using a Terminator Cycle Sequencing kit (ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA).

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 evaluate using BioEdit software [33].

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 determine by the standard methods.

RESULTS

Screening for the Antimicrobial Activities: One of the actinomycete cultures KH-4 from thirteen cultures were found exhibited various degrees of activities against 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 Al-Khurmah governorate

	* Mean values of inhibition zones (in mm) against													
	Bacteria							Fungi						
*Organism number	S. 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	Asp. niger, IMI 31276	Asp. fumigatus	Asp. flavus, IMI 111023	Fusarium oxysporum	P. chrysogenum
KH-4	24.0	22.0	22.5	22.0	22.0	21.0	20.0	022.	23.0	30.0	28.0	29.0	27.0	25.0
KH-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	0.0
KH-6	20.0	18.0	18.0	18.0	17.0	15.0	12.0	018.	18.0	30.0	27.0	26.0	25.0	23.0
KH-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	0.0
KH-16	21.0	20.0	20.0	22.0	20.0	19.0	16.0	015.	15.0	0.0	0.0	0.0	0.0	0.0
KH-18	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	30.0	27.0	26.0	25.0	23.0
KH-19	19.0	22.0	21.0	22.0	22.0	17.0	0.0	018.	19.0	25.0	22.0	23.0	21.0	19.0
KH-27	23.0	21.0	20.0	20.0	21.0	18.0	16.0	019.	20.0	30.0	27.0	28.0	26.0	25.0
KH-30	30.0	30.0	29.5	27.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
KH-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	0.0
KH-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	0.0
KH-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
KH-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

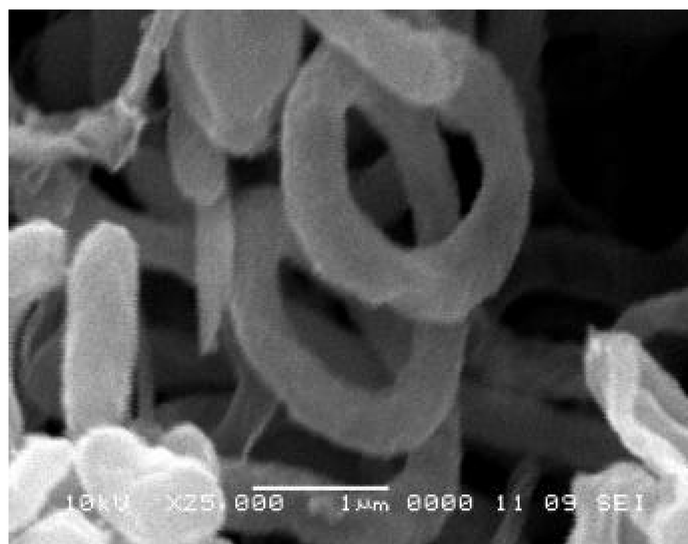

Plate 1: Scanning electron micrograph of the actinomycete isolate KH-4 growing on starch nitrate agar medium showing spore chain Spiral shape and spore surfaces warty (X25,000)

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.

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 KH-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

Table 2: The morphological, physiological and biochemical characteristics of the actinomycete isolate KH-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:Hydrolysis of:	L-Leucine	+	
	L-Histidine	+	
Starch	+	L-Phenylalanine	+
Protein	+	L-Asparagine	+
Lipid	-	L-Methionine	-
Pectin & 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	±
H2S 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 (25ug/ml) and	+
D- Galactose	+	Nalidixic acid (30 ug/ml)	+
Sucrose	++	Cefoperazone (75ug/ml)	+
L-Rhamnose	++	Gentamicin (10 ug/ml)	+
Raffinose	++	Kanamycin (30ug/ml)	+
Starch	+++	Fusidic acid (10 ug/ml)	+

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

Table 3: Culture characteristics of the actinomycete isolate KH-4.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigments
1-Starch- nitrate agar medium	Good	-L .Gray264Light gray	57-1.brlight brown	77-m.ybrmoderate 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 .Gray264Light gray	57-1.brlight brown	-
5- Glycero Asparagine agar medium (ISP-4)	Poor	-L .Gray264Light gray	57-1.brlight brown	-
6- Inorganic salts starch agar medium (ISP-5)	moderate	-L .Gray264Light gray	86-I. yellowLight yellow	-
7-Peptone yeast extract- iron agar medium (ISP-6)	moderate	-L .Gray264Light gray	57-1.brlight brown	59-d.BrDeep brown
8-Tyrosine agar medium (ISP-7)	moderate	-L .Gray264Light gray	57-1.brlight brown	59-d.BrDeep 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 KH-4 in relation to reference strain, Streptomyces torulosus. (C.F. Bergey's 1989, page 2448 & table 29-12)

Characteristics	KH-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.

alanine and L-lysine. No growth on L- valine and L-methionine. Growth in the presence of up to (5 %) NaCl. The growth is not inhibited in the presence of phenol and 45°C. The actinomycete isolate KH-4 not sensitive to Ampicillin (25ug/ml) Nalidixic acid (30 ug/ml) Cefoperazone (75ug/ml) and Fusidic acid (10 ug/ml, Gentamicin (10 ug/ml) and Kanamycin (30 ug/ml) (Table 2).

Color and Culture Characteristics: The isolate KH-4 shows the aerial mycelium is light gray; substrate mycelium is light brown and the diffusible pigment moderate yellowish brown or not produced diffusible (Table 3).

Taxonomy of Actinomycete Isolate, KH-4: This was performed basically according to the recommended international Key's viz. 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 KH-4 in relation to the most closest reference strain, viz. *Streptomyces torulosus*, it could be stated that actinomycetes isolate, KH-4 is suggestive of being likely belonging to *Streptomyces torulosus*, KH-4 (Table 4).

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'-

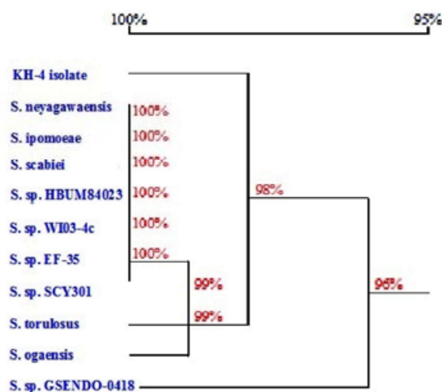

Fig. 1: The phylogenetic position of the local *Streptomyces* sp. strain among neighboring species. The phylogenetic tree was based on the pairwise comparisons of 16_S rDNA sequences

ACAAGCCCTGGAAACGGGGT-3', the product of the PCR was analyzed on 1.5% ethidium bromide gel.

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 related to *Streptomyces* sp. rather than to *Streptomyces torulosus* (Fig. 1). Multiple sequence alignment was conducted the sequences of the 16_S rDNA gene of

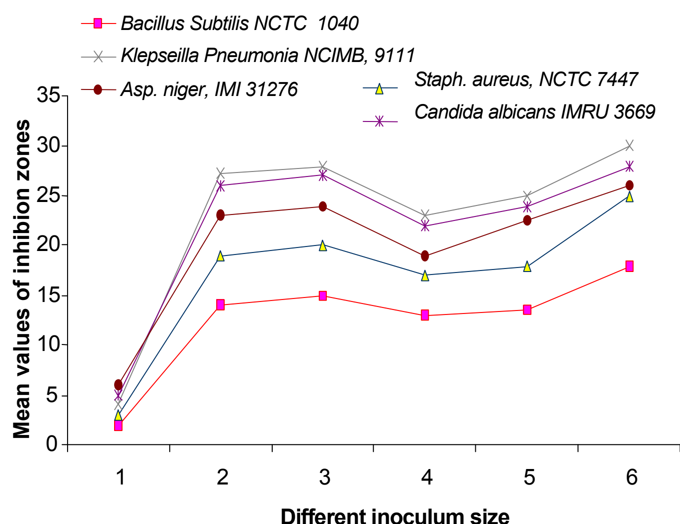

Fig. 2: Effect of different inoculum size on the antimicrobial agent(s) biosynthesis produced by *Streptomyces torulosus.* KH-4.

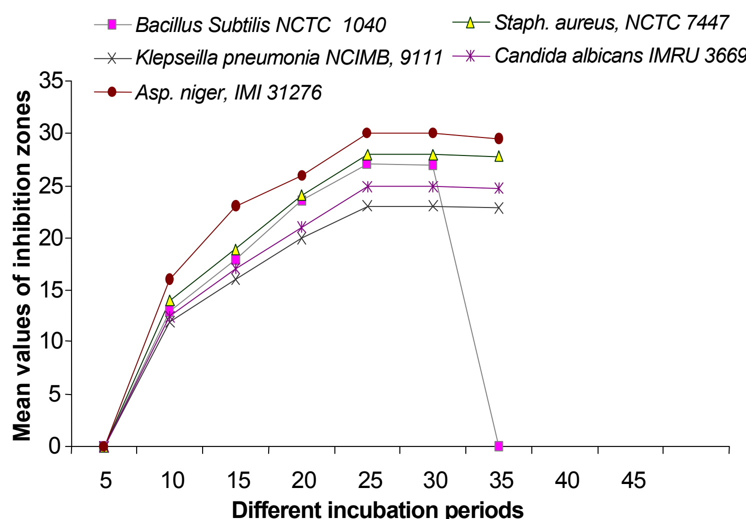

Fig. 3: Effect of different incubation periods on the antimicrobial agent(s) biosynthesis produced by *Streptomyces torulosus.* KH-4.

Streptomyces torulosus. Computer assisted DNA searches against bacterial database similarly revealed that the 16_S rDNA sequence was 98% identical *Streptomyces torulosus* (Fig. 1).

Factors Effecting on the Biosynthesis of the Antimicrobial Agent Produced by *Streptomyces Torulosus,* KH-4

Effect of Different Inoculum Size: From the results represented graphically in (Fig. 2) that, 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 *Asp. niger*, IMI 31276; *Staph. aureus,* NCTC 7447, *Bacillus subtilis* NCTC 1040, *Candida albicans* IMRU 3669 and *Klepseilla pneumonia* NCIMB, 9111, respectivily at an inoculum size of four (discs per 100 ml media).

Effect of Different Incubation Periods: Data illustrated graphically in (Fig. 3) showed the relation between antibiotic productivity and time of incubation. The level of antibiotic yield increased gradually with increasing the incubation period up to the end of 5 days, after this maximum values 30.0, 28.0, 27.0, 25.0 and 23.0 in case of *Asp. niger*, IMI 31276; *Staph. aureus, NCTC 7447, Bacillus subtilis* NCTC 1040*, Candida albicans* IMRU

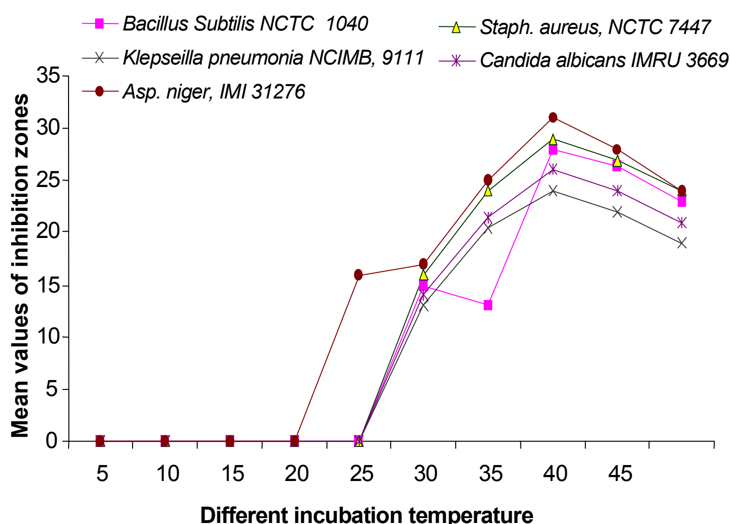

Fig. 4: Effect of different incubation temperature on the antimicrobial agent(s) biosynthesis produced by *Streptomyces torulosus.* KH-4.

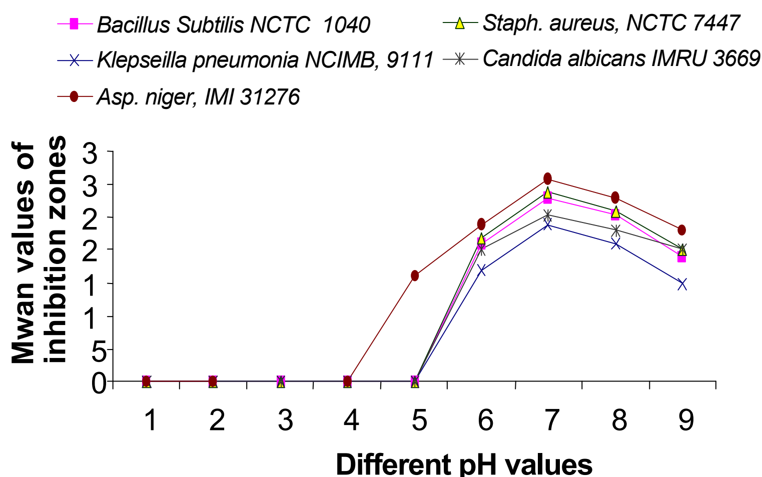

Fig. 5: Effect of different pH values on the antimicrobial agent(s) biosynthesis produced by *Streptomyces torulosus.* KH-4.

3669 and *Klepseilla pneumonia* NCIMB, 9111, respectivily at an, a steadness of antimicrobial agents productivity was observed.

Effect of Different Incubation Temperature (°C): Data represented graphically in (Fig. 4) showed that, the optimum temperature capable of promoting antimicrobial agents biosynthesis by *Streptomyces torulosus*, KH-4 was at 35°C, whereas, 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 *Asp. niger*, IMI 31276; *Staph. aureus,* NCTC 7447, *Bacillus subtilis* NCTC 1040, *Candida albicans* IMRU 3669 and *Klepseilla pneumonia* NCIMB, 9111, respectivily.

Effect of Different PH Values: The results represented graphically in (Fig. 5) that, the optimum initial pH value capable of promoting antimicrobial agents biosynthesis by *Streptomyces torulosus*, KH-4 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 *Asp. niger*, IMI 31276; *Staph. aureus,* NCTC 7447, *Bacillus subtilis* NCTC 1040, *Candida albicans* IMRU 3669 and *Klepseilla pneumonia* NCIMB, 9111, respectivily.

Effect of Different Carbon Sources: Data given in (Fig. 6) indicated that the addition of different equimolecular carbon sources for production of antimicrobial agents

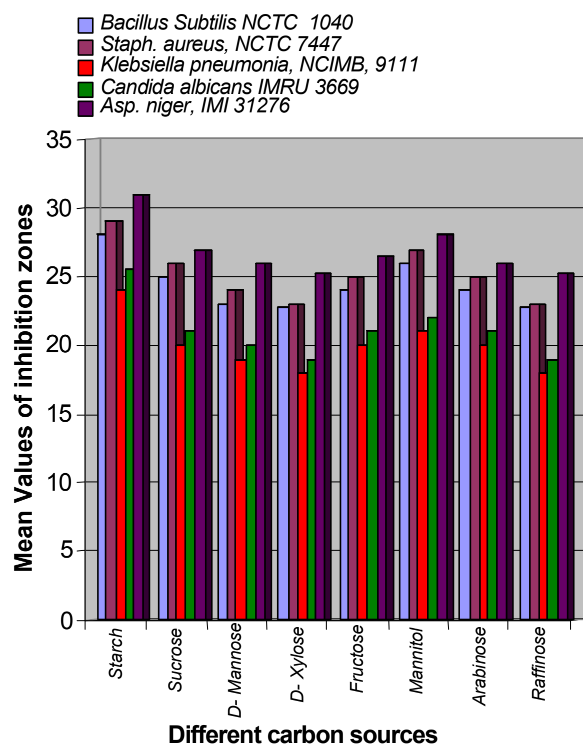

Fig. 6: Effect of different carbon sources on the antimicrobial agent(s) biosynthesis produced by *Streptomyces torulosus*. KH-4.

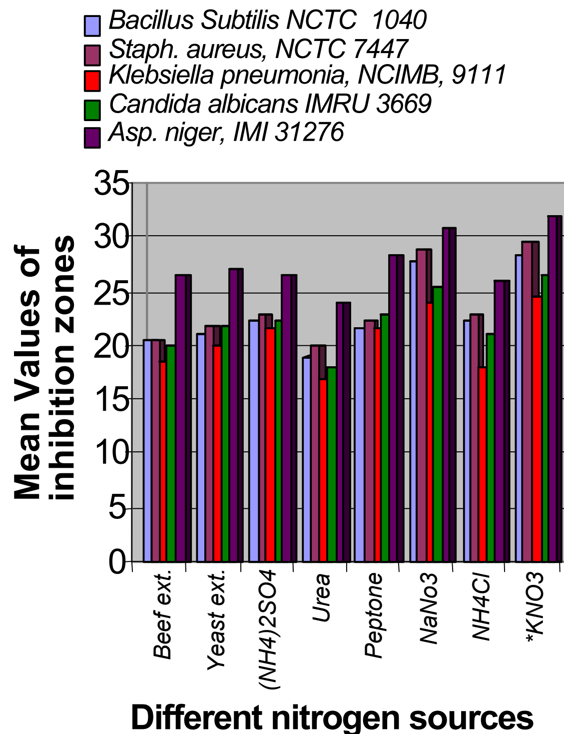

Fig. 7: Effect of different nitrogen sources on the antimicrobial agent(s) biosynthesis produced by *Streptomyces torulosus*. KH-4.

revealed that glucose is the best carbon source for biosynthesis antimicrobial substances. The effect of the used carbon sources in 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.

Effect of Different Nitrogen Sources: The nitrogen sources exhibited an increase in the level of antimicrobial agent production by *Streptomyces torulosus*, KH-4 where KNO_3 was found to be the best nitrogen source for the antimicrobial agent production (Fig. 7).

DISCUSSION

The *Streptomyces torulosus,* KH-4 was isolated from Al-Khurmah governorate. The isolate was growing on starch nitrate agar medium for investigating its potency to produce antimicrobial agents. The actinomycete isolate, KH-4 exhibited a wide spectrum antimicrobial agent [15]. Identification process has been carried out [34-36]. 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 No. 3, 4 & 5. The results of physiological, biochemical characteristics and cell wall hydrolysate of actinomycetes isolate, exhibited that the cell wall containing LL-diaminopimelic acid (DAP) and sugar pattern of cell wall hydrolysate could not detected [34 - 36]. These results emphasized that the actinomycetes isolate related to a group of *Streptomyces*. In view of all the previously recorded data, the identification of actinomycete isolate KH-4 was suggestive of being belonging to *Streptomyces torulosus,* KH-4. The resulted sequence was aligned with available almost compete sequence of type strains of family streptomycetaeae [36]. The phylogenetic tree (diagram) revealed that the local isolate is closely related *Streptomyces torulosus*, similarity matrix is 98%.

Maximum antimicrobial activity biosynthesis could be recorded that a different inoculum sizes for four discs; incubation period for five days [37]; pH 7.0 [38]; temperature 35°C [39, 40]; glucose best carbon source [41]; KNO_3 best nitrogen source [42, 43].

In conclusion, soil collected from different localities in Al- Khurmah governorate are virgin rich in actinomycetes strains that have activity against pathogenic microorganisms (Gram positive and Gram negative bacteria and unicellular and filamentous fungi) to humans, animals and plants, It may be possible to rely on these strains in the research outputs of the secondary metabolites of new use in the inhibition of microbial resistance to antibiotics such as objects of bacterial resistance to a group β-lactam antibiotics and others.

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