Production of Hygromycin-B antibiotic from *Streptomyces crystallinus*, AZ-A151: I. Isolation, Classification and phylogenetic analysis of 16S rDNA gene sequences

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Abstract: This work was carried out in the course of a screening program for specific actinomycetes bioactive substances that demonstrated inhibitory effects against some pathogenic strains. Different soil samples collected from various Egyptian localities e.g. (Assiut, Luxor and El-Minia governorates). Out of 44 soil samples collected from Assiut, Luxor and El-Minia governorates, 194 isolates of actinomycetes were isolated. The isolation of the actinomycete isolates was conducted by three different isolation media which included (starch nitrate agar medium, casein starch agar medium and glycerol asparagine agar medium). One of the actinomycete culture, symbol AZ-A151 from two cultures was found to produce a wide spectrum antimicrobial agent(s) against *Staphylococcus aureus*, NCTC 7447; *Escherichia coli*, NCTC 10416; *Klebsiella pneumonia*, NCIMB 9111; *Salmonella typhi*; *Saccharomyces cerevisiae,* ATCC 9763; *Aspergillus flavus*, IMI 111023; *Alternaria alternate* and *Fusarium verticillioides*. The nucleotide sequence of the 16s DNA gene (1.5 Kb) of the most potent strain evidenced an 92% similarity with *Streptomyces crystallinus*. From the taxonomic features, the actinomycetes isolate AZ-A151 matched with *Streptomyces crystallinus* in the morphological, physiological and biochemical characters. Thus, it was given the suggested name *Streptomyces crystallinus,* AZ-A151.

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Key words: *Streptomyces crystallinus*, Isolation, Classification and Phylogenetic analysis.

1. Introduction

Traditional methods of classification and identification of the organisms are based on morphological, physiological, biochemical developmental and nutritional characteristics. Standard references such as Bergey's Manual of Systematic Bacteriology or the Manual of Clinical Microbiology help in this type of study [Williams, 1989; Schreckenberger and von Graevenitz. 1999]. These traditional methods of bacterial identification have two major drawbacks. Firstly, this method cannot be applicable for noncultivable organisms such as Tropheryma whippelii. Secondly, occasionally biochemical characteristics of some organisms do not fit into patterns of any known genus and species [Woo *et al.,* 2000]. With the discovery of the polymerase chain reaction (PCR) and DNA sequencing methods, elucidation of closely related taxon with better authenticity has been made successfully in comparison to other conventional methods [Lane *et al.,* 1985; Bosshard *et al.,* 2003 and Clarridge, 2004]. Beside this, phylogenetic analysis of 16S rDNA gene is presently an important area of evolutionary study and sequence analysis. In addition to analyzing changes that have occurred during the evolution among different organisms, the evolution of a family of sequences may be studied. On the basis of the analysis, sequences that are most closely related can be identified by the places they occupy on neighboring branches of a tree. Phylogenetic analysis of a family of related gene sequences is a method to determine how the family might have been derived during evolution. The evolutionary relationships among the sequences are depicted by placing the sequences as outer branches on a tree. The branching relationships on the inner part of the tree reflect the degree to which different sequences are related. Two sequences that are very much alike will be located as neighboring outside branches and will be joined to a common branch beneath them. The object of phylogenetic analysis is to discover all of the branching relationships in the tree and the branch lengths. One of the most significant uses of phylogenetic analysis of sequences is to make predictions concerning the tree of life. For this purpose, 16S rDNA genes are selected as they are highly conserved in nature and universally present. At the same time it shows enough sequence variation to determine which groups of organisms share the same phylogenetic origin. Ideally, the 16S rDNA gene is also under selection & universal in distribution, meaning that as variation occurs in populations of organisms, certain sequences are not favored with a loss of the more primitive variation. A large number of

rRNA sequences from a variety of organisms were aligned and the secondary structure was deduced. Phylogenetic predictions were then made using the distance method [Woese and Fox, 1977]. On the basis of rDNA sequence signatures, or regions within the molecule that are conserved in one group of organisms but different in another [Woese and Fox, 1977], predicted that early life diverged into three main kingdoms- Archaea, Bacteria and Eukarya. Evidence for the presence of additional organisms in these groups has since been found by PCR amplification of environmental samples of RNA [Boettger, 1989 and Edwards *et al.,* 1989]. However, few studies so far have been reported on the use of rDNA sequencing for the identification of bacterial isolates in a more systematic fashion [Michel *et al.,* 2000 and Cloud *et al.,* 2002]. In the studied work, we are reporting the strain identification of our novel isolate ckm7 with the help of 16S rDNA gene sequence analysis. This partial sequencing of 16S rDNA gene was done from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh, India http:// www.mtcc.imtech.res.in). Pairwise sequence alignment of 16S rDNA gene sequence was performed to identify closely related homologs with the help of BLAST search tool available at NCBI webserver (http://www.nih.nov.ncbi).

In the present study were describe the isolation of an actinomycete strain AZ-A151 from Assiut governorate, 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 rRNA methodology. The primary bioactive substances were tested against Gram positive and Gram negative bacteria and unicellular and filamentous fungi.

2. Material and Methods

2.1. Actinomycete isolate

The actinomycete isolate AZ-A151 was isolated from soil sample collected from Assiut governorate. It was purified using the soil dilution plate technique described by [Williams and Davis, 1965].

2.2. Test organisms

2.2.1. Gram Positive: *Staphylococcus aureus*, NCTC 7447.

2.2.2. Gram Negative: *Escherichia coli*, NCTC 10416; *Klebsiella pneumonia*, NCIMB 9111; *Salmonella typhi*.

2.2.3. Unicellular fungi: *Saccharomyces cerevisiae,* ATCC 9763.

2.2.4. Filamentous fungi: *Aspergillus flavus*, IMI 111023; *Alternaria alternate* and *Fusarium verticillioides*.

2.3. Screening for antimicrobial activity

The anti- microbial activity was determined according to [Kavanagh, 1972].

2.4. Characterization studies of actinomycete isolate

2.4.1. Morphological characteristics

Morphological characteristics of aerial hyphae, spore mass, spore surface, color of aerial and substrate mycelia and soluble pigments production were conducted by growing the organism on ISP- media.

2.4.2. Physiological and biochemical characteristics

Lecithinase was detected using egg–yolk medium according to the method of [Nitsh and Kutzner, 1969]; Lipase (Elwan, *et al*., 1977); Protease [Chapman, 1952]; Pectinase [Hankin *et al*., 1971]; α-amylase [Ammar, *et al*., 1998] and Catalase Test [Jones, 1949]. Melanin pigment [Pridham, *et al.,* 1957]. Esculin broth and xanthine have been conducted according to [Gordon *et al*., 1974]. Nitrate reduction was performed according to the method of [Gordon, 1966]. Hydrogen sulphide production was carried out according to [Cowan, 1974]. The utilization of different carbon and nitrogen sources was carried out according to (Pridham and Gottlieb, 1948).

Determination of Diaminopimelic acid (DAP) and sugar pattern was carried out according to [Becker *et al*., 1964 and Lechevalier and Lechevaier, 1968].

2.4.3. Color characteristics

The ISCC-NBS color –Name Charts illustrated with centroid detection of the aerial, substrate mycelia and soluble pigments [Kenneth and Deane, 1955] was used.

2.4.4. DNA isolation and manipulation

The locally isolated actinomycete strain was grown for 6 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 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 described by [Sambrook *et al*., 1989]**.**

2.4.5. Amplification and sequencing of the 16S rDNA 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., [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 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 [Sanger *et al.,* 1977].

2.4.6. 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.

3. RESULTS

3.1. Screening for the antimicrobial activities

The metabolites of the actinomycete isolate eighty-two exhibited various degrees of activities against bacterial Gram positive and Bacteria Gram negative and unicellular and filamentous Fungi (Table 1).

3.2. Characterizations of the actinomycete isolate

3.2.1. Morphological characteristics

Spore chains are spiral and rectiflexibiles, spore masses are medium red and reddish gray, spore surfaces are smooth and reverse color light yellow to light brown; diffusible pigment production is moderate yellowish brown to deep brown (Plate 1).

3.2.2. Cell wall hydrolysate

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

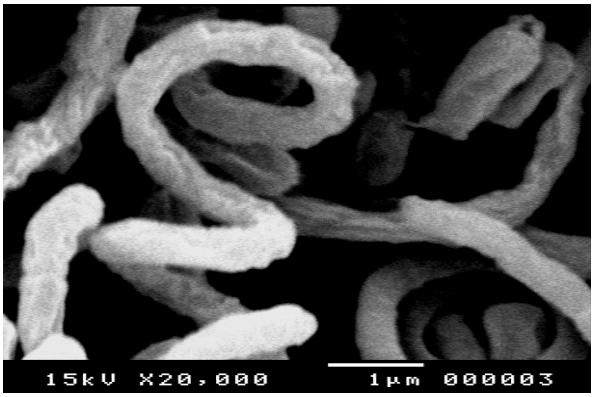

Plate (1). Scanning electron micrograph of the actinomycete isolate AZ-A151 growing on starch nitrate agar medium showing spiral spore were, rectiflexibiles, and had a smooth surface. Neither sclerotic granules, sporangia nor flagellated spores were observed (X20, 000).

3.2.3. Physiological and biochemical characteristics

The actinomycete isolate AZ-151 could hydrolyze starch and protein, whereas lipid, pectin and catalase are negative, Melanin pigment is positive, degradation of xanthin, esculin, production of H_2S, decomposition of urea, utilization of citrate and KCN are positive but nitrate reduction is negative.

The isolate under study utilizes; D- mannose, D-glucose, D- galactose, mannitol, meso-inositol, raffinose, and trehalose, but do not utilize, D- xylose, sucrose, L-rhamnose, L-arabinose, lactose, maltose, and ribose; whereas, doubtful result was obtained with D-fructose. Good growth on L-glycine, L- asparagine and L-lysine. No growth on L- valine, L-leucine L-histidine, L- phenyl alanine and L-methionine. Moreover, no growth in the presence of up to (5 %) NaCl. The growth is not inhibited in the presence of 0.1% (w/v) phenol and at 45ºC but inhibited in the presence of 0.01% (w/v) sodium azide. The actinomycete isolate is resistant to Ampicillin (25ug/ml), Nalidixic acid (30 ug/ml), Cefoperazone (75ug/ml) and Fusidic acid (10ug/ml), whereas not resistant to Polymyxin (30ug/ml), Gentamicin (10ug/ml) and Kanamycin (30ug/ml)(Table 2).

3.2.4. Color and culture characteristics

Data recorded on AZ151 declared that, the growth of this strain was disappeared in ISP-1, moderate in ISP-2, 4, 5, 6 and 7, and good growth on SNA and ISP-3. While, the aerial mycelium appeared red on all media used except SNA medium, it was reddish gray. Substrate mycelium light brown on all media used except ISP-5 has shown light yellow. Almost all media used didn't induce any diffusible pigments except SNA shown moderate yellowed brown and ISP-6, 7 showed deep brown pigments (Table 3).

3.3. Molecular phylogeny of the selected isolate

The nucleotide sequence of the 16S rDNA gene (1.5 kb) of the actinomycete isolate AZ151, and the phylogenetic tree (as displayed by the Tree View program) revealed 92% similarity with *Streptomyces crystallinus* (data not shown). Multiple sequence alignment was conducted the sequences of the 16S rDNA gene of *Streptomyces crystallinus*, and the sequencing product was determined as 1141 bp (Fig. 1).

3.4. Identification of actinomycete isolate AZ-A151

This was performed basically according to the recommended international Key's viz. (Buchanan and Gibsons, 1974; and Williams, 1989) and Numerical taxonomy of *Streptomyces* species program (PIB WIN). On the basis of above collected data, and in view of the comparative study of the recorded properties of AZ151 in relation to the most closest

reference strain, viz. *Streptomyces crystallinus* (ID Score= 0.98094**)** it could be concluded that it is identical on the basis of spore mass is medium red or reddish gray, spore chain is spiral and rectiflexibilies, and non motile spores. Cell wall hydrolysate contains LL-diaminopimelic acid, and sugar pattern not detected. Melanin pigments are produced. Utilization of D- mannose, D-glucose, D- galactose, mannitol, meso-inositol, raffinose and trehalose, but do not utilize D- xylose, sucrose, rhamnose, L-arabinose, lactose, maltose, and Ribose, whereas, doubtful with D- fructose. In view of all the previous characteristics of AZ151, it could be stated that it is suggestive of being belonging to *Streptomyces crystallinus* (Table 4).

Table 1. Antimicrobial activities of the actinomycetes strains.

Strain No.	*Mean diameter of inhibition zone (mm) against of:-								
	***Staphylococcus aureus*, NCTC 7447**	***Escherichia coli*, NCTC 10416**	***Salmon-ella typhi***	***Klebsiella pneumonia*, NCIMB 9111**	***Aspergillus flavus***	***Aspergillus flavus*, IMI 111023**	***Alternaria alternata***	***Fusarium verticillioides***	***Saccharomyce cerevisiae*, ATCC 9763**
AZ-C11	19.0±0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-C21	27.5±1.2	0.0	0.0	18.5±0.5	0.0	13.3±0.5	0.0	16.5±0.6	0.0
AZ-C22	22.0±0.0	0.0	15.0±0.0	20.5±0.6	0.0	0.0	12.8±0.5	0.0	0.0
AZ-C31	16.5±0.6	0.0	0.0	0.0	11.5±1.3	0.0	0.0	12.5±0.0	0.0
AZ-C43	17.5±0.8	0.0	0.0	0.0	0.0	0.0	13.0±0.0	0.0	0.0
AZ-C91	18.0±0.9	0.0	0.0	0.0	0.0	0.0	14.5±0.6	0.0	0.0
AZ-C131	19.5±0.0	0.0	0.0	15±0.0	0.0	0.0	10.5±0.6	0.0	0.0
AZ-C132	14.5±0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-C133	29.0±0.0	0.0	17.8±1.6	20.5±0.7	0.0	0.0	13.5±0.4	0.0	0.0
AZ-C181	21.0±1.2	18.5±0.6	0.0	0.0	0.0	20.0±0.0	19.5±0.6	0.0	12.0±0.0
AZ-C231	18.5±0.6	0.0	0.0	0.0	13.0±0.0	0.0	0.0	14.0±0.0	0.0
AZ-C242	24.5±2.6	15.0±0.0	0.0	0.0	0.0	19.0±0.0	21.0±0.0	0.0	14.0±0.0
AZ-C251	21.8±1.2	0.0	0.0	16.0±0.0	0.0	0.0	0.0	0.0	0.0
AZ-C261	17.5±0.0	0.0	12.0±0.0	16.0±1.2	0.0	0.0	0.0	0.0	0.0
AZ-C262	27.3±0.3	0.0	0.0	24.0±0.0	0.0	0.0	14.5±0.0	13.0±1.2	0.0
AZ-C281	18.8±1.0	0.0	0.0	0.0	0.0	0.0	0.0	12.5±0.3	0.0
AZ-C291	14.5±0.6	0.0	0.0	0.0	0.0	0.0	12.0±0.0	0.0	0.0
AZ-C293	26.0±0.0	0.0	15.0±0.0	20.0±0.0	0.0	0.0	0.0	14.8±0.4	0.0
AZ-C312	29.0±0.0	0.0	0.0	17.5±0.6	0.0	0.0	13.8±0.3	15.5±0.0	0.0
AZ-C321	28.3±0.9	0.0	15.8±0.3	18.5±1.0	0.0	0.0	14.0±0.0	0.0	0.0
AZ-C332	14.5±1.8	0.0	0.0	0.0	0.0	0.0	12.0±0.0	0.0	0.0
AZ-C381	17.0±1.2	0.0	0.0	0.0	0.0	10.8±1.0	0.0	0.0	0.0
AZ-C391	16.8±0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-C402	15.0±1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-C403	18.0±0.0	0.0	0.0	11.5±0.6	0.0	0.0	0.0	0.0	0.0
AZ-C411	17.0±0.0	0.0	0.0	15.0±0.0	0.0	0.0	0.0	0.0	0.0
AZ-C413	25.0±1.2	12.5±0.0	0.0	19.5±0.6	0.0	18.0±0.0	22.0±0.0	0.0	0.0
AZ-C422	16.5±1.9	0.0	13.0±0.0	0.0	0.0	0.0	11.0±0.0	0.0	15.0±0.6
AZ-C442	33.5±0.6	18.5±0.2	19.5±0.6	23.0±0.0	19.5±0.2	14.8±0.0	21.0±0.0	19.0±0.0	16.5±0.0
AZ-A22	18.5±2.3	0.0	0.0	0.0	10.5±0.5	0.0	12.0±0.0	0.0	0.0
AZ-A43	16.5±1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-A101	0.0	0.0	0.0	0.0	0.0	0.0	14.0±0.4	0.0	0.0
AZ-A111	18.0±0.0	0.0	0.0	12.5±0.6	0.0	0.0	0.0	0.0	0.0
AZ-A121	12.5±0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-A141	15.8±0.3	0.0	0.0	0.0	0.0	0.0	11.5±0.6	0.0	0.0
AZ-A151	**35.0±0.0**	**19.5±0.1**	**22.5±1.1**	**24.8±1.0**	**13.5±0.1**	**19.5±0.2**	**20.0±0.5**	**19.5±0.6**	**16.5 ±0.2**

Table 1. continued.

Strain No.	*Mean diameter of inhibition zone (mm) against of:-								
	Staphylococcus aureus, NCTC 7447	*Escherichia coli*, NCTC 10416	*Salmon-ella typhi*	*Klebsiella pneumonia*, NCIMB 9111	*Aspergillus flavus*	*Aspergillus flavus*, IMI 111023	*Alternaria alternata*	*Fusarium verticillioides*	*Saccharomyces cerevisiae*, ATCC 9763
AZ-A181	16.3±0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-A221	24.0±0.8	0.0	17.0±0.0	19.5±0.6	0.0	0.0	15.5±0.4	0.0	0.0
AZ-A251	28.0±1.8	0.0	0.0	15.8±1.0	0.0	0.0	0.0	12.5±0.8	14.0±0.0
AZ-A252	24.0±0.0	0.0	0.0	22.0±0.0	0.0	0.0	13.5±0.6	15.8±0.7	0.0
AZ-A272	27.0±0.0	0.0	12.5±0.0	14.0±0.0	0.0	0.0	13.5±0.6	0.0	0.0
AZ-A291	19.5±0.6	0.0	0.0	18.0±0.0	0.0	0.0	14.5±0.0	0.0	0.0
AZ-A292	14.5±0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-A381	13.5±0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-A382	29.0±0.7	0.0	13.5±0.0	24.0±0.0	0.0	0.0	17.0±0.0	15.5±0.6	0.0
AZ-A391	15.8±0.3	0.0	12.0±0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-A401	17.0±0.0	0.0	0.0	13.5±0.6	0.0	0.0	0.0	0.0	0.0
AZ-A421	16.0±0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-A441	14.5±0.6	0.0	0.0	11.0±0.0	0.0	0.0	0.0	0.0	0.0
AZ-N22	15.0±0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.5±0.5	0.0
AZ-N31	12.5±0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-N32	16.8±0.4	0.0	0.0	11.0±0.0	0.0	0.0	0.0	0.0	0.0
AZ-N43	17.5±0.6	0.0	14.0±0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-N44	0.0	0.0	0.0	13.0±0.0	0.0	0.0	0.0	0.0	0.0
AZ-N45	15.5±0.0	0.0	0.0	0.0	0.0	0.0	11.0±0.0	0.0	0.0
AZ-N121	16.5±0.6	0.0	0.0	9.5±0.6	0.0	0.0	0.0	0.0	0.0
AZ-N134	12.5±0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-N141	17.0±0.0	0.0	0.0	0.0	0.0	14.0±0.0	12.5±0.4	0.0	0.0
AZ-N153	0.0	0.0	0.0	0.0	0.0	0.0	11.5±0.0	0.0	0.0
AZ-N191	14.5±0.6	9.0±0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-N192	12.8±0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-N213	16.0±0.0	0.0	0.0	0.0	0.0	0.0	0.0	10.5±0.4	0.0
AZ-N222	17.5±0.6	0.0	0.0	13.0±0.0	0.0	0.0	11.5±0.6	0.0	0.0
AZ-N242	13.5±0.6	0.0	0.0	0.0	0.0	0.0	9.0±0.0	0.0	0.0
AZ-N252	15.8±1.0	0.0	12.0±0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-N293	19.0±0.0	0.0	0.0	11.5±0.4	0.0	0.0	0.0	12.5±0.0	0.0
AZ-N312	14.5±0.6	0.0	0.0	0.0	0.0	0.0	10.8±0.4	0.0	0.0
AZ-N321	0.0	0.0	0.0	0.0	0.0	0.0	0.0	12.0±0.0	0.0
AZ-N331	0.0	0.0	0.0	11.0±0.0	0.0	0.0	0.0	0.0	0.0
AZ-N361	13.0±0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
AZ-N392	0.0	0.0	0.0	0.0	0.0	0.0	15.0±0.0	0.0	0.0
AZ-N402	18.0±0.0	0.0	12.5±0.6	0.0	0.0	0.0	0.0	0.0	0.0

Table 2. The morphological, physiological and biochemical characteristics of the actinomycete isolate, AZ-A151.

Characteristic	Result
Spore chains	Spiral and Rectiflexibiles
Spore mass	Medium Red-reddish gray
Spore surface	Smooth
Color of substrate mycelium	Light brown- deep brown
Diffusible pigment	Yellowish brown
Motility	Non-motile
Diaminopimelic acid (DAP)	LL-DAP
Sugar Pattern	Not detected
Hydrolysis of: Protein and Starch	+
Lipid and Pectin	-
Catalase test	-
Production of melanin pigment on: Peptone yeast- extract iron agar	+
Tryptone – yeast extract broth	-
Degradation of: Esculin and Xanthin	+
H_2S Production	+
Nitrate reduction	-
Citrate utilization	+
Urea and KCN tests	+
Utilization of	
D-Xylose	-
D- Mannose	+
D- Glucose	+
D- Galactose	+
Rhamnose	-
Raffinose	++
Mannitol	+++
L- Arabinose	-
meso-Inositol	+++
Lactose	-
Maltose	-
Trehalose	++
D-fructose	±
Sucrose	-
D-Ribose	-
L-Cycteine	+
L-Valine	-
L-Histidine	-
L-Phenylalanine	-
L-Leucine	-
L-Asparagine	+
L-Methionine	-
L-Glycine	+
Growth with: Sodium azide (0.01)	-
Phenol (0.1)	+
Growth temperature (ºC)	30 -50 ºC
Growth at 7% NaCl concentration	+
Resistance to: Ampicillin (25ug/ml), Nalidixic acid (30 ug/ml), Cefoperazone (75ug/ml) and Fusidic acid (10 ug/ml).	+
Polymyxin (30ug/ml), Gentamicin (10 ug/ml) and Kanamycin (30 ug/ml)	-

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

Table 3. Cultural characteristics of the actinomycete isolate AZ- A151.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigments
1- Starch- nitrate agar medium (SNA)	Good	22-4-Gray Reddish gray	57-1.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)	Moderate	15- med. Red Medium red	57-1.br Light brown	-
4- Oatmeal agar medium (ISP-3)	Good	15- med. Red Medium red	57-1.br Light brown	-
5- Glycerol asparagine agar medium (ISP-4)	Moderate	15- med. Red Medium red	57-1.br Light brown	-
6- Inorganic salts starch agar medium (ISP-5)	Moderate	15- med. Red Medium red	86-I. yellow Light yellow	-
7- Peptone yeast extract- iron agar medium (ISP-6)	Moderate	15- med. Red Medium red	57-1.br Light brown	59-d.Br Deep brown
8- Tyrosine agar medium (ISP-7)	Moderate	15- med. Red Medium red	57-1.br Light brown	59-d.Br Deep brown

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

Table 4. Numerical taxonomy of *Streptomyces* species program (PIB WIN) (*Streptomyces* species) J. Gen Microbiol. 1989 13512-133 lang.

Characteristic	AZ-A151	*Streptomyces crystallinus*
Dianinopimelic acid (DAP)	LL-diaminopimelic acid	LL-diaminopimelic acid
Sugar pattern	Not detected	Not detected
Spore chain rectiflexibles	+	+
Spore mass Spiral	+	+
Spore mass red	+	+
Spore mass gray	-	-
Diffusible pigment red/orange	-	-
Diffusible pigment yellow/brown	+	+
Melanin pigment 1-Peptone yeast extract-iron agar medium (ISP-6) 2-Tyrosine agar medium (ISP-7)	 + ND	 + -
Lipolysis activity	+	+
Pectin hydrolysis	-	-
Nitrate reduction	-	-
H_2S production	+	+
Degradation of Xanthin	+	+
Growth at 45°C	+	+
Growth at NaCl 7% (w/v)	+	+
Growth with Phenol (0.1 % w/v)	+	+
Utilization of:		
L- Valine	-	-
L- phenylalanine	-	-
L- Histadine	-	±
Sucrose	-	-
meso-Inositol	+++	+
Mannitol	+++	+
Rhamnose	-	-
Raffinose	++	+

No.	Key	Source	Identification	ID Score
1	**AZ-A151**	**Assiut**	***Streptomyces crystallinus***	**0.98094**

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

```
    GGGCGTGC TAAACACATG CAAGTCGAAG GCATGAACCA CTTCGGTGGG ATTAGTGGCG
 61 AACGGGTGAG TAACACGTGG GCTTTCCTGC CTTCACTCTG GGACAAGCCC TGGTTTCGGC
121 CTCTAATACC GGATACGAGG TGGAAGCGCA TGCTTCCGGG TGGTTTGCTC CGGCGGTGTT
181 GGATGAGGGG GCGGCCTATC AGCAAGTTGG TCCCCTAATG GCCTACCAAG GCGACGACCC
241 CTAGCCGGCC TGAGAGGGCG ACCGGCCACA CTCCCACTGA GACACGGCCC AGACTCCTAC
301 GGGACCCAGC AGTGCCGAAT ATTGCACAAT GGGCGTTTGC CTGATGCAGC GACGCCGCGT
361 GAGGGATGAC GGCCTTCGGG TTGTAAACCT CTTTCAGCAG GGAAGAAGCG TTTGTGACGG
421 TACCTGCAGA AGTTGCGCCG GCTTTCTACG TGCCAGCAGC CGCGGTAATA CGTAGGGCGC
481 AAGCGTTGTC CGGAATTATT GGGCGTTTAG AGCTCGTAGG CGGCTTGTCA CGTCGGATGT
541 GAAAGCCCGG GGCTTAACCC CGGGTCTGCA TTCGATACGG GCTAGCTAGA GTGTCCTAGG
601 GGAGATCGTT ATTCCTGGTG TAGCGGTGAA ATGCGCAGAT ATCAGGAGGA ACACCGGTGG
661 CGAAGGCGGA TCTCTGGGCC ATTACTGACG CTGAGGAGCG TTTGCGTGGG GAGCGTTCAG
721 GATTAGATAC CCTGGTAGTC CACGCCGTTT TCGTTGGGAA CTAGGTGTTG GCGACATTCC
781 ACGTCGTCGG TGCCGCAGCT AACGCATTAA GTTCTCCGCC TGGGGAGTAC GGCCGCTTGG
841 CTAAAACTCT TTGGAATTGA CGCCGGCCCG CACAAGCAGC GGAGCATGTG GCTTCATTCG
901 ACGCAACGCG AAGAACCTTA CCAAGGCTTG ACATATACCG GAAAGCATTA GAGATAGTGC
961 CCCCCTTGTG GTCGGTATAC AGGTGGTGCA TGGCTGTCGT CAGCTCGTGT CGTGAGATGT
1021 TGGGTTAAGT CCCGCAACGA GCGCAACCCT TGAACTGTGT TGCCAGCATG CCCTTCCCCC
1081 TGATGGGGAC TCACAGGAGA CTGCCGCCCT CAACTCCCAG GAAGGTGGGG ACGACGTCAA
1141 GTCATCATGC CCCTTATGTC TTGGGCTGCA CACGTGCTAC AATGGCCGGT
```

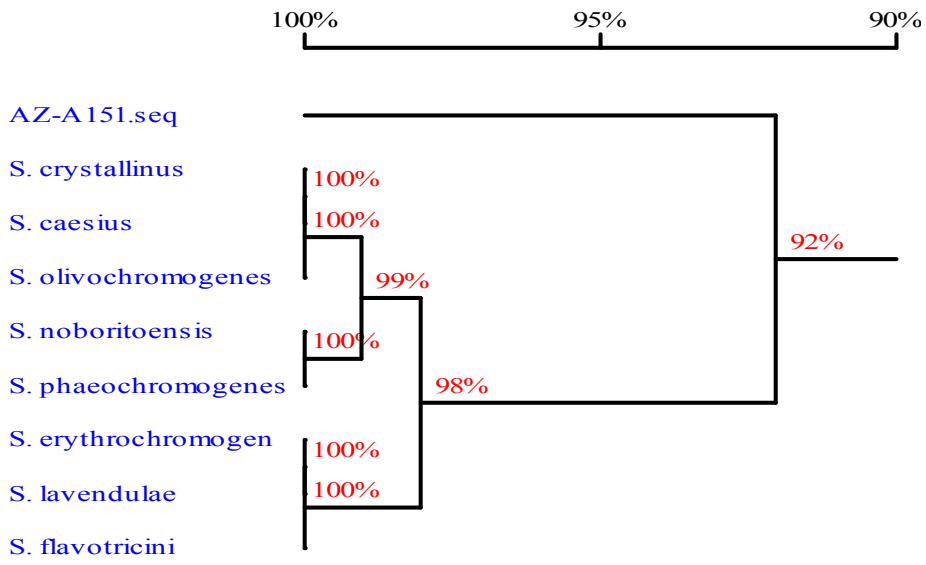

Fig. 1. The phylogenetic position of the local *Streptomyces* sp. strain among neighboring species. The phylogenetic tree was based on the pairwise comparisons of 16S rDNA sequences.

4. DISCUSSION

The antimicrobial resistance is presently an urgent focus of research and new bioactive compounds are necessary to combat these pathogens. About 70% of all known drugs have been isolated from actinomycetes bacteria of which 75% and 60% are used in medicine and agriculture, respectively [Miyadoh, 1993 & Tanaka and Mura, 1993].

The species belonging to the genus *Streptomyces* constitute 50% of the total population of soil actinomycetes and 75-80% of the commercially and medicinally useful antibiotics have been derived from this genus [Mellouli *et al*., 2003].

Selective isolation of soil actinomycetes is important for understanding their ecological properties and for finding novel strains which can produce useful bioactive secondary metabolites. Therefore, various media and techniques have been developed for selective isolation of actinomycetes [Hozzein *et al*., 2011].

Isolation and purification of actinomycete colonies (the broadest source of antibiotics) from different soil samples collected from various Egyptian localities e.g. (Assiut, Luxor and El-Minia governorates). Out of 44 soil samples collected from the previous Governorates, 194 isolates of actinomycetes were isolated. The isolation of the actinomycete isolates was conducted by three different isolation media which included (starch nitrate agar medium, casein starch agar medium and glycerol asparagine agar medium). Similarly, (Oskay *et al*., 2004) isolated 50 isolates of actinomycetes using Glycerol-Yeast Exstract Agar from 10 farming soil (Wheat, corn, vineyard, orchard, rye, vegetable, barley and cotton fields) samples. Also, In order to facilitate the discovery of novel actinomycetes from the Egyptian deserts, which can be useful as new sources for bioactive metabolites, different media for enumeration and isolation of, desert actinomycetes have been tested. For this purpose, 30 soil samples from different six sites representing the Western and Eastern deserts of Egypt were collected [Hozzein *et al*., 2011].

The initial screening for 194 actinomycete isolates were carried out against certain Gram positive and Gram negative bacteria and unicellular and filamentous fungi. Among the actinomycete isolates which were tested for their ability to produce the antimicrobial activity only 74 (38 %) of isolates produced antibiotics, included among these were broad and narrow spectrum. From this, 7.2 % (14) isolates produced antibacterial substances against gram-positive bacteria, 6.7 % (13) isolate against both gram-positive and gram-negative bacteria, 1.5 % (3) isolates produced antibacterial substances against gram-negative bacteria, 2 % (4) isolates produced antifungal substances and 20.6 % (40) isolates produced antibacterial and antifungal substances. These percentages are higher than those described by [Aghighi *et al*., 2004], studying the antifungal spectra of activity of Iranian actinomycetes strains isolated from soil Against *Alternaria solani*, *Alternaria alternata*, *Fusarium solani*, *Phytophthora megasperma*, *Verticillium dahliae* and *Saccharomyces cerevisiae*. The same authors found that, among 110 isolates screened, 13 % (14) isolates were found active at least against one of the tested fungi. However, who current percentages were lower than those described by [Barakate *et al*., 2010], they reported that, during an investigation on actinomycete isolates from rhizospheric soils from Moroccan habitats, 131 streptomycetes isolates were recovered and assessed for their antimicrobial activity. Most of isolates (83 %) were active against one or more of test organisms (one Gram-negative bacterium, three Gram-positive bacteria, three yeasts and two filamentous fungi).

The highest percentage of the 74 active isolates was obtained against *Staphylococcus aureus* 90.5 % (67) followed by *Aternaria alternata* 43.2 % (32) and *klebsiella pneumoniae*, 41.8 % (31), while the lowest percentage was obtained against *Fusarim verticillioides* 21.6% (16 isolates), *Salmonella typhi* 20.2% (15 isolates), *Escherichia coli* 9.4% (7 isolates), *Aspergillus fumigatus* 9.4% (7 isolates), *Saccharomyces cerevisiae* 8.1% (6 isolates) and *Aspergillus flavus* 6.7% (5 isolates), Similarly, [Aghighi *et al*., 2004] found that, among 110 isolates screened, approximately 13 % (14 isolates) were found active at least against one of the tested fungi. The highest percentage of the 14 active isolates was obtained against *Phytophthora megasperma* 100 % (14), followed by *Alternaria solani* and *Alternaria alternata* 78.5 % (11), while the lowest percentage was obtained against *Fusarium solani* and *Saccharomyces cerevisiae* (14.2 % (2) and 7.1 % (1) respectively). Many authors reported that, Streptomycetes isolates appear to be highly active against Gram-positive bacteria [Saadoun *et al*., 1998 and Valan Arasu *et al*., 2009] found that the *Streptomyces* sp. isolate ERI-3 was a good antibacterial and moderate antifungal compound producer.

In the present investigation, the most antimicrobial producing actinomycete isolates belonging to the yellow, gray and white colour series. Also, [Saadoun *et al*., 2002 & 2003] found that, the highest percentage of active isolates was found in red and gray series and the lowest in the green and white ones.

However, [Narayana *et al*., 2007] reported that, most antimicrobial producing species of streptomycetes were found in the gray and yellow series of no chromomeric type and no antibiotic produced by white and green series chromomeric type.

The genus *Streptomyces* was first described by [Waksman and Henrici, 1943] and then comprised hundreds of validly published species [Euzeby, 2004]. They are Gram positive, non-acid fast, aerobic

actinomycetes, non- motile spores. Catalase test are positive. Vegetative hyphae (0.5-2.0 um in diameter) produce an extensively branched mycelium that rarely fragments. The aerial mycelium at maturity forms chains of three to many spores.

Cultural criteria, such as growth on different media, the color of aerial and substrate mycelia, and the formation of soluble pigments among others, and biochemical criteria such as the utilization of carbon sources and proteolytic properties, are taken into account. Sensitivity to antibiotics and phages, serological reactions, and ecological properties has also been used for the classification of *Streptomyces* spp. [Shirling and Gottlieb, 1966].

Several methods have been developed to identify *Streptomyces* species. These include culturing methods using the selective plating technique [Kuster and Williams, 1964], construction of genetic marker systems [Wipat *et al*., 1991], a combination of chemical markers, and the presence of LL diaminopimelic acid and the absence of characteristic sugars in the cell wall [Lechevalier and Lechevalier, 1970]. In addition, 16S rRNA sequence data have proved invaluable in Streptomycetes systematics, in which they have been used to identify several newly isolated *Streptomyces* [Mehling *et al*., 1995].

According to recommended international Key's for actinomycetes viz. Bergy's Manual of Determinative Bacteriology "8th edition" [Buchanan and Gibbons, 1974]; International Journal of Systematic Bacteriology [Küster, 1972]; Journal of Fermentation Technology [Nonomura, 1974]; Bergey's Manual of Systematic Bacteriology [Williams, 1989]; Bergy's Manual of Determinative Bacteriology "9thedition" [Hensyl, 1994] and PIB WIN (1989).

The nucleotide sequence of the 16S rDNA gene (1.5 kb) of the actinomycete isolate, AZ-A151 evidenced a 92% similarity with *Streptomyces crystallinus* 16S rDNA genes. From the taxonomic features, the isolate was found to match with *Streptomyces* species in the morphological, physiological and biochemical characters. Thus the actinomycete isolate AZ-A151 was found to match with the character of *Streptomyces crystallinus* and for this, it was given the name *Streptomyces crystallinus,* AZ-A151 [Atta *et al.*, 2011].

5. Conclusion

The present study mainly involved in the isolation of Actinomycetes AZ-A151 based on its morphology and identification based on the physiology, biochemical and cultural characteristics. The nucleotide sequence of the 16s DNA gene (1.5 Kb) of the most potent strain evidenced an 92% similarity with *Streptomyces crystallinus*. *Streptomyces crystallinus,* AZ-A151 was found to produce a wide spectrum antimicrobial agent(s) against *Staphylococcus aureus*, NCTC 7447; *Escherichia coli*, NCTC 10416; *Klebsiella pneumonia*, NCIMB 9111; *Salmonella typhi*; *Saccharomyces cerevisiae,* ATCC 9763; *Aspergillus flavus*, IMI 111023; *Alternaria alternate* and *Fusarium verticillioides*.

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