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Full Length Research Paper

Screening the Egyptian desert actinomycetes as candidates for new antimicrobial compounds and identification of a new desert *Streptomyces* strain

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In a screening program to study the antimicrobial activities of desert actinomycetes as potential producers of active metabolites, 75 actinomycete strains were isolated from the Egyptian desert habitats and tested. Out of the isolated 75 organisms, 32 (42.67%) showed activity against the used test organisms. The antimicrobial activities of the active desert actinomycete strains were classified into four groups according to their spectrum of activity on different groups of test organisms and it was found that 43.75% of the active isolates have activity against Gram-positive bacteria only, 28.13% have activity against both Gram-positive and Gram-negative bacteria, 15.63% have activity against Gram-positive bacteria and yeast, 12.50% have activity against Gram-positive, Gram-negative bacteria and yeast. The most potent actinomycete strain, designated D332, was selected for further studies including its identification and isolation of its active compound. Strain D332 was identified by studying its morphology, chemotaxonomy, biochemical characteristics and phylogenetic analysis of the 16S rRNA gene sequence. All phenotypic and genotypic characteristics were consistent with the classification of strain D332 to genus *Streptomyces* where it formed a distinct phyletic line in the *Streptomyces* 16S rRNA gene tree. On the other hand, the culture broth of strain D332 was extracted with ethyl acetate after fermentation for the production of the active compound then, the crude extract was partially purified by thin layer chromatography using a solvent system composed of heptane: ethyl acetate (3: 2). The results revealed that strain D332 produced one major compound active against Gram-positive and Gram-negative bacteria and yeasts.

Key words: Desert actinomycetes, antimicrobial activities, new *Streptomyces* strain, production of the active metabolite.

INTRODUCTION

Most actinomycete species have the capability to synthesize many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, antiparasitic and enzyme inhibitors. Of these compounds, antibiotics are much more important therapeutically and commercially and approximately, two-thirds of known antibiotics have been isolated from actinomycetes (Olano et al., 2009). Strains of the genus *Streptomyces* are superior to other actinomycete strains in their ability to produce large numbers and varieties of bioactive metabolites (Tanaka and Omura, 1990). The number of antimicrobial compounds reported from species of this genus per year increased almost exponentially for about two decades, followed by a steady rise to reach a peak in the 1970s and with a substantial decline in the late 1980s and 1990s (Watve et al., 2001). The antibiotics derived from *Streptomyces* strains include erythromycin, tetra-

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cycline, streptomycin, chloramphenicol, neomycin, nystatin, amphotricin, kanamycin and cycloheximide (Sirvastava et al., 1994).

Although some novel compounds are under investigation, there is still an urgent unmet medical need for the development of novel antimicrobial compounds and microbial natural products still appear as the most promising source for drug identification (Luzhetskyy et al., 2007; Okoro et al., 2009). The antibiotic resistance and decrease in the rate of discovery of new antimicrobial compounds draws the attention of scientists to try to investigate unexplored habitats for novel actinomycetes as possible candidates of new antimicrobials. The recent discovery of novel primary and secondary metabolites from taxonomically unique populations of extremophilic actinomycetes suggest that, these organisms could add a new dimension to microbial natural product research (Thumar et al., 2010).

In Egypt, the desert habitats representing about 94% of the land and are less studied. Therefore, we were interested to screen the Egyptian desert actinomycetes as a new source for production of novel active compounds (Hozzein, 2000; Ali et al., 2002; Hozzein et al., 2007). The present investigation highlights the screening of some actinomycete strains isolated from the Egyptian desert habitats for antimicrobial activities, taxonomic identification of the most active strain and partial purification of its antimicrobial agent.

MATERIALS AND METHODS

Collection and processing of the soil samples

Soil samples were collected in clean sterile plastic bags from the eastern and western desert soils in Beni-Suef and Giza Governorates of Egypt. The soil samples were air-dried at room temperature.

Isolation of desert actinomycetes

The desert actinomycetes were isolated from the air dried desert soil samples by soil dilution plate technique on the minimal medium (MM) recommended by Hozzein et al. (2008) for isolation of actinomycete strains from the desert environments.

Screening for antimicrobial activities

All the isolated actinomycete strains were inoculated on ISP2 agar plates and incubated at 30°C for 7 days. Agar discs of well grown cultures were placed on plates seeded with the following test strains; *Bacillus subtilis, Sarcina lutea, Staphylococcus aureus*, Methicillin-resistant *S. aureus* (MRSA), *Escherichia coli, Mycobacterium pheli, Salmonella typhi, Salmonella paratyphi, Shigella* spp., *Candida albicans* and *Candida kruzei*. The plates were then, incubated at 37°C for 24 h for bacteria and at 30°C for 48 h for *Candida* strains. Diameters of the inhibition zones around the actinomycete discs were measured as an indication of the antimicrobial activities.

Taxonomic identification of the most potent actinomycete strain D332

Phylogenetic analysis of the 16S rRNA gene sequence

Extraction of genomic DNA, PCR amplification, direct sequencing of 16S rRNA from strain D332 and the phylogenetic analysis were carried out as described earlier (Hozzein and Goodfellow, 2007).

Chemotaxonomy

Standard analytical procedures were used to extract and analyse the isomeric forms of diaminopimelic acid (Hasegawa et al., 1983), whole-organism sugars (Staneck and Roberts, 1974) and polar lipids (Minnikin et al., 1984).

Cultural and morphological characteristics

The cultural characteristics of strain D332 were recorded as described by Shirling and Gottlieb (1966). The morphological features were observed after examining the cover slip cultures by the light and scanning electron microscopes using a Cambridge Steroscan 240 scanning electron microscope and the procedure described by O'Donnell et al. (1993).

Phenotypic tests

Strain D332 was examined for a range of standard phenotypic tests following established methods (Williams et al., 1983).

Production of the antimicrobial compound(s)

A loopful of 7 days old culture of strain D332 grown on MM agar was used to inoculate a 500 ml Erlenmeyer flasks each containing 100 ml of ISP2 medium and incubated at 30°C on a shaking incubator at 200 rpm for 2 days. Then, 10% of the actinomycete inoculum was transferred to starch nitrate production medium (Tadashi, 1975) and incubated at 30°C on a shaking incubator at 200 rpm for 5 days. The fermentation broth was collected and examined for the antimicrobial activity by using the agar well diffusion method in nutrient agar plate seeded with *B. subtilis*.

Extraction and partial purification of the antimicrobial compound(s)

The fermentation broth was filtered and extracted with 1:1 (v/v) ethyl acetate. The organic ethyl acetate phase was collected and evaporated to dryness under reduced pressure by using a rotary evaporator. The antimicrobial activity of the crude extract was tested by using the filter paper disc diffusion method assay on *B. subtilis*. The crude extract was then, partially purified by thin layer chromatography (precoated silica gel TLC plates) using a solvent system composed of heptane: ethyl acetate (3: 2). The activity at the partial purification stage was checked using the bioautography method in which the developed chromatogram strip was placed on the surface of a *B. subtilis* seeded agar plate. The plate was then, incubated at 37°C for 24 h. The presence of inhibition zones around the active compound(s) was determined and the distance from the starting point, where the crude extract was loaded to the TLC strip, to the centre of the clear zones were measured.

RESULTS AND DISCUSSION

The desert habitats in Egypt can be considered as an

Table 1. The antimicrobial activities of the biologically active desert actinomycete isolates expressed as inhibition zones of growth against the used test organisms in mm.

Organism code	Test isolate*										
	Bs	**Mp**	**MRSA**	**Sa**	**Sl**	**Ec**	**Sh sp.**	**Sp**	**St**	**Ca**	**Ck**
S11	32	30	26	28	35	-	-	-	-	-	-
S14	22	26	23	23	28	-	-	-	-	-	-
S133	-	-	16	16	18	-	-	15	14	-	-
S134	18	26	17	19	20	17	-	16	15	-	-
S135	-	-	15	-	-	-	-	23	18	-	-
S139	16	-	-	14	18	-	-	18	17	-	-
S22	25	19	20	26	23	-	-	-	-	-	-
S212	24	-	22	23	27	-	-	-	-	-	-
S215	-	-	-	35	-	21	24	16	17	23	32
S230	17	-	18	20	27	-	-	-	-	-	-
S36	18	-	-	-	-	-	-	-	-	-	-
S38	-	17	-	-	25	-	-	-	-	16	-
S39	20	-	-	-	16	-	-	-	-	-	-
S310	25	-	-	23	20	-	-	-	-	30	19
S319	-	-	-	-	20	-	-	-	-	28	34
S320	-	-	19	-	-	-	20	17	22	30	28
S322	-	-	-	-	21	-	-	-	-	-	-
S323	25	-	16	18	20	-	-	-	-	35	30
S325	16	-	-	23	16	27	-	-	-	-	-
S328	-	-	-	32	-	18	-	-	-	16	16
S329	-	-	-	-	16	-	-	-	-	-	-
S331	-	-	-	-	16	20	-	-	-	-	-
D13	18	20	-	-	26	-	-	-	-	-	-
D17	19	-	-	-	-	-	-	-	-	19	-
D137	14	16	-	-	16	-	-	18	16	-	-
D211	27	30	-	21	25	-	-	-	-	-	-
D217	15	-	-	18	16	-	-	-	-	-	-
D221	17	-	-	-	20	17	-	-	-	-	-
D224	-	-	-	18	18	16	19	-	-	-	-
D226	16	-	-	-	18	-	-	-	-	-	-
D327	-	-	-	-	16	-	-	-	-	-	-
D332	35	-	-	32	31	25	23	39	40	30	35

*Bs, *B. subtilis*; Mp, *M. pheli*; MRSA, Methicillin-resistant *S. aureus*; Sa, *S. aureus*; Sl, *S. lutea*; Ec, *E. coli*; Sh sp., *Shigella* spp; Sp, *S. paratyphi*; St, *S. typhi*; Ca, *C. albicans* and Ck, *C. kruzei.*

inexhaustible resource for biotechnology that has not been well exploited. Although, previous studies on actinomycetes isolated from the Egyptian deserts are very few, their antimicrobial potential was encouraging (Hozzein et al., 2007). In the present study, 75 dissimilar desert actinomycete strains were isolated from the collected soil samples. The results of the antimicrobial screening program revealed that, 32 out of the isolated 75 strains were active against the tested pathogenic organisms as shown in Table 1. This means that 42.67% of the isolated desert actinomycetes are biologically active. The antimicrobial activities of the active strains could be divided according to the spectrum of their activity into four groups (Figure 1) as follows: Group I, includes the desert actinomycete isolates capable of producing antimicrobial activities against Gram-positive bacteria only. This group contains 14 isolates (43.75% of the active strains); S11, S14, S22, S212, S230, S36, S39, S322, S329, D13, D211, D217, D226, and D327; Group II, includes the isolates that have the ability of producing antimicrobial activities against both Gram-positive and Gram-negative bacteria. This group includes 9 isolates (28.13%); S133, S134, S135, S139, S325, S331, D137, D221, and D224; Group III, includes the isolates inhibited the growth of Gram-positive bacteria and also, the tested yeast strains. This group harbors 5 isolates (15.63%); S38, S310, S319, S323 and D17; Group IV, includes the isolates showing antimicrobial activities against Gram-positive

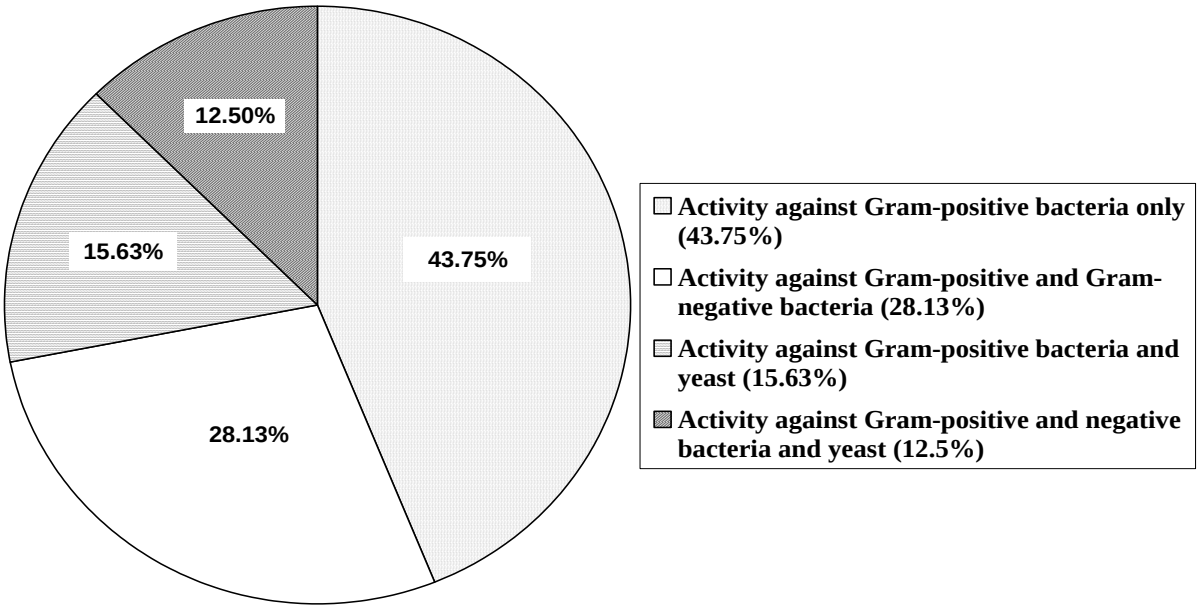

Figure 1. Classification of the active desert actinomycete isolates according to their spectrum of activity.

and Gram-negative bacteria and yeast strains. This group has 4 isolates (12.50%); S215, S320, S328 and D332.

It is obvious from the results that the activities against Gram-positive bacteria were more frequent than against Gram-negative bacteria and yeast. This frequency of activities against Gram-positive bacteria is similar to previous results reported by Basilio et al. (2003), Oskay et al. (2004), Anansiriwattana et al. (2006) and Charoensopharat et al. (2008).

The desert actinomycete strain D332 was selected for its broad spectrum and high antimicrobial activity, as clear from the results shown in Table 1, for further studies including its classification and partial purification of its antimicrobial compound(s).

The taxonomical position of strain D332 was obtained after a stepwise phylogenetic analysis of the 16S rRNA gene sequence with the closely related similar sequences. It was found that it belongs to genus *Streptomyces* as obvious from the phylogenetic tree (Figure 2). The similarity values between strain D332 and the selected closely related *Streptomyces* species ranges from 99.7 to 98.5%. It is evident also from the tree that strain D332 formed a phyletic line in the *Streptomyces* 16S rRNA gene tree and it could represent a new species of the genus *Streptomyces*.

The affiliation of strain D332 to genus *Streptomyces* was supported by its chemotaxonomical characteristics. It contained LL-diaminopimelic acid as the characteristic diamino acid of the peptidoglycan in the whole-cell hydrolysate and glucose and galactose as whole-organism sugars (wall chemotype I) (Lechevalier and Lechevalier, 1970). The polar lipid pattern revealed the presence of phosphatidyl ethanolamine, phosphatidyl inositol mannosides, diphosphatidyl glycerol (phospholipid type II) (Lechevalier et al., 1977). This chemical profile is clearly consistent with the assignment of strain D332 to genus *Streptomyces* (Williams et al., 1989).

Strain D332 showed abundant growth on all ISP media used with aerial mycelium color varied from grayish white to moderate grey. The substrate mycelium color varied from grayish white to reddish brown. No soluble pigments were produced on any medium. It formed aerial mycelium with short to long spore chains with coiled ends and smooth-surfaced spores (Figure 3).

The most potent desert actinomycete strain D332 showed good utilization of fructose, cellobiose, mannitol and starch, while moderate utilization of glucose, sucrose, salicin, galactose, mannose and lactose was recorded. Weak utilization was observed with sodium acetate, raffinose, sorbitol and maltose, while no utilization was recorded with inositol. It produced amylase, catalase and gelatinase enzymes and degraded casein but did not degrade any of xanthine, xylan, hypoxanthine, L-tyrosine or guanine. It grew well at a temperature range from 15 to 45°C. It showed good growth at different NaCl concentrations up to 7%. All of the mentioned morphological, cultural and phenotypic characteristics are similar to those reported as members of genus *Streptomyces*.

To confirm that strain D332 is a new species of the genus *Streptomyces*, further comparative studies with the closest phylogenetic neighbors including DNA-DNA hybridization should be carried out (Manfio et al., 1995).

The crude supernatant obtained from the culture broth of strain D332 was tested by the agar well diffusion method for its antimicrobial activity (Figure 4a) before proceeding with the extraction of the active metabolite. Then, the fermentation broth was extracted by using ethyl acetate and the crude extract was examined by the

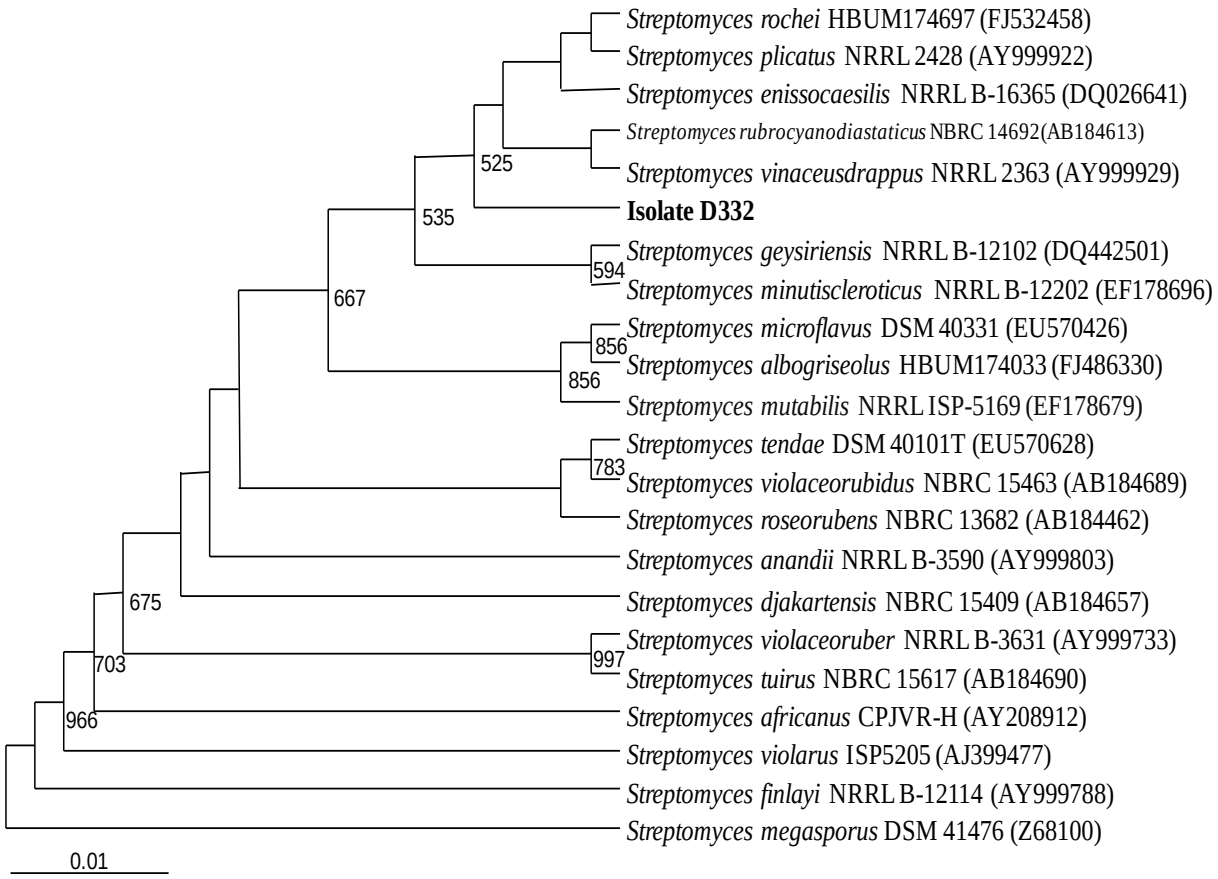

Figure 2. Phylogenetic relationships between strain D332 and closely related members of the genus *Streptomyces* based on nearly complete 16S rRNA gene sequences. Numbers at nodes indicate levels of bootstrap support based on a neighbor joining analysis of 1000 resampled datasets; only values above 50% are given. Bar 0.01 substitutions per nucleotide position.

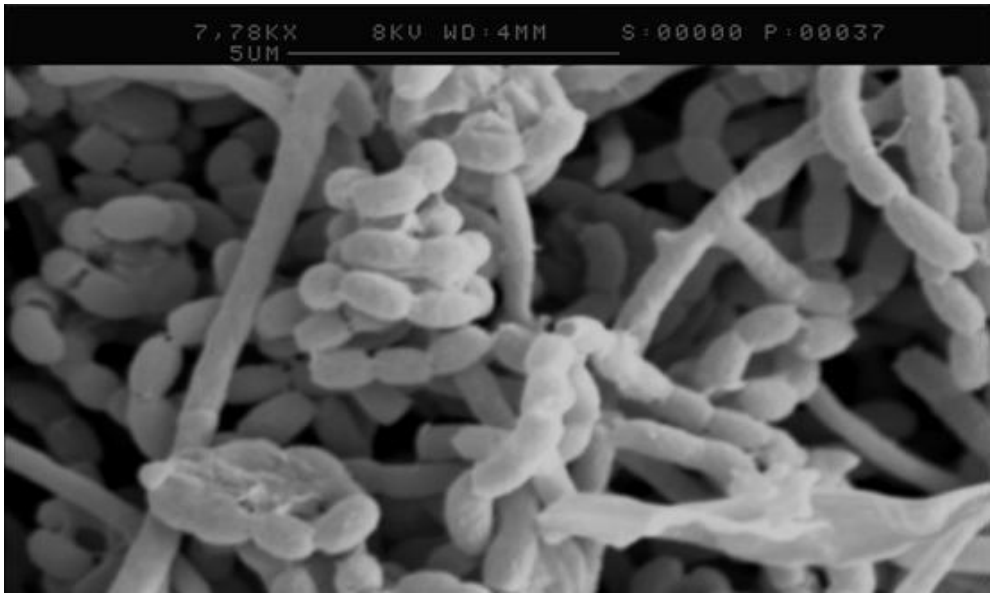

Figure 3. An electron micrograph of strain D332 showing the spore chains forming coil ends with smooth-surfaced spores.

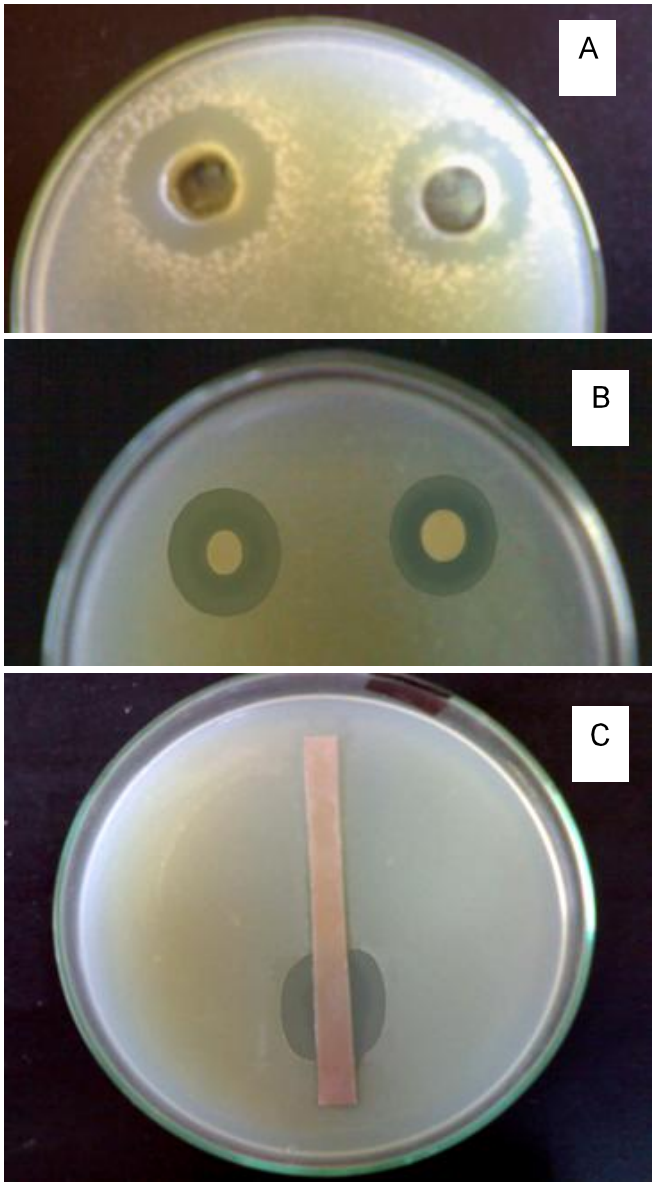

Figure 4. (a) A plate showing the activity of the most potent desert actinomycete, *Streptomyces* strain D332, using the agar well diffusion method; (b) a plate showing the activity of the crude extract using the disc diffusion method; (c) the bioautography bioassay plate showing the single inhibition zone of the active compound; all on *B. subtilis* seeded plate.

filter paper disc method (Figure 4b). Partial purification of the active compound has been carried out by thin layer chromatography. Only one band was detected on the thin layer chromatography (TLC) plate. Moreover, a single sharp inhibition zone in the bioautography assay revealed that, the *Streptomyces* strain D332 produce only a single active compound (Figure 4c). The results of the bio-autography showed also that, the R_f value was 0.28. Similar results were obtained by El-Naggar et al. (2006) who reported that, *Streptomyces violaceusniger* strain HAL64 produces only one major compound in its culture filtrate that strongly inhibits the growth of Gram-positive bacteria, but the inhibition against Gram-negative bacteria and yeast was lower. However, further purification and analysis with high performance liquid chromatography (HPLC) should be conducted before characterization of the pure active metabolite produced by the *Streptomyces* strain D332.

Ahmed (2007) reported the production of an antimicrobial compound from *Streptomyces violachromogenes* isolated from soil habitat of Yemen. The filtrate was extracted with ethyl acetate and purification of the active metabolite has been carried out by thin layer chromatography, and also recorded one spot only. Recently, Arasu et al. (2009) reported the isolation of *Streptomyces* ERI-3 from a forest rock soil sample, which was a good antibacterial and moderate antifungal compound producer.

In the present study, the results showed that 42.67% of the isolated desert actinomycetes are biologically active. These results are very encouraging to continue screening more actinomycete strains from the desert habitats and strongly support the idea that species of actinomycetes from underexploited environments could be a very fruitful source of novel bioactive secondary metabolites.

The results showed also that the *Streptomyces* strain D332, which was isolated from the Egyptian desert soil produced one biologically active compound with high broad spectrum activity against Gram-positive and Gram-negative bacteria and yeasts suggesting that this strain is a promising producer of an antimicrobial compound.

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