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## Antimicrobial Activities of Some Actinomycete Strains Isolated from the Sinai Egypt Soils

Abdel-Rahman<sup>1</sup>, A.G.; B.M. Refaat<sup>2</sup>; M. Helal<sup>2,3</sup> and A.A.Kobisi<sup>1\*</sup>

<sup>1</sup>Plant Protection Department, Desert Research Center, Mataria, Cairo, Egypt.

<sup>2</sup>Botany and Microbiology Department, Faculty of Science (Boys), Al-Azhar University, Cairo, Egypt.

Email: [ag\\_rahman@outlook.com](mailto:ag_rahman@outlook.com)

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### ABSTRACT

Seventy three pure actinomycete colonies were isolated from 48 rhizospheric soil samples revealing different locations in Sinai. These isolates were tested for their antimicrobial activities against *Bacillus subtilis* and *Staphylococcus aureus* as gram-positive bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* as gram-negative bacteria, *Candida albicans* as an unicellular fungi and *Aspergillus niger*, *Aspergillus flavus*, *Rhizoctonia solani* and *Fusarium oxysporum* as filamentous fungi. Isolates S6, S13 and S35 were the most effective against the tested bacteria and fungi. They had the lowest Minimum Inhibitory Concentration (MIC) values and showed the highest and widest spectrum of antibacterial and antifungal activities. Factors controlling actinomycetes active metabolite(s) productivity were studied.

### INTRODUCTION

Botanical and microbial pesticides are having advantage over chemical pesticides by its highly effective, safe, and ecologically acceptable nature. Actinomycetes have the capability to synthesize many different biological active secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic, and enzymes like cellulase and xylanase used in waste treatment (Oskay *et.al.*, 2004). The search for new antibiotics continues to be most important in research programs around the world because of the increase of resistant pathogens and toxicity of some used antibiotics (Berdy, 1989). Lam *et. al.*, (1989) reported that, Production of secondary metabolites by microorganisms differs qualitatively and quantitatively that depends on the species and strains of microorganisms used as well as on their nutritional and cultural conditions.

Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of antimicrobial that will inhibit the visible growth of a micro-organism after overnight incubation. MICs are considered the 'gold standard' for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. MICs are used in diagnostic laboratories to confirm unusual resistance, to give a definitive answer when a borderline result is obtained by other methods of testing, or when disc diffusion methods are not appropriate (Andrews, 2001).

The present study aimed to investigate the antimicrobial activity of some soil actinomycetes isolates against bacteria and fungi and spot the light on the factors affecting the productivity of their active metabolite(s)

## MATERIALS AND METHODS

### Sample collection and preparation:

Forty eight soil samples were collected from fifteen different localities distributed through South and North Sinai Governorates, Egypt. The samples were collected aseptically from a 10-15 cm depth in clean plastic bags with a sterile spatula. The samples were immediately brought to the laboratory of the Plant Protection Department, DRC. Soil samples were air-dried and were sieved through a 2 mm sieve. To reduce the vegetative bacterial cells and allow the spores of actinomycetes to survive the sieved soils were mixed before plating with Ca CO<sub>3</sub> in a 10:1 (w/w) ratio.

### Isolation of actinomycetes colonies:

One gram of the prepared soil was suspended with 10 ml of sterile distilled water and incubated at room temperature (25 ± 2°C) for 1h on a rotary incubator shaker with vigorous shaking. Soil suspension (100 µl) was spread on starch nitrate agar (SCA) Petri-dishes and incubated at 30°C for 4 days. Colonies of actinomycetes were picked up using sterile toothpicks, placed onto SCA

plates and incubated for 7 days. Pure colonies of actinomycetes were then subcultured onto SCA slants and incubated for 7 days at 30°C (Sandeepa and Menaka, 2014).

### Preparation of actinomycete filtrates :

Actinomycetes filtrates were prepared by cutting 3 discs (9 mm in diameter) of pure actinomycete colony, inoculated in a 250 ml Erlenmeyer flask containing 100 ml of a liquid medium (starch nitrate broth), and incubated on a shaker (200 rpm) at 30 °C. for 7 days (Walker *et. al.*, 1966). At the end of the incubation period, the culture was centrifuged at 15,000 rpm (1260 g) for 20 min. and the supernatant was stored at 4 °C. until used.

### Microorganisms used for testing of antimicrobial activity:

Gram-positive bacteria: (*Bacillus subtilis* and *Staphylococcus aureus*), Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), unicellular fungi: (*Candida albicans*) and filamentous fungi: (*Aspergillus niger*, *Aspergillus flavus*), *Rhizoctonia solani* and *Fusarium oxysporum*.

### Screening of actinomycete filtrates for their antimicrobial activity.

#### 1. Diffusion plate method:

Estimation of antimicrobial activity of actinomycete filtrates was carried out using the agar well diffusion method (Cooper, 1963) based on the observation of inhibition clearing zone of microbial growth on an agar medium. The seeded plates with target test organisms were cut by a sterile cork borer to make holes (9 mm in diameter and 2 cm apart). Only 0.1 ml of each actinomycete filtrate was transferred into a hole under aseptic conditions and allowed to diffuse at room temperature for 2 hrs. then the plates were incubated at 37°C for 24 h. Clear inhibition zone around each hole was measured in mm.

#### 2. Minimal inhibition concentration method:

The most potent isolates were further investigated for their minimal inhibitory concentration (MIC). Minimal inhibitory concentration (MIC) value was defined as the lowest concentration of each natural product which completely inhibited microbial growth. Chosen filtrates were dried under reduced pressure and series of concentrations (100, 50, 25, 12.5, 6.25 and 3.125 mg/ml) were prepared and the minimal inhibitory concentration was determined by the disk diffusion method as described by Klančnik *et al.*, (2010).

**Factors affecting the productivity of actinomycete (*Streptomyces lavendulae* SST-32) active metabolite(s):**

Several factors were tested for their role on the productivity of bioactive substance(s) by the actinomycetes *Streptomyces lavendulae*.

1. Suitable nutritional media (starch-nitrate, yeast extract-malt extract and Czapek's medium).
2. Cultivation technique (Static and Shaking).
3. Incubation period (up to 10 days)
4. pH values: The initial pH values of the media were adjusted to cover the range from 4 to 10 before sterilization.
5. Incubation temperature: 20, 25, 30, 35, 40 and 45 °C.

6. Nutritional requirements:

Starch nitrate medium lacking its carbon or nitrogen sources was supplemented with different types of sugars or nitrogen sources at equi-molecular amounts.

**A-** Carbon source: The carbon sources were represented by, D-glucose, D-fructose, soluble starch, rhaminose, maltose, sucrose, mannose, D-xylose and meso-Inositol.

**B-** Nitrogen source: KNO<sub>3</sub>, NaNO<sub>3</sub>, (NH<sub>4</sub>)<sub>2</sub>.SO<sub>4</sub>, NH<sub>4</sub>NO<sub>3</sub>, urea, casein, yeast extract and peptone). The nitrogen sources were added in an equi-molecular amount of nitrogen except peptone and yeast extract which used at the concentration of 0.2% for each.

**RESULTS and DISCUSSIONS**

**Primary Screening Test:**

Forty eight soil samples were collected from 15 sites in north and south Sinai. From them seventy three actinomycete isolates were obtained and purified. Soil types, associated plants and number of isolates from each site were described and tabulated in a previous study (Abdel-Rahman *et.al.*, 2017). Actinomycete isolates were subjected to primary screening for their antimicrobial activities against soil bacteria (*Staphylococcus aureus* and *Escherichia coli*) and fungi (*Candida albicans* and *Aspergillus flavus*). Isolates which caused  $\geq$  10 mm inhibition zone on tested bacteria and fungi were considered as active isolates and selected for secondary screenings. Of these 73 isolates only 23 isolates (Table 1) were considered as active isolates. Twelve of them (S1, S2, S6, S7, S8, S13, S19, S28, S35, S36, S41 and S46) had excellent antimicrobial action against the four tested organisms, while the other 11 isolates (S3, S4, S5, S14, S16, S20, S21, S22, S23, S27 and S40) came in the second category. On the contrary twenty six actinomycete isolates failed to exhibit any antimicrobial activity, while the remainder twenty four isolates were exhibited moderate to slight effects.

**Antibacterial screening of selected active isolates:**

Of the total 73 isolates 23 isolates were selected as active isolates and were tested for their antibacterial action against *B. subtilis* and *S. aureus* as Gram-positive bacteria and *E. coli* and *P. aeruginosa* as Gram-negative bacteria. As represented in Table (2), *P. aeruginosa* was found to be the most resistant species in comparison to the other tested bacteria as only 6 isolates (S3, S6, S13, S27, S35 and S46) could inhibit its growth, while on the other hand *B. subtilis* bacteria was the most sensitive species as all the tested isolates harmed its growth. The other two species (*S. aureus* and *E. coli*) were in between. Isolates number S4, S19, S21 and S40 were found to possess strong activity against Gram negative bacteria as they completely inhibited the growth of the two tested species

(*E. coli* and *P. aeruginosa*). On the basis of the broad spectrum and highly inhibition zone only five actinomycete isolates (S3, S6, S13, S35 and S46) had the most wide antibacterial spectrum as they evidently

inhibited the growth of the four tested bacteria. These five most potent isolates were further investigated for their minimal inhibitory concentration (MIC).

Table (1): Primary antimicrobial screening of the 73 actinomycetes isolates.

No.	Highly active isolates	Active isolates	Moderate and slightly active	Not active
1	S1	S3	S9	S11
2	S2	S4	S10	S24
3	S6	S5	S12	S25
4	S7	S14	S15	S26
5	S8	S16	S17	S29
6	S13	S20	S18	S31
7	S19	S21	S30	S32
8	S28	S22	S33	S37
9	S35	S23	S34	S42
10	S36	S27	S38	S43
11	S41	S40	S39	S44
12	S46	---	S49	S45
13	---	---	S53	S47
14	---	---	S55	S48
15	---	---	S56	S50
16	---	---	S57	S51
17	---	---	S58	S52
18	---	---	S63	S54
19	---	---	S66	S59
20	---	---	S68	S60
21	---	---	S70	S61
22	---	---	S71	S62
23	---	---	S72	S64
24	---	---	S73	S65
25	---	---	---	S67
26	---	---	---	S69

Table (2): Anti-bacterial activity of the selected 23 actinomycete filtrates

Isolate No.	Mean diameter of inhibition zone (mm)			
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
S1	15	11.5	0	12.1
S2	11.8	15.5	0	10.6
<b>S3</b>	<b>12.7</b>	<b>11</b>	<b>16</b>	<b>10.7</b>
S4	11	16	0	0
S5	18	14	0	11.2
<b>S6</b>	<b>24.6</b>	<b>21.3</b>	<b>19.2</b>	<b>20.7</b>
S7	12.5	16	0	11
S8	12.8	12.5	0	14.2
<b>S13</b>	<b>19.3</b>	<b>18.4</b>	<b>13.2</b>	<b>15.8</b>
S14	15	14.4	0	10.5
S16	16	0	0	13
S19	14.5	12.7	0	0
S20	13.6	0	0	12.9
S21	14.3	14.2	0	0
S22	12.5	16	0	11
S23	11	0	0	12.5
S27	11.2	0	10.2	0
S28	14	14	0	10.5
<b>S35</b>	<b>16</b>	<b>16.0</b>	<b>13.5</b>	<b>18.3</b>
S36	13.3	0	0	12.2
S40	11.5	16	0	0
S41	15	14	0	10.5
<b>S46</b>	<b>13</b>	<b>14</b>	<b>10.5</b>	<b>11.9</b>

#### Minimum Inhibitory Concentration (MIC) of the most potent isolates against tested bacteria:

Series of concentrations (100, 50, 25, 12.5, 6.25 and 3.125 mg/ml) of the most potent isolates were prepared and tested for their MIC against four tested bacteria (*B. subtilis* and *S. aureus* as  $G^+$  bacteria and *E. coli* and *P. aeruginosa* as  $G^-$  bacteria). As shown in Table (3) the MIC value of isolate S6 against *B. subtilis* was 6.25 mg/ml as compared with 12.5 mg/ml for the other four actinomycete isolates. The same trend was

observed from isolate S6 against the other tested bacteria which meant that isolate S6 was the most potent isolate as it prevent the growth of *E. coli* at a concentration of 3.125 mg/ml and showed a MIC value 6.25 mg/ml against the other three tested bacteria. On the same way, results in that table also indicated that the minimum concentrations needed to inhibit the visible growth of all the four tested bacteria were 12.5, 6.25, 25, 25 and 25 mg/ml from isolates S3, S6, S13, S35 and S46, respectively.

Table (3): MIC (mg/ml) of selected actinomycete isolates against the 4 tested bacteria.

Isolate No.	Tested bacteria			
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
S3	12.5	12.5	12.5	6.25
<b>S6</b>	<b>6.25</b>	<b>6.25</b>	<b>6.25</b>	<b>3.125</b>
S13	12.5	12.5	25	12.5
S35	12.5	25	25	12.5
S46	12.5	25	25	12.5

#### Antifungal screening of the selected active isolates:

Of the total 73 isolates 23 isolates were selected as active isolates and were tested for their antifungal action against *C. albicans*, *A. niger*, *A. flavus*, *R. solani* and *F. oxysporum*. Results represented in Table (4) showed that isolates S6, S13 and S35 were the most potent against *F. oxysporum*, *R. solani* and *C. albicans* but they were less effective against *A. niger*. Isolates S4, S19 and S23 were the most effective against this fungus. On the same way isolates S1, S6, S13 and S23 had the greatest values of inhibition zones of *A. flavus* radial growth. On the other hand seven of the 23 tested isolates had no visual harm effects on the growth of *A. niger* fungus.

On the basis of the broad spectrum and highly inhibition zone results in Table (4) showed that only five actinomycete isolates (S1, S6, S13, S35 and S36) had the most wide antifungal spectrum as they evidently harmed the growth of all the five tested fungi. These five most potent isolates were further investigated for their minimal inhibitory concentration (MIC).

#### Minimum Inhibitory Concentration (MIC) of the most potent isolates against the tested fungi:

Results in Table (5) showed that the minimum concentration needed to inhibit the visible growth of all the five tested fungi was obtained when they were treated with isolate

S6 filtrate at a concentration of 6.25 mg/ml. Filtrates of isolates S13 and S35 came in the following category with 12.5 mg/ml MIC value. On the other hand isolates S1 and S36 had the least MIC value with 25 mg/ml. These results are in harmony with Deshmukh and Vidhale, (2014) who noticed that in a primary screening out of 147 actinomycete isolates 50 isolates (34.01%) showed an activity against 2 test bacteria such as *Staph. aureus* and *E.coli* by agar overlay technique. In secondary screening, out of 50 primary isolates 19 actinomycete isolates were preceded for an antibacterial activity against *Staphy. aureus* (MTCC 7443), *B. subtilis* (MTCC 441), *E.coli* (MTCC 443) and *P. aeruginosa* (MTCC 424) by agar well diffusion method. Similar results have been reported by Attimarad *et.al.*, (2012) who showed that only six isolates namely ACT-A2, ACT-A3, ACT-A4, ACT-A5, ACTA7 and ACT-A15 showed significant antibacterial activity against both gram-positive and gram-negative bacteria. Alpana *et.al.* (2010) showed that among 316 isolates, 98 (31.01%) isolates exhibited antifungal activity against one or more pathogens. Out of 98 active isolates, 19, 67, 42, 37, 18 and 25 isolates showed activity against *C. albicans*, *Trichophyton rubrum*, *Microsporium canis*, *M. gyseum*, *A. flavus*, *A. fumigatus* respectively, while 7 isolates showed activity against all the fungal pathogens.

The previous results with bacteria and fungi studies confirmed that isolate S6 was the most efficient with the highest antimicrobial activity. This actinomycete isolate was isolated from the rhizosphere of

*Tamarix nilatic* plants grown in a sandy soil at El-Tor area - Sinai and taxonomically was identified as *Streptomyces lavendulae* SST-32 (Abdel-Rahman *et.al.*, 2017).

Table (4): Anti-fungal activity of the selected 23 actinomycete filtrates

Isolate No.	Mean diameter of inhibition zone (mm)				
	<i>F. oxysporium</i>	<i>R.solani</i>	<i>C. albicans</i>	<i>A. niger</i>	<i>A. flavus</i>
<b>1</b>	<b>11.0</b>	<b>10.2</b>	<b>16.5</b>	<b>14.0</b>	<b>14.3</b>
2	10.2	11.5	12.3	0.0	10.2
<b>3</b>	<b>12.0</b>	<b>14.0</b>	<b>11.9</b>	<b>0.0</b>	<b>0.0</b>
4	13.3	0.0	10.4	19.0	13.0
5	10.5	11.6	0.0	12.5	11.5
<b>6</b>	<b>18.5</b>	<b>17.5</b>	<b>25.2</b>	<b>15.2</b>	<b>17.8</b>
7	10.5	14.5	12.24	0.0	13.8
8	11.8	0.0	12.2	0.0	11.5
<b>13</b>	<b>20.5</b>	<b>18.8</b>	<b>16.5</b>	<b>16.0</b>	<b>18.4</b>
14	0.0	15.4	9.9	13.0	0.0
16	11.5	0.0	10.6	0.0	11.8
19	0.0	13.5	14.2	19.0	12.0
20	14.0	12.6	0.0	13.6	12.8
21	9.9	12.4	14.5	19.0	0.0
22	15.2	14.5	0.0	15.4	11.5
23	12.5	13.7	0.0	11.5	16.5
27	12.8	11.8	14.3	16.0	0.0
28	0.0	14.2	13.7	10.2	10.0
<b>35</b>	<b>17.2</b>	<b>16.8</b>	<b>18.7</b>	<b>16.7</b>	<b>11.0</b>
<b>36</b>	<b>14.6</b>	<b>13.5</b>	<b>13.5</b>	<b>12.0</b>	<b>12.2</b>
40	12.5	0.0	15.8	0.0	12.0
41	10.9	0.0	11.6	13.0	0.0
46	0.0	17.1	13.2	0.0	9.8

Table (5): MIC (mg/ml) of selected actinomycete isolates against the five tested fungi.

Isolate No.	Tested fungi				
	<i>F. oxysporium</i>	<i>R.solani</i>	<i>C. albicans</i>	<i>A. niger</i>	<i>A. flavus</i>
1	25	25	25	25	25
<b>6</b>	<b>6.25</b>	<b>6.25</b>	<b>6.25</b>	<b>6.25</b>	<b>6.25</b>
13	12.6	12.5	12.5	12.5	12.5
35	12.5	12.5	12.5	12.5	12.5
36	25	25	25	25	25

#### Factors affecting the productivity of actinomycete (*Streptomyces lavendulae* SST-32) active metabolite(s).

Three media named starch-nitrate, yeast extract-malt extract and Czapek's medium were tested to detect the suitable nutritional media needed for the production of the maximum yield of the bioactive

metabolite(s) by *S. lavendulae* SST-32. As shown in Table (6) starch-nitrate medium was the best medium for the production of the bioactive metabolite as compared with the two other media and that shaking cultivation technique was more suitable than the static one. Concerning to the suitable incubation period and the initial pH value

of the medium, results revealed that the yield of the active metabolite(s) increased gradually with increasing the incubation period up to the seventh day and began to decrease after that. By the same way adjusting the initial pH of the medium at 7 gave the maximum inhibition zone (23.3 mm) and yielded the highest mycelium dry weight (0.36 mg/50 ml). Adjusting initial pH at values below or above 7 caused gradual decrease in activity. The suitable incubation temperature for maximizing active metabolite(s) productivity by *S. lavendulae* was between 30°C. and 35°C. At 30°C. the produced metabolites caused 23.5 mm inhibition zone and yielded 0.32 mg/50 ml mycelium dry weight while at 35°C. it caused 22.2 mm inhibition zone and yielded 0.28 mg/50 ml mycelium dry weight.

When *S. lavendulae* SST-32, was allowed to grow on a growth medium containing different carbon sources data represented in Table (6) revealed that, the highest active metabolite(s) productivity could be obtained in the presence of soluble starch followed by D-glucose as carbon source. By the same way when *S. lavendulae* SST-32, was allowed to grow on a growth medium containing different nitrogen sources results showed that inorganic forms of nitrogen ( $\text{KNO}_3$ ,  $\text{NaNO}_3$ ,  $(\text{NH}_4)_2\text{SO}_4$  and  $\text{NH}_4\text{NO}_3$ ) were more suitable than the organic forms (urea, casein, yeast extract and peptone) and that sodium nitrate in the medium produced the highest active metabolites.

Similar results were obtained by Aly *et. al.* (2006) who reported that, maximum antibacterial recorded against *B. subtilis* found at 30°C. Jicheng *et.al.* (2007) noticed that the range of pH for the proper growth of *Streptomyces* is between 7 and 7.5. As well, the initial pH influences the apparent decomposition of antibiotic, and pH changes also influence culture conditions for most fermentation processes (Parente *et. al.* 1994). Yang *et. al.* (2006) found that,  $\text{KNO}_3$ , and  $(\text{NH}_4)_2\text{SO}_4$  favored antibiotic production by *Xenorhabdus spp.*

As a conclusion, the most parameters affecting the productivity of the actinomycete *Streptomyces lavendulae* SST-32 active metabolite(s) were growing the actinomycete on starch-nitrate medium containing soluble starch and sodium nitrate as carbon and nitrogen sources, adjusting initial pH on 7 and incubation in a shaking system at 30°C for seven days.

This study proves that the Egyptian soil is rich in microorganisms which can be a valuable source for different biological control agents. This would be a bright direction to develop new bioactive pesticides. The study also lights the scope on the antimicrobial activities of actinomycete metabolites and the ability to use them as environmentally and friendly alternatives for the extensive use of chemical pesticides in integrated pest management programs.

Table (6): Factors affecting the productivity of *Streptomyces lavendulae* SST-32 active metabolite(s).

Tested Factor		inhibition Zone (mm)	Dry Wight (mg/50 ml)
Nutritional media	Starch- nitrate medium	18.4	0.35
	Yeast- malt extract medium	14.2	0.27
	Czapeks medium	11.8	0.24
Fermentation technique	Static	15.6	0.25
	Shaking	18.5	0.36
Incubation period	1	1	0.03
	2	8	0.06
	3	9	0.13
	4	10	0.14
	5	12	0.21
	6	15	0.27
	7	18	0.36
	8	14	0.25
	9	12	0.16
Initial pH	4	10.2	0.18
	5	11.2	0.2
	6	12	0.25
	7	23.3	0.34
	8	15.5	0.22
	9	12.1	0.16
Incubation Temp.	25	15.8	0.12
	30	23.5	0.32
	35	22.2	0.28
	40	12.2	0.13
Nitrogen source	KNO <sub>3</sub>	19	0.28
	NaNO <sub>3</sub>	23	0.34
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	18.8	0.27
	NH <sub>4</sub> NO <sub>3</sub>	18.6	0.28
	Urea	15	0.21
	Casein	14.4	0.22
	Yeast extract	16.2	0.19
	Peptone	14.7	0.18
Carbon source	D-glucose	19.2	0.28
	D-fructose	17.7	0.27
	Soluble starch	20.2	0.34
	rhaminose	18.5	0.32
	maltose	17.3	0.28
	sucrose	15.2	0.22
	mannose	15.4	0.24
	D-xylose	15	0.19
	meso-Inositol	13.5	0.2

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## ARABIC SUMMARY

لنشاط المضاد للميكروبات لبعض سلالات الأكتينومايسيت المعزولة من تربة سيناء المصرية

عبدالرحمن جمال الدين عبدالرحمن<sup>١</sup> - بهجت محمد رفعت<sup>٢</sup> - محمد هلال السيد<sup>٢</sup> - عبدالناصر أحمد قبيصي<sup>١</sup>

١- قسم وقاية النبات - مركز بحوث الصحراء - المطرية - القاهرة - مصر

٢- قسم النبات والميكروبيولوجي - كلية العلوم (بنين) - جامعة الأزهر - القاهرة - مصر

تم الحصول على ٧٣ عزلة نقية من الأكتينومايسيت تم عزلها من ٤٨ عينة تربة من منطقة الرايزوسفير ممثلة لمختلف الأماكن بسيناء. هذه العزلات تم اختبار نشاطها المضاد للميكروبات على بكتريا *Staphylococcus aureus* الموجبة لصبغة جرام و البكتريا *Escherichia coli* وبكتريا *Pseudomonas aeruginosa* السالبة لصبغة جرام و فطريات *Candida albicans* و *Aspergillus niger* و *Aspergillus flavus* و *Rhizoctonia solani* و *Fusarium oxysporum*. كانت العزلات S6 – S13 – S35 هي الأعلى كفاءة ضد انواع الفطريات و البكتريا المختبرة حيث اعطت أقل قيم في اختبار اقل تركيز مثبط للنمو (MIC) و لمداهها الابادى الأكبر لأنواع البكتريا و الفطريات المختبرة. تم دراسة العوامل المؤثرة على انتاج المواد الفعالة حيويًا بواسطة الاكتينومايسيتيس.