

Isolation and Screening of some Actinomycetes from Soil from Damietta and Mansoura and its Antimicrobial Activities

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ABSTRACT

Sixty two isolates of actinomycetes were isolated from different eight sandy and clay soils from various locations of Egypt (Damietta and Mansoura) during the year 2016. All isolates of actinomycetes were screened against known different types of bacteria and fungi like: three Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*), two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and one fungus (*Candida albicans*). About nineteen strains of actinomycetes were isolated which had different antibacterial and antifungal activity. Two potent actinomycetes isolates were selected to be identified according to their morphological, physiological and biochemical characterization and identified as *Streptomyces rimosus*.

Keywords: Antibacteria, anticandida, *Streptomyces rimosus*.

INTRODUCTION

Recently, there is an urgent need to find new drugs, especially antibiotics, to control the spread of antibiotic resistant pathogenic microorganisms (Spellberg *et al.*, 2008; Fischbach and Walsh, 2009). Soil microorganisms provide an excellent resource for the isolation and identification of therapeutically important products. Consequently, actinomycetes remain at the top of the natural antibiotic producers (Magarvey, *et al.*, 2004). Actinomycetes are aerobic, filamentous, spore forming gram-positive bacteria, characterized by substrate and aerial mycelia growth (Lacey, 1997). The bio-active secondary metabolites produced by actinomycetes include antibiotics, immunosuppressive agents, antitumor agents and enzymes. The secondary metabolites from actinomycetes are known to possess antibacterial, antifungal, antioxidant, anti-cancer, anti-algal and anti-inflammatory (Kekuda *et al.*, 2010; Ravikumar *et al.*, 2011). *Streptomyces* spp. is the largest genus of actinomycetes and the type genus of the family Streptomycetaceae (Hong *et al.*, 2009). Streptomycetes are filamentous Gram-positive bacteria that belong to the phylum of Actinobacteria. These bacteria produce over 60% of all known antibiotics and many other bioactive natural products (Hopwood, 2007; Barka, 2016). 10,000 known antibiotics, about 45–55% were produced by *Streptomyces* spp. (Lazzarini *et al.*, 2000). This study was done to isolate actinomycetes from soil samples collected from Egypt and test their ability against some bacteria and yeast, and searching for microorganisms which produces some antimicrobial compound for treating some diseases.

MATERIALS AND METHODS

Isolation of actinomycetes

According to (Johnson *et al.*, 1960) actinomycetes strains were isolated from eight soil samples coded from A to H code. Soil samples were taken from the soil surface with the depth of 15 to 20 cm. 10 gm of the soil sample were added into conical flask containing 90 ml sterile distilled water. By using an orbital shaker at room temperature soil samples were shaking well for 15 minutes. Isolation of actinomycetes was made by using the dilution methods techniques which range from 10⁻¹ to 10⁻⁶ by transferring 1ml from each dilution into another dilution respectively (Elliah. *et al.*, 2004). Under sterilized conditions one ml of each dilution was transferred into sterilized petri dishes, then approximately 20 ml of starch-nitrate agar medium (Starch 10.0 gm; KNO₃ 3.0gm; NaCl 2.0gm; K₂HPO₄ 1.0gm; MgSO₄.7H₂O 0.05gm; CaCO₃ 0.02gm; FeSO₄.7H₂O 0.01gm; Agar 12.0gm; water 1000ml) was poured to this sterilized petri dishes, the plates were incubated at 30°C and

noticed after 3, 5 and 7 days until the colonies had appeared. The growing colonies were subcultured and purified.

Screening of isolates on solid media

All isolates of actinomycetes were grown on starch-nitrate agar media for 7 days at 30°C. Agar discs were cut off by a sterilized cork-borer (0.9 cm in diameter) by using agar plate diffusion method (Wu, 1984), and transferred into the surface of nutrient agar plates inoculated with spore suspension of tested bacteria and yeast as follow: three Gram-negative bacteria namely (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*), two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and yeast (*Candida albicans*). Antibacterial and anticandida activities were determined by measuring the inhibition zone diameter (mm).

Identification of the most potential isolate of actinomycetes

The selected isolates G2 and G10 of actinomycetes were identified according to the International Streptomyces Project (ISP) by Shirling and Gottlieb (1968a; 1968b; 1969 and 1972); Pridham and Tresner, (1974) and Bergey's Manual of systematic Bacteriology (Williams *et al.*, 1989).

Cultural properties

The cultural properties of the selected strains of actinomycetes were studied, namely the type of growth, color of aerial and substrate mycelium and growth intensity. These cultural properties were studied on different type of media as follow: starch-nitrate agar, starch-ammonium sulphate agar, Dox agar, glucose-nitrate agar, glycerol-nitrate agar, glycerol-asparagine agar, oatmeal agar and yeast malt extract agar.

Morphological properties

The selected isolates of actinomycetes were morphologically identified to describe spore formation, aerial and substrate mycelium by using cover-slip method under light microscope and Scanning Electron Microscope on JEOL- JSM- 6510 LV system at Electron Microscope Unit on faculty of Agriculture, Mansoura University.

Physiological properties

Physiological characteristics included starch hydrolysis, liquification of gelatin, urea hydrolysis, melanoid pigment, coagulation of milk, Lecithovitellin (LV) Reaction, decomposition of cellulose, reduction of nitrates to nitrites, hydrogen sulfide production, esculin hydrolysis, using of different carbon sources and nitrogen sources, resistance to antibiotics were carried out for the tested isolates.

Chemotaxonomic analysis

Hydrolysate analysis of actinomycetes whole cell

Determination of the cell wall analysis composition which including diaminopimelic acid (DAP) and type of sugars were carried out according to Stanek and Roberts (1974).

RESULTS

Sixty two isolates of actinomycetes were collected and isolated by using starch-nitrate agar medium from different eight sandy and clay soils (A to H) from various locations of Egypt (Damietta and Mansoura) within the year 2016. All isolates of actinomycetes from soil samples were arranged in Table 1 according to its color (white, grey, orange, pink and pinkish white), code of soil, site of isolation and plant cover were also listed. Actinomycetes were appeared in the form of colonies characterized by earthy odor, powdery and even pinpoint colonies with different colors (Table 1).

Table 1. Actinomycetes colonies isolated from soil samples:

Isolate number	Isolate code	Isolate color	Cover plant \Tree	Location
1	A 1	White	<i>Medicago sativum</i> and <i>Psedium guajava</i>	Damietta
2	A 2	Grey	Same as above	Damietta
3	A 3	Orange	Same as above	Damietta
4	A 4	White	Same as above	Damietta
5	A 5	White	Same as above	Damietta
6	A 6	White	Same as above	Damietta
7	A 7	Grey	Same as above	Damietta
8	A 8	White	Same as above	Damietta
9	A 9	White	Same as above	Damietta
10	A 10	Grey	Same as above	Damietta
11	B 1	White	<i>Solanum lycopersicum</i>	Damietta
12	B 2	Pink	Same as above	Damietta
13	B 3	White	Same as above	Damietta
14	B 4	White	Same as above	Damietta
15	B 5	White	Same as above	Damietta
16	B 6	Grey	Same as above	Damietta
17	C 1	Grey	<i>Medicago sativum</i>	Damietta
18	C 2	White	Same as above	Damietta
19	C 3	White	Same as above	Damietta
20	C 4	White	Same as above	Damietta
21	C 5	Grey	Same as above	Damietta
22	C 6	White	Same as above	Damietta
23	C 7	Grey	Same as above	Damietta
24	D 1	White	<i>Solanum tuberosum</i>	Mansoura
25	D 2	white	Same as above	Mansoura
26	E 1	pink	<i>Lactuca sativa</i>	Mansoura
27	E 2	pink	Same as above	Mansoura
28	E 3	white	Same as above	Mansoura
29	E 4	grey	Same as above	Mansoura
30	E 5	white	Same as above	Mansoura
31	E 6	white	Same as above	Mansoura
32	E 7	white	Same as above	Mansoura
33	E 8	white	Same as above	Mansoura
34	F 1	white	<i>Psidium guajava</i>	Damietta
35	F 2	grey	Same as above	Damietta
36	F 3	white	Same as above	Damietta
37	F 4	white	Same as above	Damietta
38	F 5	white	Same as above	Damietta
39	G 1	white	<i>Abelmoschus esculentus</i>	Damietta
40	G 2	Pinkish white	Same as above	Damietta
41	G 3	orange	Same as above	Damietta
42	G 4	white	Same as above	Damietta
43	G 5	Pinkish white	Same as above	Damietta
44	G 6	grey	Same as above	Damietta
45	G 7	white	Same as above	Damietta
46	G 8	grey	Same as above	Damietta
47	G 9	Pinkish white	Same as above	Damietta
48	G 10	Pinkish white	Same as above	Damietta
49	G 11	grey	Same as above	Damietta
50	G 12	white	Same as above	Damietta
51	H 1	white	<i>Ficus retusa</i>	Damietta
52	H 2	white	Same as above	Damietta
53	H 3	grey	Same as above	Damietta
54	H 4	grey	Same as above	Damietta
55	H 5	pink	Same as above	Damietta
56	H 6	white	Same as above	Damietta
57	H 7	white	Same as above	Damietta
58	H 8	white	Same as above	Damietta
59	H 9	pink	Same as above	Damietta
60	H 10	grey	Same as above	Damietta
61	H 11	white	Same as above	Damietta
62	H 12	white	Same as above	Damietta

Screening of isolates on solid media

All actinomycetes isolates were screened against *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus cereus* and *Candida albicans*. The results showed that about nineteen of

actinomycetes isolates had different antibacterial and antifungal activity as result expressed in table (2) showed that the width of clear inhibition zones. It was clear that the antagonism against Gram-positive bacteria was greater than Gram-negative. All actinomycetes isolates were not have antibacterial activity against *Pseudomonas aeruginosa* and only one isolate has antibacterial activity against *Klebsiella pneumoniae*. Eleven isolates had anticandidal activity against yeast *Candida albicans*. We chose the isolates G2 and G10 because they had high antibacterial and anticandidal activity with height inhibition zones against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Candida albicans*.

Table 2. Antibacterial and antifungal activity of the total isolates of actinomycetes (Inhibition zone by mm)

Isolate number	Isolate code	<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Klebsiella pneumoniae</i>	<i>Candida albicans</i>
1	A1	-	-	-	-	-	-
2	A2	-	13	-	-	-	14
3	A3	-	-	-	-	-	-
4	A4	-	-	15	-	-	-
5	A5	23	-	20	-	-	-
6	A6	-	-	-	-	-	-
7	A7	-	-	-	-	-	-
8	A8	-	-	-	-	-	-
9	A9	-	-	-	-	-	-
10	A10	14	-	-	-	-	-
11	B1	-	-	-	-	-	-
12	B2	-	12	-	-	-	10
13	B3	-	14	-	-	-	-
14	B4	-	-	-	-	-	-
15	B5	-	-	-	-	-	-
16	B6	-	-	-	-	-	-
17	C1	-	10	-	-	-	11
18	C2	-	-	-	-	-	-
19	C3	-	-	-	-	-	-
20	C4	13	-	17	-	-	-
21	C5	-	-	-	-	-	-
22	C6	-	-	-	-	-	-
23	C7	-	-	-	-	-	-
24	D1	-	-	-	-	-	-
25	D2	-	12	-	-	-	16
26	E1	14	14	18	-	-	14
27	E2	-	-	-	-	-	-
28	E3	-	-	-	-	-	-
29	E4	-	-	-	-	-	-
30	E5	-	-	-	-	-	-
31	E6	-	-	-	-	-	-
32	E7	-	-	-	-	-	-
33	E8	-	-	-	-	-	-
34	F1	-	-	-	-	-	-
35	F2	-	-	-	-	-	-
36	F3	-	-	-	-	-	-
37	F4	-	-	-	-	-	-
38	F5	-	-	-	-	-	-
39	G1	-	15	-	-	-	12
40	G2	28	14	20	-	-	12
41	G3	-	-	-	-	-	-
42	G4	-	-	-	-	-	-
43	G5	30	14	15	-	-	-
44	G6	-	-	-	-	-	-
45	G7	30	12	20	-	-	-
46	G8	-	20	-	-	11	15
47	G9	-	-	23	-	-	10
48	G10	32	15	16	-	-	11
49	G11	-	-	-	-	-	-
50	G12	-	-	-	-	-	-
51	H1	-	-	-	-	-	-
52	H2	16	13	-	-	-	12
53	H3	-	-	-	-	-	-
54	H4	10	-	-	-	-	-
55	H5	-	-	-	-	-	-
56	H6	-	-	-	-	-	-
57	H7	-	-	-	-	-	-
58	H8	-	-	-	-	-	-
59	H9	-	-	-	-	-	-
60	H10	-	-	-	-	-	-
61	H11	-	-	-	-	-	-
62	H12	-	-	-	-	-	-

Identification the most active actinomycetes isolate

Cultural properties of the most potent actinomycetes isolate

The selected actinomycetes isolates G2 and G10 did not grow on yeast-malt agar media but grow well at starch-nitrate agar, starch ammonium sulphate agar, glycerol nitrate

agar, oatmeal agar and glucose-nitrate agar media. From Table (3) it was observed that the color of medium in all cultures was non-pigmented, the substrate mycelium was creamy color but in Dox agar medium was buff to brown. The type of growth on glycerol asparagine agar medium was leathery but on other cultures was powdery.

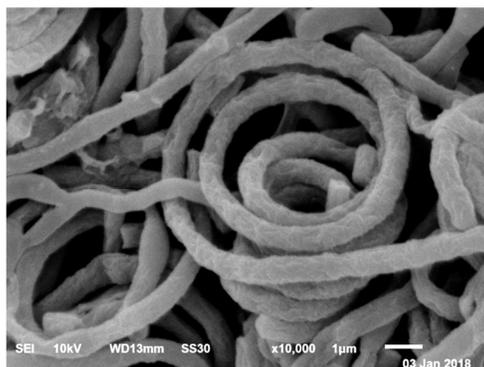
Table 3. Cultural properties of the most potent Actinomycetes isolates (G2 &G10)

Medium	Type of growth	Color of aerial mycelium	Color of substrate mycelium	Color of medium	Intensity of growth
1-Starch-nitrate agar	Powdery, good aerial mycelium	Pink with white	Creamy	Non-pigmented	+++
2-Starch-ammonium sulphate agar	Powdery, good aerial mycelium	White	Creamy	Non pigmented	+++
3-Glycerol –nitrate agar	Powdery, good aerial mycelium	White	Creamy	Non pigmented	+++
4-yeast-malt agar	-	-	-	-	-
5-Oatmeal agar	Powdery, good aerial mycelium	Pink	Creamy	Non-pigmented	+++
6-Dox agar	Powdery, good aerial mycelium	White to grey	Buff to brown	Non-pigmented	++
7-Glycerol asparagine agar	Leathery, good aerial mycelium	Whitish to creamy	Creamy	Non-pigmented	++
8-glucose- nitrate agar	Powdery, good aerial mycelium	White to pink	Creamy	Non –pigmented	+++

+++ : very good growth, ++: good growth, _ : no growth.

Morphology of spore chain of actinomycetes isolate

The most potent isolates (G2 &G10) was examined under light microscope and scanning electron microscope which showed the spiral and coiled sporophore and smooth spore surface as in figure 1 (A &B)



A



B

Figure 1. Sporophore morphology and spore surface of actinomycetes isolate (G2&G10) using scanning electron microscope

Physiological and biochemical properties of the most potent actinomycetes isolates

The selected actinomycetes isolates can hydrolyze starch and urea but can not coagulate milk and produce melanoid pigments as presented in (Table 4). In addition, the tested isolate had the ability to utilize many carbon sources

such as maltose, starch, glucose, fructose, arabinose, sucrose, lactose, rhamnose, xylose, raffinose and sodium acetate. Also, Data in Table 4 showed that the selected isolate could be utilized all nitrogen sources except L-methionine. For antibiotics resistance the isolates of actinomycetes were very sensitive against six different types of antibiotics as mentioned in Table 5 such as Penicillin (PEN), Lincomycin (LCN), Rifampicin (RAM), Levofloxacin (LEV), Tetracycline (TE) and Gentamycin (HLG). On the other side the selected isolate was not resistance to Cephalexin (CL), Amoxicillin (AMC), Cefotaxime (CTX) and Cefepime (FEP).

Table 4. Physiological properties of the most potent actinomycetes isolates (G2&G10)

Test	Result	Test	Result
Starch hydrolysis	+	Esculin hydrolysis	+
Liquification of gelatin	-	Urea hydrolysis	+
Melanoid pigmentation	-	Lecithovitellin reaction (LV)	+
Coagulation of milk	-	Decomposition of cellulose	-
Reduction of nitrates to nitrites	-	Production of hydrogen sulphide	+
Carbon utilization			
Maltose	+++	D-galactose	-
Starch	+++	D-mannitol	-
D-glucose	++	Myoinositol	-
D-fructose	++	cellulose	-
Arabinose	++	Sodium acetate	+
Sucrose	++	D- xylose	+
Lactose	+	Raffinose	++
Rhamnose	+		
Nitrogen utilization			
Potassium nitrate	+++	L-Proline	+++
L- Histidine	+	Peptone	++
L-Methionine	-	L-Valine	+++
phenylalanine	++	Hydroxyproline	+++
Casein	+++	L-Serine	+++
L-Tyrosine	+++	Cysteine	++
L-Threonine	++		

+++ Very high growth, ++ High growth, + Good growth and - No growth

Chemotaxonomic properties of the most potent isolates of actinomycetes (G2&G10)

The results of the chemotaxonomic properties indicated that the two isolates had characterized LL- DAP and no characteristic sugars.

According to cultural, morphological, physiological and chemotaxonomical properties, the most active actinomycetes isolates were identified as *Streptomyces rimosus*.

Table 5. Antibiotic resistance of most potent actinomycetes isolates (G2&G10)

Antibiotics	Inhibition zones by millimeters
Penicillin PEN	16 mm
Cephalexin CL	-
Lincomycin LCN	43 mm
Amoxicillin AMC	-
Cefotaxime CTX	-
Rifampicin RAM	57 mm
Cefepime FEP	-
Levofloxacin LEV	41 mm
Tetracycline TE	11 mm
Gentamycin HLG	40 m

-: not resistance

DISCUSSION

Actinomycetes have been identified primarily on morphological criteria for over a hundred years. Actinomycetes are the most widely distributed group of microorganisms in nature (Oskay et al., 2004). Actinomycetales are an important group of microorganisms which present in different soils (Bérdy, 2005). About 75% of the known commercially and medically useful antibiotics are produced by *Streptomyces* (Sujatha et al., 2005). Actinomycetes produced visible colony from 3rd day to 7th day with diversity and different colors like white, grey, orange, pink and pinkish white, noticed that all colonies possessed an earthy odour due to its ability to produce a wide range of metabolites such as enzymes, inorganic and organic acids, hydrogen sulphide and biopigments (Kubik, 2010).

Sixty two isolates were screened against known different types of bacteria and fungi as follow: *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Candida albicans*. The results indicated that about nineteen isolates of actinomycetes had different antibacterial and anticandidal activities after measuring the inhibition zone of the activity. The selected isolates (G2) and (G10) showed the highest antibacterial and anticandidal activity than other isolates as indicated against with *Escherichia coli*, *Staphylococcus aureus*, *Bacillus cereus* and *Candida albicans*. Bachiega et al., (2005) reported that 20.3% of the actinomycete isolates studied was active against *Candida albicans*. In addition, (Gandotra et al. 2012) observed that 33.3 of *Streptomyces spp.* analyzed showed some degree of activity against *Candida spp.* isolates. One similar to those the ones reported by (Kavithambigai, 2006), in this study, about 18% of actinomycetes isolates were active against *Candida albicans*, and 21% of actinomycetes isolates were active against *Bacillus cereus*.

The reasons for differential sensitivity to Gram negative and positive bacteria could be described to the physiological activities in these organisms. Gram negative bacteria have an outer membrane consisting of lipopolysaccharide compounds making the cell wall impermeable to lipophilic solutes. The Gram positive are more susceptible due to the presence of outer peptidoglycan layer which is not an effective permeability barrier (Pandey et al., 2005). The most potent of actinomycetes isolate had

the ability to utilize many carbon and nitrogen sources and resistance to many antibiotics.

According to the results of Hoare and Work (1957) reported that chemotaxonomy is that the study of chemical variation in organisms and also the use of chemical characters within the classification and identification. The two isolates G2& G10 of actinomycetes gave the same results in the composition of cell wall analysis and contained LL- isomer of diaminopimelic acid (DAP) and there were no characteristic sugar pattern. The presence of LL-diaminopimelic acid in the cell-wall preparations indicated that these organisms may be more closely related to some of the streptomycetes. Finally the tested isolates gave the same results typically and there was no significant variation between them and classified as *Streptomyces rimosus*.

Streptomyces rimosus is a known industrial producer of oxytetracycline and was originally isolated from soil (Finley et al., 1950). Most antibiotics are excreted as secondary metabolites when the producers are grown in rich media, therefore, the production and presence of antibiotics are likely to be limited to a few microhabitats where conditions are favorable (Williams, 1982).

CONCLUSION

In this study, sixty two isolates of actinomycetes were collected and isolated from different eight sandy and clay soils. All isolated strains of actinomycetes were screened against some of pathogenic bacteria and fungi. Nineteen of actinomycetes isolates had different antibacterial and anticandidal activities. *Streptomyces rimosus* was chosen and identified based on the physiological, morphological and chemotaxonomic characterizations. *Streptomyces rimosus* was characterized by production antimicrobial activity.

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عزل وعملية مسح لبعض الأكتينومايسيتات من التربة من دمياط والمنصورة و نشاطها ضد ميكروبي محمد اسماعيل أبو ديار¹، محمد محمد عبده موسى¹، محمد نجيب عبدالقنى حسنين¹ و سماح محمد نبيه² ¹ قسم النبات والميكروبيولوجي- كلية العلوم- جامعة دمياط جمهورية مصر العربية ² قسم النبات- كلية العلوم- جامعة المنصورة- جمهورية مصر العربية

اجريت هذه الدراسة بهدف الكشف عن كائنات دقيقة لها نشاط ضد ميكروبي وتم عزل اثنان وستون عزلة من الأكتينومايسيتات من تربة طينية ورملية مختلفة من المنصورة ودمياط بمصر كما تم عمل مسح ميكروبي لكل العزلات ضد بعض أنواع البكتريا الموجبة والسالبة لصبغة جرام وفطر الكانديدا وهي ايشريشيا كولاي، بسيدوموناس ايرجنوزا، كلبيسيلا نومينا، استافيلوكوكس اوريس، باسيلس سيريس، والخميرة كانديدا البيكانز. ومن اهم النتائج التي حصلنا عليها بعد المسح الميكروبي وجود تسعة عشر عزلة لها نشاط ضد بكتيري وضد الخميرة. وتم اختيار عزلتين لتعريفهما طبقا للصفات المزرعية والمورفولوجية والفسولوجية والتصنيف الكيميائي وتبين أنهم لكائن واحد وقد تم تعريفه: استربتوميسيس رايموسيزس (*Streptomyces rimosus*)