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RARE ACTINOMYCETES FROM EGYPTIAN HABITATS: ISOLATION AND SCREENING FOR ANTIMICROBIAL ACTIVITIES

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ABSTRACT

Sixty-six of rare actinomycetes were isolated from samples of soil, fresh water and decayed plants collected from different Egyptian localities. They were recovered on humic-vitamen B agar medium using dilution, several antibiotics as selective agents and mild heat techniques. They assessed for their antimicrobial activity using diffusion assay methods against seven Gram-positive bacteria, seven Gram-negative bacteria, two yeasts and two filamentous fungi. Among the 66 isolates, 35 (53%) strains showed an activity against some of the test organisms. The polyphasic identification of the active isolates revealed that they are commonly *Micromonospora* (23 isolate, 65.71%), less commonly *Actinoplanes* (11 isolates, 31.43%) and rarely *Dactylosporangium* (1 strain, 2.86%) genera.

Keywords: Antimicrobial activity, decayed plants, rare actinomycetes, soil, water.

INTRODUCTION

Screening of microorganisms for the production of novel antibiotics has been intensively pursued for many years by scientists. Actinomycetes have the capability to synthesise many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic and enzymes like cellulase and xylanase (Waksman, 1961; Lacey, 1973; Ouhdouch *et al*., 2001, Saadoun and Gharaibeh, 2003; Rifaat *et al*., 2007).

The term rare actinomycete was introduced by many workers to denote actinomycetes less frequently found in soil. Several genera of rare actinomycetes such as *Actinomadura, Actinoplanes, Amycolatopsis, Dactylosporangium, Microbispora* and *Micromonospora* have been described from which many enzymes and antibiotics have been discovered (Hacene *et al*., 1994; Lazzarini *et al*., 2000; Ouhdouch *et al*., 2001; Mazza *et al*., 2003).

The present investigation carried out for isolation of rare actinomycetes from Egyptian habitats as well as the determination of antagonistic spectra of whole isolates. The active rare actinomycetes isolates were also identified.

MATERIALS AND METHODS

1- Samples and bacterial strain isolation

Five cultivated rhizosphere soil samples from Qalubiya (S.Q), Sharkiya (S.S), Ismaelliya (S.I), Dakahliya (S.D) and Gharbiya (S.G); 5 fresh water samples from River Nile at Cairo (W.C1 & W.C2), Giza (W.G1 & W.G2) and El-Fayoum (W.F) as well as 5 different decayed plant samples from papyrus of the Gold Island (D.P1 & D.P2), Ward El-Neel at Sharkiya (D.W1 & D.W2) and maize at Kalubiya (D.M) were collected under aseptic conditions.

Bacterial strains were isolated from the soil samples by dilution techniques on humic-vitamin B agar medium which was recommended for isolation of rare actinomycetes (Hayakawa and Nonomura, 1984). For water samples, mild heat was applied for pre-treatment steps to reduce the dominance of fast growing actinomycetes and to facilitate the recovery of slow growing and relatively less competitive types (Cross, 1981). 0.2 ml of the pre-treatment sample was spread on the same previous medium. In case of decayed plant samples, small pieces of approximately 3 cm^2 are cut out and each piece transferred to a 100 ml conical flask containing 25 ml of sterile water. After 2 min. of agitation on rotary shaker, 0.1 ml of the leaf washing liquid was spread on the surface of the previous agar medium.

The antifungal cycloheximide (50 µg/ml) was used to inhibit the development of invasive fungi. Also, one of the following antibacterial agents was added to the isolation media: cycloserine, gentamycin and streptomycin (10 µg/ml). These antibiotics were chosen on the basis of good results obtained previously during the selective isolation of rare actinomycetes (Sabaon *et al*., 1998). The plates were incubated at 30°C for 3-4 weak and all colonies were examined directly by light microscopy to detect the rare actinomycetes isolates.

2- Antimicrobial assay

For determining the antimicrobial spectrum of the isolated rare actinomycetes, the diffusion assay method was used Bauer *et al*., 1966). Inhibition zones were measured after

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Table 1. Initial screening of rare actinomycetes strains.

Sample No.	Origin	Number of actinomycetes isolates	Number of active actinomycetes
S.Q	Soil of Qalubiya	8	5
S.S	Soil of Sharkiya	5	3
S.I	Soil of Ismaelliya	4	4
S.D	Soil of Dakahliya	4	2
S.G	Soil of Gharbiya	5	2
W.C1	Fresh water from Cairo	4	2
W.C2	Fresh water from Cairo	4	2
W.G3	Fresh water from Giza	3	1
W.G4	Fresh water from Giza	5	3
W.F	Fresh water from El-Fayoum	2	0
D.P1	Decayed plant of Papyrus	8	5
D.P2	Decayed plant of Papyrus	4	2
D.W1	Decayed plant of Ward El-Neel	3	3
D.W2	Decayed plant of Ward El-Neel	4	1
D.M	Decayed plant of Maize	3	0
Total		66	35

incubation at 30^0 C for 24 hours for bacteria and yeast and 48 hours for filamentous fungi.

The used target organisms were: (i) Gram-positive bacteria as *Staphylococcus aureus*, *Streptococcus thermophilus*, *Streptococcus pneumoniae*, *Micrococcus luteus, Enterococcus faecalis, Bacillus cereus* and *Bacillus subtilis,* (ii) Gram-negative bacteria as *Escherichia coli, Pseudomonas aeruginosae, Klebsiella pneumonia, Proteus* sp., *Shigella dysenteriae, Serratia marcescens* and *Acinetobacter calcoaceticus,* (iii) yeast as *Candida albicans* and *Saccharomyces cerevisiae* and (iv) fungi as *Aspergillus niger* and *Mucor racemosus*.

3- Morphological and cultural characteristics of the isolates

Morphological and cultural characteristics of isolated rare actinomycetes were examined according to the method described by Shiriling and Gottleib (1966) and Holt *et al*. (1994).

4- Chemotaxonomical analysis

Isolates were grown on yeast -malt extract broth for seven days at 30^0 C. Mycelia were harvested by centrifugation and washed by distilled water. These were used for chemical analysis of diaminopimelic acid (DAP) isomer and whole cell sugars according to the method of Hasegawa *et al*. (1983).

RESULTS AND DISCUSSION

1. Isolation of rare actinomycetes

Rare actinomycetes are present in small quantity in the various habitats and cannot be isolated by the current methods used in microbiology. Their selection must pass through elimination of other microorganisms which obstruct the growth of actinomycetes. Actinomycetes/ microorganisms ratio of a sample increases by (i) the use of certain sources of carbon and nitrogen making the cultures media less favourable to the growth of bacteria (Hayakawa and Nonomura, 1984; Cavalla and Eberlin, 1994), (ii) by the use of antibiotic substances which inhibit the development of bacteria and fungi (Larpent and Larpent-Gouragand, 1990) and (iii) by physical pre-treatment such as mild heat (Cross, 1981).

In the present work, results presented in Table 1 reveal a considerable variation of the numbers of rare actinomycetes strains isolated from each sample on the selective medium. Among the 66 isolated strains of rare actinomycetes, 26 were isolated from soil sample (39.5%), 18 from water sample (27%) and 22 from decayed plants (33.5%). The presence of a high number of rare actinomycetes in the soil and decayed plants is in agreement with the bibliographical data which let appear the soil as the principle reservoir of actinomycetes (Lemriss *et al*., 2003).

Table 2. Antimicrobial activity of isolated rare actinomycetes.

Sample No.	*Staphy-lococcus aureus*	*Strepto-coccus thermophilus*	*Strepto-coccus pneumoniae*	*Micrococ-ccus luteus*	*Enteroco-ccus faecalis*	*Bacillus cereus*	*Bacillus subtilis*	*Escherichia coli*	*Pseudomonas aeruginosae*
S.Q1	-	-	-	-	-	-	-	+	++
S.Q2	-	-	-	-	-	-	-	+	++
S.Q3	-	-	-	-	-	-	-	+++	+++
S.Q6	-	-	-	-	-	-	-	+++	+++
S.Q8	-	-	-	-	-	-	-	+++	+++
S.S2	-	-	-	-	-	-	-	+	++
S.S3	++	+	+	-	+	+	+	+	+++
S.S4	++	+	+	-	+	+	+	+	++
S.I1	++	+	+	-	+	+	+	+	++
S.I2	++	+	+	-	+	+	+	±	++
S.I3	-	-	-	-	-	-	-	+	++
S.I4	-	-	-	-	-	-	-	+++	+++
S.D1	-	-	-	-	-	-	-	+++	+++
S.D3	-	-	-	-	-	-	-	+++	+++
S.G3	-	-	-	-	-	-	-	+++	+++
S.G5	-	-	-	-	-	-	-	+	++
W.C1.2	-	-	-	-	-	-	-	+++	+++
W.C1.3	++	+	+	-	+	+	+	±	++
W.C2.1	-	-	-	-	-	-	-	++	++
W.C2.2	+	+	+	-	±	+	+	+	+
W.G1	++	+	+	-	+	+	+	+	+++
W.G2.1	-	-	-	-	-	-	-	+	+++
W.G2.2	-	-	-	-	-	-	-	+++	++
W.G2.5	-	-	-	-	-	-	-	++	++
D.P1.1	-	-	-	-	-	-	-	++	+++
D.P1.2	-	-	-	-	-	-	-	++	++
D.P1.4	-	+	+	-	+	+	++	++	-
D.P1.6	-	-	-	-	-	-	-	+	+++
D.P1.7	-	-	-	-	-	-	-	++	+
D.P2.3	-	+	+	-	±	+	+	+	-
D.P2.4	-	-	-	-	-	-	-	-	++
D.W1.1	-	+	±	-	+	+	++	+	-
D.W1.2	-	±	+	-	+	+	++	++	-
D.W1.3	-	-	-	-	-	-	-	-	+
D.W2.4	++	++	+	++	+	+	+	++++	++++

2. Antibiotic production

Among the rare actinomycetes isolates, only 35 (53%) were active against at least one of the tested microorganisms (Table 1). No active rare actinomycetes were isolated from fresh water sample of El-Fayoum (W.F) and sample from decayed plant of maize (D.M). Lemriss *et al*. (2003) indicated that the isolation of actinomycetes with antimicrobial activity is higher than 40% while Jiang and Xu (1996) mentioned that it is less than 10%. Figure 1 showed the percentage of the active strains of rare actinomycetes against the target test organisms. The percentage of isolates showed anti Gram-negative and anti yeast of 32.71%, while 23.37% showed anti fungal activity. In addition, only 11.21% showed anti Gram-positive bacteria. Rare actinomycetes with antibacterial and antifungal activities were 43.9 and 56.1% respectively.

Table. 2. Cont.

Sample No.	*Klebsiella pneumonia*	*Proteus* sp.	*Shigella dysenteriae*	*Serratia marcescens*	*Acinetobacter calcoaceticus*	*Candida albicans*	*Saccharomyces cerevisiae*	*Aspergillus niger*	*Mucor racemosus*
S.Q1	++	+	+	+	++	-	+	+	+
S.Q2	++	+	+	+	++	-	+	+	+
S.Q3	++	++	++	++	++	-	+	+	+
S.Q6	++	++	++	++	++	-	+	+	+
S.Q8	++	++	++	++	++	-	+	+	+
S.S2	++	+	+	+	++	-	+	+	+
S.S3	+++	+++	+	++	++	-	+	+	+
S.S4	++	+	±	+	++	-	+	±	±
S.I1	+	++	+	+	++	±	+	+	±
S.I2	++	++	+	+	+	-	+	+	+
S.I3	++	+	+	+	++	-	+	+	+
S.I4	++	++	++	++	++	-	+	+	+
S.D1	++	++	++	++	++	-	+	+	+
S.D3	++	++	++	++	++	-	+	+	+
S.G3	++	++	++	++	++	-	+	+	+
S.G5	++	+	+	+	++	-	+	+	+
W.C1.2	++	++	++	++	++	-	+	+	+
W.C1.3	+++	+++	+	++	++	-	+	+	+
W.C2.1	++	+	++	+	++	-	+	+	+
W.C2.2	++	+++	±	++	++	-	+	±	+
W.G1	+++	++	++	+	+	-	+	+	±
W.G2.1	++	++	++	++	+	-	+	+	+
W.G2.2	++	+	++	++	+	-	+	+	+
W.G2.5	+	+	+	+	++	-	+	±	+
D.P1.1	++	+	+	++	-	-	+	-	-
D.P1.2	+	+	+	+	-	-	+	-	-
D.P1.4	+	+	±	++	++	-	++	-	-
DP.1.6	++	+	+	++	-	-	+	-	-
DP.1.7	++	+	+	++	-	-	+	-	-
D.P2.3	±	+	+	++	++	-	++	-	-
D.P2.4	++	+	+	+	-	-	+	-	-
D.W1.1	+	±	+	+	+	-	+	-	-
D.W1.2	+	+	+	++	++	-	++	-	-
D.W1.3	+	+	+	++	-	-	+	-	-
D.W2.4	+	+	+	++++	++++	+	+	+	+

The activity of the isolated strains against the tested organisms is illustrated in Table 2. All strains isolated from soil, water together with the majority of decayed plants reveal high to moderate activity towards *Escherichia coli*, *Pseudomonas aeruginosae*, *Klebsiella pneumonia*, *Proteus* sp., *Shigella dysenteriae*, *Serratia marcescens*, *Acinetobacter calcoaceticus* and *Saccharomyces cerevisiae*. In addition, all strains isolated from soil and water samples showing moderate activity towards *Aspergillus niger* and *Mucor racemosus*. Some strains from the different studied habitats are moderately active against *Staphylococcus aureus*, *Streptococcus thermophilus*, *Streptococcus pneumoniae*, *Micrococcus luteus*, *Enterococcus faecalis*, *Bacillus cereus* and *Bacillus subtilis*. All isolates from the different samples (except one from decayed plants) have no activity against *Micrococcus luteus* and *Candida albicans*. The strain isolated from the decayed plant of Ward El-Neel (sample D.W2.4) is active all over the tested organisms and showing strong activity towards *Escherichia coli*, *Pseudomonas aeruginosae*, *Serratia marcescens* and *Acinetobacter calcoaceticus*.

Table. 3. Morphological and physiological characteristics of the active rare actinomycetes isolates.

Sample No.	Diagnostic mycelial Pigment	Diffusible pigment	Growth on		Growth utilisation							Nitrate reduction	Maximum NaCl tolerance (% w/v)
			Czabek-sucrose agar	Potato slice	α- Melibiose	Raffinose	D-Mannitol	L- Rhamnose	Glycerol	Inositol	D-Ribose		
S.Q1	-	Olive	+	-	-	-	-	-	-	-	+	-	3
S.Q2	-	Olive	+	-	-	-	-	-	-	-	+	-	3
S.Q3	-	Green	+	-	-	-	-	-	-	-	+	-	3
S.Q6	-	Olive	+	-	-	-	-	-	-	-	+	-	3
S.Q8	-	Green	+	-	-	-	-	-	-	-	+	-	3
S.S2	-	Dark brown	+	-	+	+	-	-	+	-	-	-	1.5
S.S3	Orange	-	+	-	+	-	+	-	-	-	+	-	3
S.S4	Orange	-	+	-	+	-	+	-	-	-	+	-	3
S.I1	Orange	-	+	-	+	-	+	-	-	-	+	-	3
S.I2	Orange	-	+	-	+	-	+	-	-	-	+	-	3
S.I3	-	Dark brown	+	-	+	+	-	-	+	-	-	-	1.5
S.I4	-	Brown	+	-	+	+	-	-	+	-	-	-	1.5
S.D1	-	Dark brown	+	-	+	+	-	-	+	-	-	-	1.5
S.D3	-	Dark brown	+	-	+	+	-	-	+	-	-	-	1.5
S.G3	-	Dark brown	+	-	+	+	-	-	+	-	-	-	1.5
S.G5	-	Brown	+	-	+	+	-	-	+	-	-	-	1.5
W.C1.2	-	Dark brown	+	-	+	+	-	-	+	-	-	-	1.5
W.C1.3	Orange	-	+	-	+	-	+	-	-	-	+	-	3
W.C2.1	-	Dark brown	+	-	+	+	-	-	+	-	-	-	1.5
W.C2.2	Orange	-	+	-	+	-	+	-	-	-	+	-	3
W.G1	Orange	-	+	-	+	-	+	-	-	-	+	-	3
W.G2.1	-	Dark brown	+	-	+	+	-	-	+	-	-	-	1.5
W.G2.2	-	Dark brown	+	-	+	+	-	-	+	-	-	-	1.5
W.G2.5	-	Dark brown	+	-	+	+	-	-	+	-	-	-	1.5
D.P1.1	Purple	-	+	-	-	-	-	+	-	-	-	+	3
D.P1.2	Purple	-	+	-	-	-	-	+	-	-	-	+	3
D.P1.4	Orange	Brown	+	-	+	+	-	-	-	-	+	-	1.5
D.P1.6	Purple	-	+	-	-	-	-	+	-	-	-	+	3
D.P1.7	Purple	-	+	-	-	-	-	+	-	-	-	+	3
D.P2.3	Orange	Brown	+	-	+	+	-	-	-	-	+	-	1.5
D.P2.4	Purple	-	+	-	-	-	-	+	-	-	-	+	3
D.W1.1	Orange	Brown	+	-	+	+	-	-	-	-	+	-	1.5
D.W1.2	Orange	Brown	+	-	+	+	-	-	-	-	+	-	1.5
D.W1.3	Purple	-	+	-	-	-	-	+	-	-	-	+	3
D.W2.4	-	-	+	-	+	+	+	+	-	-	-	-	3

3. Morphological characteristics

The morphological characteristics of the active strains are presented in Table 3 from which the following criteria can be indicated:

a. Half of the strains have no diagnostic mycelial pigment, while the rest have orange or purple colour.
b. Fourteen strains have no diffusible pigment, while the others have brown to dark brown and rarely olive or green colours.
c. All isolates grow on the Czapek-sucrose agar and none of them grow on potato slice.
d. The utilization with different carbon sources show unlike patterns.
e. Approximately all isolates can not reduce nitrate.
f. Half of the isolates can tolerate the presence of NaCl till 3%, while others tolerate till 1.5%.

Table.4. Taxonomic groups of active rare actinomycetes isolates

Sample No.	Locality	Identified rare actinomycetes
S.Q1	Soil of Qalubiya	*Micromonospora olivasterospora*
S.Q2	Soil of Qalubiya	*Micromonospora olivasterospora*
S.Q3	Soil of Qalubiya	*Micromonospora olivasterospora*
S.Q6	Soil of Qalubiya	*Micromonospora olivasterospora*
S.Q8	Soil of Qalubiya	*Micromonospora olivasterospora*
S.S2	Soil of Sharkiya	*Micromonospora purpurea*
S.S3	Soil of Sharkiya	*Actinoplanes tecichomyceticus*
S.S4	Soil of Sharkiya	*Actinoplanes tecichomyceticus*
S.I1	Soil of Ismaelliya	*Actinoplanes tecichomyceticus*
S.I2	Soil of Ismaelliya	*Actinoplanes tecichomyceticus*
S.I3	Soil of Ismaelliya	*Micromonospora purpurea*
S.I4	Soil of Ismaelliya	*Micromonospora purpurea*
S.D1	Soil of Dakahliya	*Micromonospora purpurea*
S.D3	Soil of Dakahliya	*Micromonospora purpurea*
S.G3	Soil of Gharbiya	*Micromonospora purpurea*
S.G5	Soil of Gharbiya	*Micromonospora purpurea*
W.C1.2	Fresh water from Cairo	*Micromonospora purpurea*
W.C1.3	Fresh water from Cairo	*Actinoplanes tecichomyceticus*
W.C2.1	Fresh water from Cairo	*Micromonospora purpurea*
W.C2.2	Fresh water from Cairo	*Actinoplanes tecichomyceticus*
W.G1	Fresh water from Giza	*Actinoplanes tecichomyceticus*
W.G2.1	Fresh water from Giza	*Micromonospora purpurea*
W.G2.2	Fresh water from Giza	*Micromonospora purpurea*
W.G2.5	Fresh water from Giza	*Micromonospora purpurea*
D.P1.1	Decayed plant of Papyrus	*Micromonospora echinospora*
D.P1.2	Decayed plant of Papyrus	*Micromonospora echinospora*
D.P1.4	Decayed plant of Papyrus	*Actinoplanes capillaceus*
D.P1.6	Decayed plant of Papyrus	*Micromonospora echinospora*
D.P1.7	Decayed plant of Papyrus	*Micromonospora echinospora*
D.P2.3	Decayed plant of Papyrus	*Actinoplanes capillaceus*
D.P2.4	Decayed plant of Papyrus	*Micromonospora echinospora*
D.W1.1	Decayed plant of Ward El-Neel	*Actinoplanes capillaceus*
D.W1.2	Decayed plant of Ward El-Neel	*Actinoplanes capillaceus*
D.W1.3	Decayed plant of Ward El-Neel	*Micromonospora echinospora*
D.W2.4	Decayed plant of Ward El-Neel	*Dactylosporangium aurantiacum*

4. Chemotaxonomical analysis

All of the studied isolates showed meso DAP in their cell wall as well as xylose and arabinose as a characteristic sugars in cell hydrolysates.

5. Identification of rare actinomycetes isolates

Results obtained after the polyphasic identification of isolated rare actinomycetes are presented in Table 4 from which, it is clear that the active actinomycetes belong generally to *Micromonospora* (65.7%), less commonly *Actinoplanes* (31.3%) and rarely to *Dactylosporangium* (3%) genera.

The soil isolates revealed the presence of three different phena. The first was identified as *Micromonospora olivasterospora*, while the second belongs to *Micromonospora purpurea*. The third phena was identified as *Actinoplanes teichomyceticeus*. In addition, rare actinomycetes in water samples are commonly

Micromonospora purpurea followed by *Actinoplanes teichomyceticeus*.

Several workers emphasised that *Micromonospora olivasterospora*, *Micromonospora purpurea* and *Actinoplanes teichomyceticeus* showed antimicrobial activity (e. g. Bardone *et al*., 1978; Wagman and Weinstein, 1980; Borghi *et al*., 1984; Lazzarini *et al*., 2000).

In the case of decayed plants samples, the dominant group (6 strains) were identified as *Micromonospora echinospora*, followed by the presence of *Actinoplanes capillaceus* (4 isolates). These species reported as potential producers of new antibiotic (Lazzarini *et al*., 2000). One strain could be identified as *Dactylosporangium aurantiacum*.

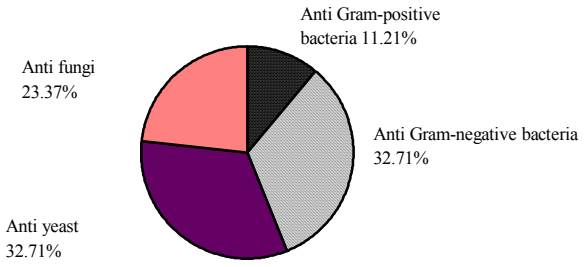

Fig. 1. Percentage of the active strains of rare actinomycetes against the target organisms.

CONCLUSIONS

In the course of screening for new antibiotics, sixty six rare actinomycetes strains were isolated from different Egyptian habitats using humic-vitamin B agar medium. Antibiotic production of the isolates has been tested against Gram-positive, Gram-negative, yeast and fungi. Thirty five isolates showed activity against the tested organisms. Approximately all the isolates were active against Gram-negative bacteria. Isolates from soil and fresh water samples were active towards fungi and one tested yeast. The isolates showed different pattern of activity against Gram-positive bacteria. The taxonomic study revealed that the active isolates belonging to *Micromonospora*, *Actinoplanes* and *Dactylosporangium* genera. Screening of rare actinomycetes will hopefully generate new leads.

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