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Diversity and antimicrobial activity of Red Sea mangrove actinomycetes (Egypt).

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ABSTRACT

A total of 85 actinomycetes isolates were obtained from 60 sediment samples collected from mangrove rhizosphere at the Egyptian Red Sea coast. A set of 23 isolates representing all morphological diversity of the isolates were chosen for numerical classification studies based on their various taxonomic characteristics. The strains were assigned to one major cluster comprising 5 strains; four minor clusters (2-3 strains) and 8 single clustered strains. Most of the isolates exhibited antimicrobial activity against at least one of the tested pathogenic indicators. The results of partial 16S rDNA gene sequencing of representative strains showed the affiliation of most strains to the genus *Streptomyces*. The results indicate that mangrove environment is a potential source for novel actinomycetes that could be useful in the discovery of novel antimicrobial secondary metabolites. Characterization of the bioactive metabolites produced by the most potent strains is being conducted.

1. INTRODUCTION

Actinomycetes are a group of Gram-positive bacteria, often filamentous, characterized by high guanine and cytosine (≥55%) in their DNA (Zotchev, 2012). Actinomycetes belong to Phylum Actinobacteria (Orderactinomycetales) that represents one of the largest taxonomic units among the 18 major lineages currently recognized within the domain Bacteria (Olano*et al*., 2009). It is important to get knowledge about the taxonomic and ecological positions of the antibiotic producing actinomycetes in order to get insight on their activities and secondary metabolites produced by them (Adegboye and Babalola, 2012). Several advanced approaches such as numerical taxonomy, chemotaxonomy and molecular taxonomy have been introduced to complement morphological observations and help in species differentiation (Goodfellow *et al*., 1990).

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Actinomycetes have been known as arich sources of biologically active metabolites and still contribute to therapeutically active, novel, natural products (Jensen and Fenical, 2000), in addition to extracellular enzymes of considerable economic importance (Schrempf, 2001; George *et al.*, 2012). The secondary metabolites produced by actinomycetes are of diverse biological activities such as antibacterial, antifungal, antioxidant, antitumor, antiviral and enzyme inhibitors (Lam, 2006; Bull and Stach, 2007; George *et al*., 2012). Since 1950s, actinomycetes derived from terrestrial sources have yielded many important anti-infective and anti-cancer compounds. However, in recent years, the frequency of rediscovery of already known compounds from terrestrial actinomycetes has increased, while the frequency of discovery new compounds has decreased (Donadio *et al*., 2010; Zotchev, 2012). Moreover, the excessive use of antibiotics has excreted selective pressure on microbial populations leading to the emergence and spread of microorganisms resistant to the commonly used antibiotics. The previously commensals such as *Staphylococcus aureus*, *Enterococci* and *Escherichia coli* have become potent human pathogens through acquisition of multiple antibiotic resistances and simultaneous increase in virulence. The opportunistic pathogens such as pan drug-resistant Gram-negative strains of *Pseudomonas aeruginosa, Acinetobacterbaumannii, Burkholderiacepacia* and *Stenotrophomonasmaltophilia* are now threatening an ever growing population of susceptible patients with compromised immune systems (Alekshun and Levy**,** 2006; Wright, 2007). Therefore, new ecosystems should be investigated for the isolation of actinomycetes as a source of novel antibiotics and other therapeutic agents (Bull *et al*., 2000). Recently, marine actinomycetes have been recognized as a source of novel compounds with biological activities, indicating that marine actinomycetes are important sources for the discovery of novel secondary metabolites (Magarvey *et al*., 2004; Fiedler *et al.*, 2005; Jensen *et al*., 2005_a). Many of these compounds possess unique structural features rarely or never found among the compounds isolated from the terrestrial sources.

Mangrove environment is a rich source for new species of *Streptomyces, Nocardiopsis* and various strains of actinomycetes. The nutritional and geographical conditions of mangrove ecosystem affect the diversity of genetic and metabolic features of mangrove actinomycetes and subsequently the production of new metabolites (Arifuzzaman *et al*., 2010**)**. Actinomycetes from mangrove environment are rich source of antiviral, antibiotics antifungal and anticancer agent, and enzyme inhibitor (Subathra *et al*., 2012).Mangrove forests in Egypt are estimated to cover about 525 ha (Kairo and Hegazy, 2003).

The present study was designed to assess the biodiversity and elucidate the bioactivity of mangrove actinomycetes along the Egyptian Red Sea coast.

2. MATERIALS AND METHODS

2.1 Sampling and locations

Sediment samples were collected by inserting a polyvinyl corer (10cm diameter, previously sterilized with alcohol) into the sediments. The corer is sterilized with alcohol before sampling at each station. The central portion of the top 2 cm sediment sample was taken out with the help of a sterile spatula, transferred to a sterile polythene bag and transported immediately to the laboratory (Sahu *et al.*, 2005). A total of 60 sediment samples were collected from foursites of the mangrove environments along the Egyptian Red Sea as shown in (Fig. 1). All studied areas of the mangrove vegetation are represented by a single community dominated by *Avicennia marina*.

2.2 Isolation and purification of actinomycetes

Approximately 50 mg of each wet sediment sample was used to inoculate anNaST21Cx agar plate. What man no.1 sterile filter paper disks (precut to fit the agar surface) were placed on the agar and the sediments was dispersed evenly on the surfaces of the cellulose disks. The plate was incubated in a humidified chamber at 30°C for up to 30 days. Following incubation, selected colonies were streaked on ISP 2 medium containing (25 mg/ml) of eachantibiotic of cycloheximide and nalidixic acid (Magarvey *et al.*, 2004).

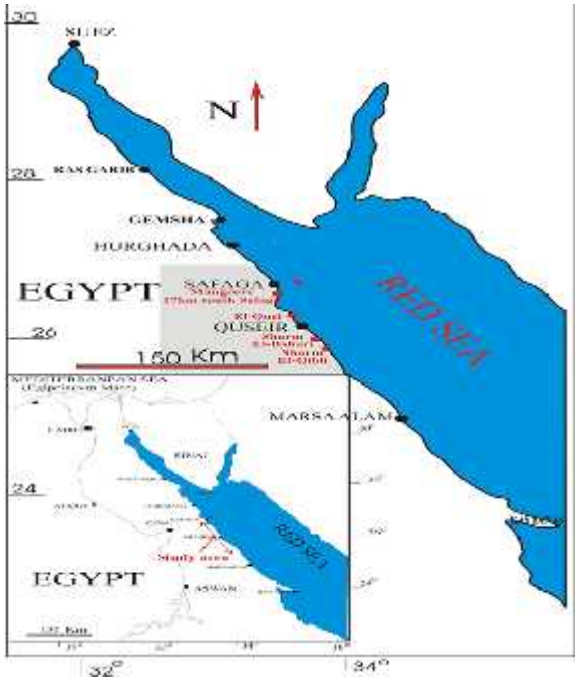

Fig. 1: The study area and sampling sites (Madkour, 2004).

2.3 Morphology, physiology and biochemical characterization

Out of85 isolates,23 strains were selected depending on the variations of their colors and pigmentation after cultivation on starch casein agar medium and incubation (2-6) weeks at 30^0C. The ability of strains to grow on ISP2, ISP3, ISP4, ISP5, ISP6 (Shirlingand Gottlieb, 1966),starch casein nitrate agar,nitrate agar, modified nutrient agar, Czapek's agar, Bennett's modified agarand Waksman glucose agar was tested (Suthindhiran and Kannabiran, 2009). Cellular morphology, aerial hyphae, diffusible and substrate mycelial pigments, and the presence of spore mass, growth at different temperatures (20-50ºC), and the effect of different pH (5-9) were examined as described by Shirling and Gottlieb (1966). Isolates were screened for sodium chloridetolerance (0-10 %) according to Tresner *et al.* (1968). The utilization of different carbon and nitrogen sources was carried out according to Pridham and Gottlieb (1948). All tests were performed at 30ºC for up to 3 weeks.

Additional phenotypic characterization were performed using the standard procedures, catalase and urease production were examined by the method of Weyland *et al.* (1970), nitrate reduction (Williams *et al.*, 1983), sulphide precipitation (Cheesbrough, 1985), lipase and protease (Hankin and Anagnostakis, 1975), amylase (Meena *et al.*, 2013), carboxy methyl cellulose degradation was detected according to Magarvey *et al.* (2004). Resistance against phenol (0.01- 1.2 w/v), crystal violet (0.0001- 0.003 w/v), sodium azide (0.07- 0.4w/v) were detected according to Koli and Kulkarni (2012). Antimicrobial susceptibility test was carried out by disk diffusion assay using Nalidixic acid 30mcg, Erythromycin 15mcg, Ampicillin 10mcg, Amoxcillin 5mcg,

Tetracyclin 30mcg, Amikacin 30mcg, Gentamycin 10mcg, Ofloxacin 5mcg, Streptomycin 10mcg, Levofloxacin 5mcg, according to Jorgensen and Turnidge (2007). Carbohydrate utilization was carried out according to Shirling and Gottlieb (1966).

2.4 Screening the antimicrobial activity of the isolates

2.4.1 Microbial indicators and preparation of inoculums

The following microbial indicators were used to study the antimicrobial activity of the isolates: *Escherichia coli, Vibrio damsela, Vibrio* sp., *Aspergillusniger* and *Fusarium* sp. The inoculums of bacterial indicators were prepared by growing the cells in nutrient broth and then, incubated at 32 °C for 24h. The cells were standardized to OD_{600nm} 0.1 and stored at 4 °C until ready for use (Cwala *et al*., 2011). Fungal inoculums were prepared according to Wayne (2002), fungal cultures were maintained in 0.2% dextrose medium and the optical density 0.10 at 530 nm was adjusted using spectrophotometer.

2.4.2 Antimicrobial assay

For purpose of producing the antimicrobial agents from the isolates using shaking flasks, broth media were used. Organisms under study were inoculated in flasks (250 ml) containing 50 ml (Waksman's glucose medium). Seeded flasks were incubated at 30-32 °C on a rotary shaker at 200 rpm. Samples were taken after 7 days. At the end of the incubation period, the cultures were harvested and the broths were filtered, to be used as antimicrobial agents. The well-cut diffusion technique was used to test the ability of the different isolates to inhibit the growth of indicator microorganisms. Solidmedia inoculated with indicator microorganism were used. Fifty milliliters of sea water agar medium inoculated with indicator microorganisms were poured after solidification into plates. After solidifies, wells were punched out using 0.5 cm cork borer, and each of their bottoms was then sealed with two drops of sterile water agar. One hundred micro-liters of tested filtrates were transferred into each well after sterilizing by ultra-filtration using 0.22 filters. All plats were incubated at 30 °C for 24 h for bacteria and 48 h for fungi, the detection of clear inhibition zones around the wells is an indication of antimicrobial activities of the different isolates (El-Masry *et al.*, 2002).

2.4.3 Numerical analysis

Taxonomic characters were coded in a binary form of the presence/absence type. A cluster analysis and dendogram were performed using PRIMER V5 software.

2.5 Molecular characterization of the strains

Genomic DNA of the actinomycetes isolates was prepared in accordance with the methods described by Sambrook *et al*. (1989). The 16S ribosomal DNA gene was partially amplified by PCR method with Taq DNA polymerase and primers 27F (5' AGT TTG ATC CTG GCT CAG 3') and 1492 R (5' ACG GCT ACC TTG TTA CGA CTT 3'). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94˚C for four minutes, followed by 30 cycles at 94˚C for one minute, primer annealing at 52˚C for one minute, and primer extension at 72˚C for one minute. At the end of the cycling, the reaction mixture was held at 72˚C for 10 min and then cooled to 4˚C. The PCR product obtained was sequenced by an automated sequencer (Genetic Analyser 3130, Applied Biosystems, USA) in Macrogen® (908 world meridian venture center, #60-24, Gasan-dong, Geumchun-gu, Seoul 153-781, Korea)

2.6 Construction of phylogenetic tree

A phylogenetic tree was generated using Maximum Likelihood method based on the Tamura 3-parameter model (Tamura, 1992) with bootstrap testing (Felsenstein, 1985) of 500 replicates, in MEGA6 (Tamura *et al*.,2013).

2.7 Electron microscopy studies

For electron microscopy studies, starch nitrate agar medium was inoculated with spores of the strain and incubated for 7 days at 32 °C. A plug of the culture was removed and fixed in glutaraldehyde (2.5 %, v/v), washed with water and post-fixed in osmium

tetroxide (1 %, w/v) for 1h. The sample was washed twice with water and dehydrated in ascending ethanol (30 %, 50 %, 70 %, 90 % and 100 %), and finally coated in gold (Higginbotham and Murphy, 2010) and examined at 15-20 KV in JEOL JSM 5400 LV, scanning electron microscope, Japan at the electron microscope unit of Sohag University.

3. RESULTS

3.1 Isolation and purification of actinomycetes

A total of 60 sediment samples (fifteen from each site) collected from four mangrove locations along the Egyptian Red Sea coast were processed for actinomycetes isolation. NaST21Cx agar plates covered with cellulose filter paper were inoculated with about 50 mg of wet sediment sample and incubated in a humid chamber for 30 days. We found that this method is more efficient for actinomycetes isolation than inoculation from sediment suspension, suggesting that they were associated to sediment particles. Representatives of all actinomycetes-smorphotypes observed from each sample were picked from primary isolation plates and purified on ISP2 medium. Out of 60 samples processed, only 50(83%) yielded actinomycetes growth and resulted in the isolation of 85 actinomycete isolates. These isolates were initially grouped into 23 groups according to their colors on starch casein nitrate agar medium. One isolate was chosen from each group for numerical classification studies.

3.2 Cluster analysis

The cluster analysis of 23 actinomycetes isolates was carried out using PRIMER v5.2 software according to their morphological, physiological and biochemical characteristics. At 88% similarity level, about 65% (15 isolates) of the isolates were grouped into five clusters (A, B, C, D and E) and eight (about 35%) of the isolates were grouped separately and formed eight single clusters at this level (Fig. 2). Each of the three clusters (A, B and C) contained only two isolate and were clustered at 88%, 89% and 90% similarity level respectively. Cluster (D) comprised three isolates clustered at 88% similarity level. The major cluster (E) contained five isolates clustered at 88% similarity level. The characteristic features of isolates comprising the different clusters and the single clustered isolates are indicated in (Table 1).

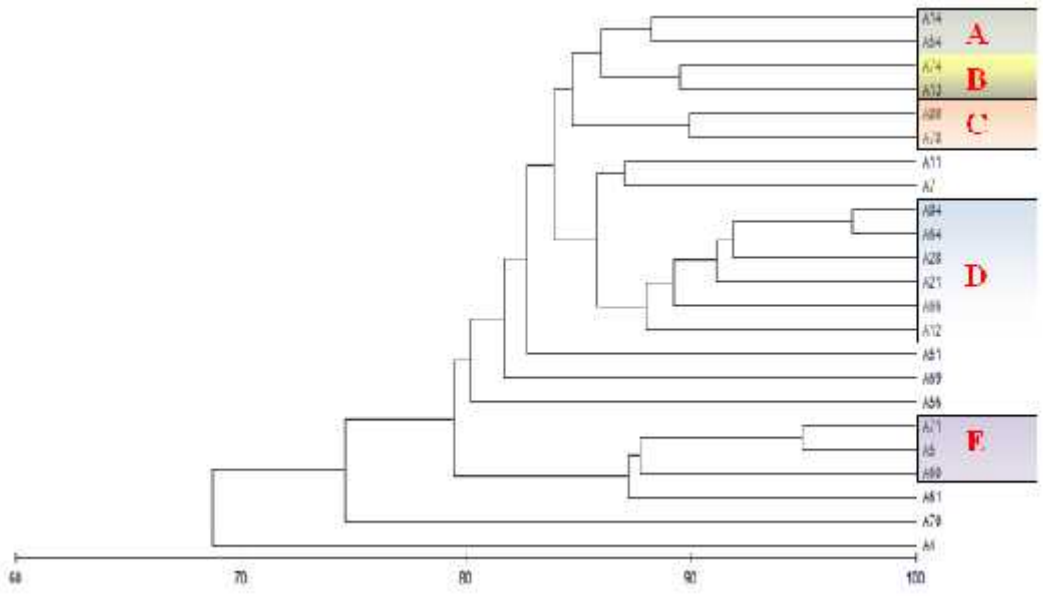

Fig. 2: Simplified dendogram showing relationship between actinomycetes isolates using PRIMER V5software.

Table 1: Phenotypic characteristics and the percentage of positive results

Cluster	**A**			**B**			**C**			**D**							**E**				**Single clustered isolates**							
Strains	**A14**	**A54**	**% positive**	**A74**	**A13**	**% positive**	**A78**	**A80**	**% positive**	**A84**	**A64**	**A21**	**A28**	**A66**	**A12**	**% positive**	**A5**	**A71**	**A60**	**% positive**	**A11**	**A7**	**A51**	**A69**	**A56**	**A61**	**A4**	**A70**
Growth on																												
ISP2	-	+	50	+	+	100	+	+	100	+	-	+	+	+	+	83.3	+	+	+	100	+	+	-	+	-	+	-	-
ISP3	+	+	100	+	+	100	-	+	50	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
ISP4	-	-	0	-	-	0	-	-	0	+	+	+	+	+	+	100	-	-	-	100	-	-	-	+	-	-	-	-
ISP5	+	+	100	-	+	50	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
ISP6	+	+	100	+	+	100	+	+	100	+	+	+	-	+	+	83.3	+	+	+	100	+	+	+	+	+	+	-	+
SCNA[1]	+	+	100	+	+	100	+	+	100	+	+	+	-	+	+	83.30	+	+	+	100	+	+	+	-	+	+	+	+
MNA[2]	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	-	+	+	+	+
CZA[3]	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	-	+	+	+	+
BMA[4]	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	-	+	+	+	-
NA[5]	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
WGA[6]	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
Substrate mycelium																												
Yellow	+	+	100	+	-	50	+	+	100	+	+	+	+	+	+	100	+	-	-	33.3	-	+	-	+	-	+	+	+
Brown	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	+	-	33.3	-	-	+	-	-	-	-	-
Grey	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	+	33.3	-	-	-	-	-	-	-	-
Cream	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	-	-
Pink	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	-	-
White	-	-	0	-	+	50	-	-	0	-	-	-	-	-	-	0	-	-	-	0	+	-	-	-	-	-	-	-
Green	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	-	0	-	-	-	-	+	-	-	-
Aerial mycelium																												
White	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	-	0	+	-	-	-	-	-	-	-
Cream	-	-	0	-	-	0	+	+	100	+	+	-	-	+	+	66.7	-	-	-	0	-	+	+	+	-	+	+	+

1-starch casein nitrate agar2- modified nutrient agar 3-Czapek's agar 4-Bennet's modified agar 5- Nitrate agar 6-Waksman glucose agar

Table 1: continued

Cream	-	-	0	-	-	0	+	+	100	+	+	-	-	+	+	66.7	-	-	-	0	-	+	+	+	-	+	+	+
Grey	-	-	0	-	-	0	-	-	0	-	-	+	-	-	-	16.7	+	+	+	100	-	-	-	-	-	-	-	-
Yellow	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	-	-
Pink	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	-	-
Violet	-	-	0	-	+	50	-	-	0	-	-	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	-	-
Green	+	+	100	+	-	50	-	-	0	-	-	-	+	-	-	16.7	-	-	-	0	-	-	-	-	+	-	-	-
Diffusible pigment																												
Beige	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	-	-
Yellow	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	-	-
Dark brown	-	-	0	-	-	0	-	-	0	-	-	-	+	-	-	16.7	-	-	-	0	-	-	+	-	-	-	-	-
Violet	-	-	0	-	-	0	-	-	0	-	-	-	-	-	+	16.7	-	-	-	0	-	-	-	-	-	-	-	-
Growth at (0C)																												
10	-	-	0	-	+	50	-	-	0	-	-	-	-	-	-	0	+	+	-	66.7	-	-	-	-	-	-	-	-
20	+	+	100	+	+	100	+	+	100	-	-	+	-	-	+	33.3	+	+	+	100	+	+	+	+	-	+	-	+
30	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
40	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
50	+	-	50	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
55	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	-	-
Growth at different pH																												
5	-	-	0	-	+	50	-	-	0	+	+	-	-	+	+	66.7	+	+	+	100	+	+	-	-	+	+	+	-
6	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
7	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
8	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
9	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	-	+	+	+	+	+
Growth in the presence of NaCl (%)																												
0	+	+	100	+	+	100	+	+	100	+	-	+	+	+	+	83.3	+	+	+	100	+	+	+	+	+	+	-	+
2	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
4	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	-	-

Table 1: continued

6	+	-	50	+	+	100	+	-	50	+	+	+	+	+	+	100	-	+	-	33.3	+	+	+	+	-	+	-	-
8	+	-	50	+	-	50	-	-	0	+	+	+	+	+	+	100	-	-	-	0	+	+	+	+	-	+	-	-
10	+	-	50	+	-	50	-	-	0	+	+	+	+	+	+	100	-	-	-	0	+	+	-	-	-	+	-	-
Utilization of																												
Glucose	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	-	+	-	+
Starch	+	+	100	+	+	100	-	-	0	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	-	+
Lactose	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	+	+	+	100	-	-	-	-	+	+	-	-
Maltose	+	+	100	+	+	100	-	-	0	+	+	+	+	-	+	83.3	+	+	+	100	+	+	-	+	+	+	-	+
D-xylose	+	+	100	+	-	50	-	+	50	-	-	-	-	-	-	0	+	+	-	66.7	-	-	-	-	-	+	+	+
Fructose	-	-	0	-	-	0	-	+	50	+	-	+	+	-	+	66.70	+	+	-	66.7	+	+	-	-	+	-	+	-
D-arabinose	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	-	0	-	-	-	-	+	-	+	-
Sucrose	+	+	100	+	+	100	+	-	50	+	+	+	+	-	+	83.3	+	+	+	100	+	+	+	-	+	+	+	+
Biochemical tests																												
Lipase	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
Urease	+	+	100	+	+	100	+	+	100	+	+	+	+	-	+	83.3	+	+	+	100	+	+	+	+	+	+	+	+
Protease	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	-	+	+	+	+	-	+
Catalase	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
Nitrate reduction	-	-	0	+	-	50	-	-	0	+	-	-	+	+	+	66.7	+	-	+	66.7	-	-	+	-	+	-	+	+
Hydrogen sulphide production	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	-	-
Degradation of																												
Carboxymethyl cellulose	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	-	+	+	-	+	-	+
Gelatin	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	-	66	-	-	+	+	+	+	-	+
Starch	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	-	+
Antibiotic resistance (mcg)																												
Nalidixic acid 30	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
Erythromycin 15	+	-	50	-	-	0	+	+	100	-	-	-	-	-	-	0	+	-	-	33.3	-	+	+	-	-	+	+	-
Ampicillin 10	+	+	100	-	+	50	+	+	100	+	+	+	+	+	+	100	+	+	+	100	-	+	+	+	-	+	+	-
Amoxicillin 5	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	-	+	+	+	+	+	-	-

Table 1: continued

Tetracyclin30	-	+	50	-	+	50	+	-	50	+	+	-	+	-	-	50	+	+	-	66.7	-	-	-	-	-	+	-	+
Amikacin30	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	-	0	-	-	-	-	-	-	-	+
Gentamycin 10	-	+	50	-	-	0	-	+	50	-	-	-	-	-	-	0	-	+	-	33.3	-	-	-	+	-	+	-	+
Ofloxacin5	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
Streptomycin 10	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	+	-	33	-	+	-	-	-	-	-	-
Levofloxacin 5	+	+	100	-	-	0	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	-	-	-	+	-	-
Phenol resistance (g/l)																												
0.1-0.4	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
0.5	+	+	100	-	-	0	+	+	100	-	-	-	+	+	+	50	+	+	+	100	+	-	-	-	+	+	+	+
0.6	-	-	0	-	-	0	+	+	100	-	-	-	-	+	+	33.3	+	+	+	100	+	-	-	-	-	+	+	+
0.7	-	-	0	-	-	0	-	+	50	-	-	-	-	-	+	16.7	+	+	+	100	-	-	-	-	-	+	+	+
0.8	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	+	+	+	100	-	-	-	-	-	+	+	-
0.9	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	+	+	+	100	-	-	-	-	-	+	+	-
1	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	+	+	+	100	-	-	-	-	-	+	+	-
1.1	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	+	+	+	100	-	-	-	-	-	+	+	-
1.2	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	+	-	+	66.7	-	-	-	-	-	+	-	-
Resistance to sodium azide (g/l)																												
0.07	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	+
0.08	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	-
0.09	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	-
0.1	-	-	0	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	+	-
0.2	-	-	0	+	+	100	+	+	100	+	+	+	+	+	-	83.3	+	+	+	100	+	+	-	+	+	+	+	-
0.3	-	-	0	+	+	100	+	+	100	-	-	+	-	-	-	0	+	+	+	100	+	+	-	+	-	+	+	-
0.4	-	-	0	-	-	0	-	-	0	-	-	-	-	-	-	0	-	-	-	0	+	+	-	-	-	-	-	-
Resistance to crystal Violet (g/l)																												
0.0001	+	+	100	+	+	100	+	+	100	+	+	+	+	+	+	100	+	+	+	100	+	+	+	+	+	+	-	+
0. 001	+	+	100	+	+	100	+	-	50	+	+	+	+	+	+	100	+	+	+	100	+	-	+	-	+	+	-	-
0. 002	-	+	50	+	+	100	-	-	0	-	-	-	-	-	+	16.7	+	+	+	100	-	-	-	-	+	-	-	-
0. 003	-	+	50	+	+	100	-	-	0	-	-	-	-	-	+	16.7	+	+	+	100	-	-	-	-	+	-	-	-

3.3 Antimicrobial activity of actinomycetes

All of the isolates (23) were examined to determine their antimicrobial activity toward *Escherichia coli*, *Salmonella* sp., *Vibrio damsella*, *Vibrio* sp., *Aspergillusniger* and *Fusarium* sp. Atotal of 18 (78.26%) strains showed antimicrobial activity. Of these, three strains (13%) showed activity against *Escherichia coli*, *Salmonella* sp. and *Aspergillusniger*; 13 strains (56.52%) against *Vibriodamsela*; 15 strains (65.2%) against *Vibrio*sp. and 2 strains (8.69%) against *Fusarium* sp. (Fig. 3). Isolates A5, A71 and A70 were the most promising. They exhibited antimicrobial activity against most of the testedpathogenic organisms (Table 2).

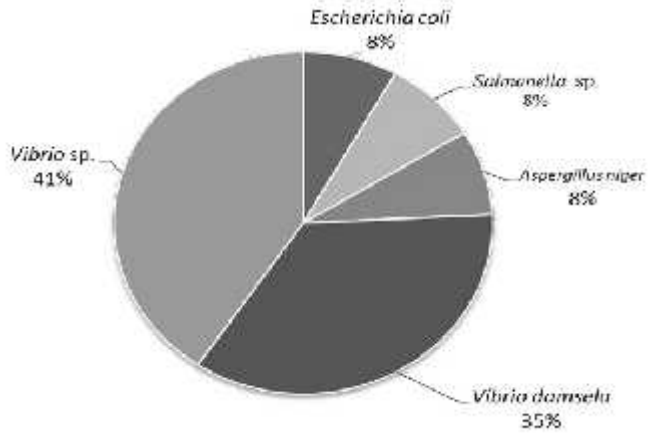

Fig. 3: Percentage of isolates exhibiting antagonistic activity against different pathogens.

Table 2: Antimicrobial activity profile of actinomycetes isolates

	Microbial indicators					
Isolate code	***Escherichia coli***	***Salmonella* sp.**	***Vibrio damsela***	***Vibrio* sp.**	***Aspergillusniger***	***Fusariumsp***
A54	-	-	+	-	-	-
A7	-	-	+	+	-	-
A5	+	+	-	+	+	+
A51	-	-	+	-	-	-
A21	-	-	-	-	-	-
A66	-	-	+	+	-	-
A4	-	-	-	+	-	-
A71	+	+	-	+	+	+
A13	-	-	+	-	-	-
A74	-	-	-	-	-	-
A11	-	-	+	+	-	-
A78	-	-	+	+	-	-
A64	-	-	+	+	-	-
A80	-	-	+	+	-	-
A69	-	-	-	-	-	-
A56	-	-	+	+	-	-
A60	-	-	-	-	-	-
A61	-	-	-	-	-	-
A12	-	-	-	+	-	-
A70	+	+	+	+	+	-
A14	-	-	-	+	-	-
A28	-	-	+	+	-	-
A84	-	-	+	+	-	-

3.4 Phylogenetic analysis

On random basis, six isolates were selected for phylogenetic analysis as one isolate from each five clusters (A, B, C, D and E) plus one selected isolate from the single clusters. Phylogenetic analysis of the six strains as representatives of the diversity revealed the predominance of the genus Streptomyces. As indicated in (Table 3), A5 strain had 99% similarity to *Streptomyces*

rochei strain AL14. Micromorphological examination of this isolate revealed the spiral spore chain and the smooth spore surface (Fig. 5). Strain 54 and strain A28 had 99% homology with *Streptomyces* sp. AML554, a species isolated from the Gulf of California. A51 strain had 99% similarity to *Streptomyces* sp. SB1(2014), a strain isolated from mangrove soil. A13 strain had 99% similarity to *Streptomyces thermolilacinus* strain K5.

Table 3: Identification, similarities and gene bank accession numbers of the 16S rDNA sequences of the six selected strains

Strain code	Identification	Details of similar species	Accession N0.	Identity (%)
A5	*Streptomyces*sp Nyr04	*Streptomyces rochei* strain AL14	KT074931	99
A54	*Streptomyces*sp Nyr06	*Streptomyces*sp AML554	KT192568	99
A51	*Streptomyces*sp Nyr03	*Streptomyces*sp SB1(2014)	KT074930	99
A28	*Streptomyces*sp Nyr01	*Streptomyces*spAML554	KT074933	99
A13	*Streptomyces*sp Nyr02	*Streptomyces thermolilacinus* strain K5	KT074934	99
A11	*Nocardiopsis*sp Nyr05	*Nocardiopsislucentensis* strain DD7	KT074932	93

Only one strain (A11) was found to be non- streptomycete and demonstrated 93% similarity to *Nocardiopsislucentensis* strain DD7, a marine *Nocardiopsis* strain isolated from India. Maximum Likelihood method based on the Tamura 3-parameter model produced tree topologies that grouped partially 16S rDNA gene sequences obtained from all six strains into a single clade and further divided into four subclades. However, the partial 16S rDNA gene sequences of Strain A11 (*Nocardiopsis*sp Nyr05) showed low homology with all clade members (Fig. 4). This supports the results obtained from cluster analysis based on phenotypic characteristics where A11 strain was among the single clustered isolates (Fig. 2).

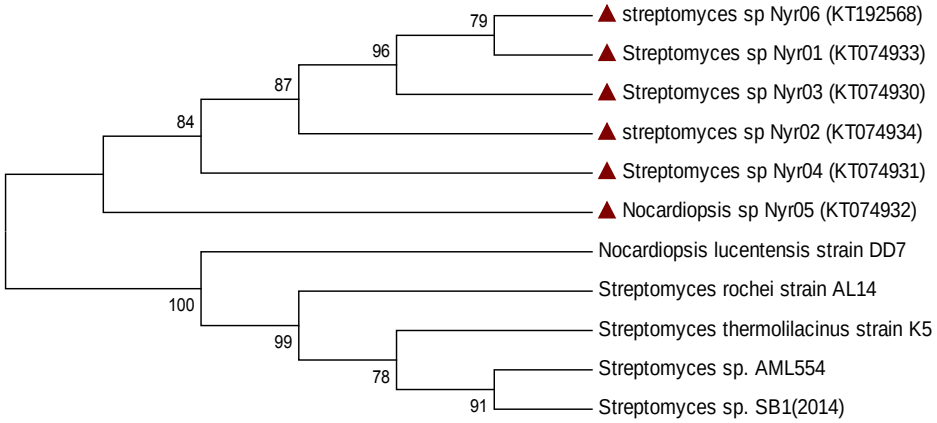

Fig. 4: Unrooted Maximum-Likelihood (ML) tree of actinomycetesstrains based on 16S rDNA sequences. The evolutionary history was inferred using Maximum Likelihood method based on the Tamura 3-parameter model. Numbers at nodes represent bootstrap values from 500 resampled datasets; the marked taxa are the selected strains in present investigation.

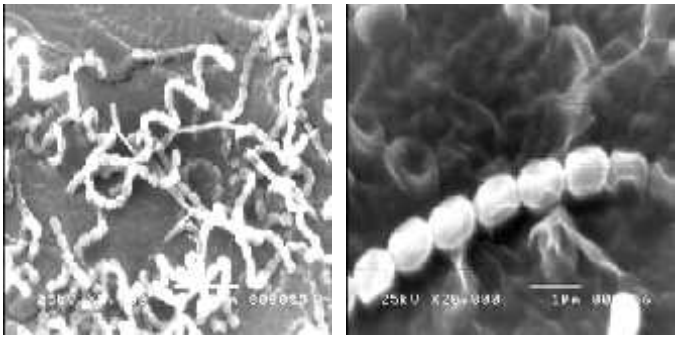

Fig. 5: Scaning electron micrograph of A5 isolate growing on starch casein agar.(A) Spore chain spiral (X5.000) and (B), spore surface smooth (X20.000).

4. DISCUSSION

Numerical taxonomy was introduced to allow the simultaneous evaluation of a large number of phenotypic traits (Sneath and Johnson, 1972). Numerical taxonomy for streptomycetes and related genera was conducted using phenetic characteristics and yielded major, minor and single strains clusters. Many of the minor clusters were consisted of less than five strains and were regarded as species. Major cluster varied from (6-71) strains and were regarded as single species despite the high diversity observed within each cluster (Williams *et al*., 1983).The use of 16S rDNA sequencing has been significant in the systematic of bacteria and actinomycetes (Yokota, 1997). Phylogenetic trees based on 16S rDNA sequencing have allowed the investigation of evolution and provided basis for identification of actinomycetes (Hapwood *et al*., 1985). CLUSTAL W version embedded in MEGA 6 was used as the default parameter for constructing the phylogenetic tree as recommended by Hall (2013). Phylogenetic analysis of actinomycetes isolated during the present investigation revealed that five strains had sequence identity of 99% (query sequence coverage 99%) to *Streptomyces*species sequences found in the database. According to Stackebrandt and Ebers (2006), these isolatesshould betested for genomic uniqness as novel strains. One strain (A11) exhibited identity of 93% to *Nocardiopsislucentensis* strain DD7. This isolate most likely could be considered as a new taxon according to Drancourt *et al*. (2000) who indicate the criteria for identification using 16S rDNA sequence analysis where, identification to the species level was defined as16S rDNA sequence similarity of more than or equal to 99% with that of the prototype strain sequence in the gene bank; identification to the genus level was defined as a 16S rDNA sequence similarity of more than or equal 97% with that of the prototype strain sequence in the gene bank. A failure to identify was defined as a 16S rDNA sequence similarity score of lower than 97% with those deposited in the gene bank at the time of analysis. All of six isolates were included within one separate clade in the constructed phylogenetic tree. Since the isolates were all isolated from the same environment, this observation could be attributed to lateral gene transfere. It has been shown that exchange of DNA fragments, including partsof the taxonomically important 16S rDNA gene, is a possible phenomenon in some prokaryotes (Schouls *et al*., 2003). Actinomycetes derived from natural sources have been known for their secondary metabolites with potent antimicrobial activities (Takahashi and Omura, 2003). The search for novel actinomycetes from poorly explored habitats has received considerable attention as a premise for raising the prospect of discovering new bioactive compounds (Bredholt *et al*., 2008; Eccleston *et al*., 2008; Okoro *et al*., 2009). This assumption has been found true as actinomycetes obtained from unexplored marine habitats are proving to be valuable source for new bioactive compounds (Fiedler *et al*., 2005; Bull and Stach, 2007). The recent tremendous increase in the rate of isolation of novel

compounds from microorganisms living in mangrove forest have stimulated the exploration of actinomycetes living in such habitat (Subathra *et al*., 2012). Mangrove sediments are potential source for the isolation of actinomycetes rather than any other marine source (Kathiresan *et al*., 2005). In the present study, 60 mangrove sediment samples were processed for actinomycetes isolation. Of these 50 (85%) yielded actinomycetes growth and resulted in the isolation of 85 isolates. The high number of actinomycetes obtained from mangrove sediment could be attributed to the great productivity of mangrove ecosystem due to the high input of organic matter in the form of detritus. The decomposition of detritus material provides nutrients for saprophytic actinomycetes (Kathiresan *et al*., 2005; Eccleston *et al*., 2008). Most of the actinomycetes obtained during the present study belongtoa single genus, *Streptomyces.* In addition, a number of *Nocardiopsis* were also observed in the present investigation. The dominance of *Streptomyces* in any actinomycetes population is a well-known fact (Alexander, 1971). The predominance of *Streptomyces* in actinomycetes populations recovered from marine sediments have been frequently reported (Kokare *et al*., $2004_{a,b}$; Hans-Peter *et al*., 2005; Jensen *et al*., 2005). The antagonistic activity is considered to be the rapid and feasible method for the prediction of bioactive potential of environmental microbes (Patil *et al*., 2011). Most of the strains isolated during the present study showed antagonistic activity against most of the tested microbial indicators. The presence of bioactive compounds producing actinomycetes, especially antibiotics, in mangrove habitat has been observed (Jensen *et al*., 1991; Sujatha *et al*., 2005; Ara *et al*., 2007). It is hypothesized that, the antibiotic production by mangrove actinomycetes is evolved in response to selective pressure created during competition for resources and the involvement in biogeochemical cycles (Williams and Davies, 1965; Long and Azam, 2001).

It could be concluded that mangrove region of the Egyptian Red Sea coast is a rich source forwell-active actinomycetes. Some of the investigated isolates exhibited broad spectrum activity against the bacterial indicators. Further investigations are needed in order to determine the active metabolites of these isolates.

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