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Antimicrobial activities of some marine Streptomycetes

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Abstract

Twenty marine actinomycetes were isolated from sediments and rhizosphere of some halophyte plants from coastal regions of North Delta, Egypt. Four isolates which showed a wide range of antimicrobial activities (inhibition for both Gram-positive and Gram-negative bacteria, and fungi) were selected and identified on the basis of their cellular morphology, physiological and chemotaxonomic characterization. The isolates were identified and named as *Streptomyces albus* strain DEG18, *Streptomyces canaries* strain REB9, *Streptomyces* sp strain REB5 and *Streptomyces* sp strain G12. Extraction of metabolites filtrate and biomass were carried out by ethyl acetate and acetone, respectively. In secondary screening, all four *Streptomyces* strains showed antibacterial activity against *Enterobacter cloaca* and antifungal activity toward *Fusarium oxysporum*, three strains out of them showed antibacterial activity against *Bacillus cereus*, whereas two showed activity against *Bacillus subtilis* and *Staphylococcus aureus*, whereas some strains showed activity toward *Klebsiella pneumoniae* and *Alternaria alternata*.

Keywords: marine Streptomyces, antimicrobial activity.

Introduction

The continuous search for new antimicrobial compounds or new microorganism strains producing antimicrobial agents becomes necessary because of the increase of antibiotic resistant pathogens and toxicity of some chemical antibiotics. Marine biotechnology environment has opened up unexpected new horizons for finding novel organisms for trapping their potential resources (Ravenschlay *et al*., 1999; Stach *et al*., 2003; Jensen *et al*., 2005; Lam, 2006). However, culturally independent methods have demonstrated that marine sediments contain a wide range of unique microorganisms. Actinomycetes have a profound role in the marine environment apart from antibiotic production (Das *et al*., 2006). Actinomycetes are aerobic, spore forming gram positive bacteria, characterized by substrate and aerial mycelia growth (Lechevalier and Lechevalier, 1981). Actinomycetes are the most economically and biotechnologically valuable microorganisms due to their potential in antimicrobial activity. They have produced a wide range of secondary metabolites of various medical importance such as antibiotics, antagonistic agents**,** including antibacterials, antifungals, antiprotozoans as well as antivirals, pharmacological agents, including antitumorals, immunomodulators, neurological agents and enzyme inhibitors, agrobiologicals, including insecticides, pesticides and herbicides, and compounds with regulatory activities, such as growth factors, siderophores or morphogenic

agents and immunosuppressant (Adegboye and Bablola, 2013). A large number of antibiotics were obtained and reported from the members of the genus *Streptomyces* only (Alan and James, 2007; Lyudmila *et al*., 2008; Junker *et al*., 2009; Koch and Loffler, 2009; Hotam *et al*., 2013). Pharmacological and agricultural screens are increasingly being used in combination with antimicrobial tests, to detect simultaneous bioactivities for a given compound. This has revealed several novel therapeutic and agrobiological agents and previously unknown biological activities for antibiotics (Berdy, 2005). Actinomycetes in marine environments are often under extreme conditions of temperature, pressure, salinity and depletion of micronutrients, with survival and proliferation often depending on their ability to produce biologically active compounds (Bull *et al*., 2000). It is believed that marine actinomycetes may have different characteristics from terrestrial actinomycetes and therefore might produce novel bioactive metabolites and new antibiotics (Ramesh and Mathivanan, 2009; Hames-Kocabas and Uzel, 2012), so, marine actinomycetes have attracted great attention to search novel antibiotics derived from new microorganisms (Carte, 1996; Kijjoa and Sawangwong, 2004). The research to date supports this hypothesis and it has been shown that marine actinomycetes produce novel types of new secondary metabolites (Lam 2006; Fenical and Jensen, 2006). Many of these metabolites possess novel biological activities and have the potential to be developed as therapeutic agents (Feling *et al*., 2003; Maldonado *et al*., 2005**).** However, this work aimed to isolate and identify antimicrobial producing marine actinomycetes as a potential source for production of antimicrobial agents.

Materials and Methods

Collection of Samples

Soil samples were collected at a depth of 10-20 cm from sediments of several different sites from coastal regions of North Delta, Egypt. Some samples were isolated from plant rhizosphere and a mucilaginous layer of algae that are grown on marine rocks. The collection sites and locations of the sampling were Ras El-Bar, El-Sheikh Dergham, Manzala Lake bank, Damietta El-Gededa and Gamasa. Physical properties of water samples, including pH, total dissolved salts (TDS) and electric conductivity (EC) were recorded.

Isolation of actinomycetes

Two gm of samples or five parts of one inch plant root samples included soil particles were added in 18 ml of sterile sea water, vortexed and diluted with sterile sea water as in dilution agar plating method (Johnson *et al.*, 1959). Aliquots (150µl) of each dilution were respectively spread on the surface of the starch casein agar medium (starch 10gm; casein 2gm; NaCl 6gm; KH_2PO_4 0.5gm; $MgSO_4$ 0.5gm; agar 18gm and sterile sea water 1000ml). The pH was adjusted to 7.2 -7.4 prior to autoclaving with the addition of sterile nalidixic acid and cycloheximide at 10µg/ml and 20 µg/ml, respectively to diminish the growth of marine bacteria and fungi. The plates were incubated at 28± 2ºC for 14 - 21 days. The purified actinomycetes were preserved on starch-casein agar slopes at 4°C and in glycerol (40% v/v) at -80°C for longer storage periods.

Identification of the most potential actinomycetes

- Morphological and physiological characteristics

After selection of actinomycetes isolates according to their antagonistic and antimicrobial activities, their identification was carried out by studying their morphological, cultural and physiological characteristics. Streptomycetes species used in this investigation was identified according to the International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1968a; 1968b; 1969; 1972; Pridham and Tresner, 1974a; 1974b; Bergey's Manual of Systematic Bacteriology (Williams *et al*., 1989). Their morphological characters such as colony characteristics, type of aerial hyphae, their branching, growth of vegetative hyphae and spore formation were examined by light microscope and by JSM-5300, Jed Scanning electron microscope at Alexandria University. The physiological characteristics included gel liquefaction, utilization of starch, coagulation of milk, decomposition of cellulose and utilization of sugar.

- Cultural characters

Determination of the actinomycetes isolates colour; the colour of growth, sporulation aerial hyphae, substrate hyphae and diffusion of their pigment into the media were assayed on different media such as: starch casein agar, starch nitrate

agar, starch-ammonium sulphate agar, Czapek-Dox agar, glycerol - asparagine agar, glycerol yeast agar and CM-1 agar media.

- *Chemotaxonomic analysis of the selected Streptomyces isolates*

Determination of the cell wall composition, including diaminopimelic acid (DAP) isomers and sugars was based upon the methods of Becker *et al*. (1964; 1965), Stanek and Roberts (1974).

Bioactivity of isolates in primary screening

The antimicrobial activities of the isolated actinomycetes were detected against six local bacteria: *Enterobacter cloacae, Klebsiella pneumoniae* and *Escherichia coli* (Gram-negative bacteria) *Bacillus cereus, B. subtilis* and *Staphylococcus aureus* (Gram-positive bacteria) and four local fungi: *Fusarium oxysporum, Aspergillus niger, Aspergillus flavus* and *Alternaria alternata* by using diffusion method.

- *Screening on solid media*

The streptomycetes isolates were grown on starch casein agar media for 10 days at 28 ±2ºC. By using an agar plate diffusion method (Wu, 1984), agar discs were cut off by a sterilized cork-borer (1 cm diameter) and transferred into the surface of agar plates previously inoculated with tested microorganisms. The bacteria were grown on nutrient agar while fungi were grown on potato dextrose agar media. The antagonistic activity was determined by measuring the inhibition zone diameter (mm).

- *Screening using liquid media*

The isolates were grown on starch casein broth and adjusted the pH to 7.2- 7.4. The cultures were incubated on a rotary shaker (150 rpm) at 28 ± 2ºC for 9 days. Metabolites were centrifuged and the supernatants were filtered by using sterilized 45µm Millipore filter paper. By using sterile cork-borer (1 cm diameter), the hollow pores in inoculated nutrient agar and potato dextrose agar (PDA) were made and filled with 300µl of cell-free supernatant for each pore. The antimicrobial activities were assayed by measuring the inhibition zones diameter (mm) of bacteria and fungi.

Bioactivity of strains in secondary screening

Agar diffusion method was used to determine the antibacterial and antifungal activity of the ethyl acetate extract of metabolite filtrates and biomass acetone extracts as inhibition zone (mm). This was compared with the crude metabolites as described above.

Extraction of antimicrobial agents from metabolites and biomass

Extraction of antimicrobial agents from metabolites and biomass of most potential actinomycetes were carried out by growing them on a starch casein broth. The medium was adjusted to pH 7.2 ± 0.2 by 1N NaOH and 1N HCl, distributed into 250 ml conical flask containing 50 ml and inoculated using spore suspensions. Flasks were incubated at 28º ± 2º C for 9 days on rotary–shaker at 150 rpm. After fermentation, the antimicrobial compounds were extracted by using ethyl acetate. The culture broth was centrifuged at 5,000 rpm for 10 minutes and filtered to remove biomass. The cell-free supernatant was transferred to a separating flask. Ethyl acetate was added with a ratio of 1:1 (v/v) and shaken vigorously for 10 minutes. The top layer is transferred to a clean glass tube. Ethyl acetate extraction was done twice. The supernatant was collected and passed throughout a column containing traces of sodium sulphate and the filtrate was evaporated to dryness. One mg dry extract were dissolved in 10ml of methanol and the antimicrobial activity was bioassayed using only 200µl. Acetone was added for biomass and shaken vigorously for 10 minutes. The top layer of the extract is transferred to a clean glass tube. The antimicrobial activity was bioassayed using 200µl (Lin and Liu, 2010).

Results and Discussion

Twenty marine actinomycetes species were isolated from different marine sites in coastal regions of North Delta, Egypt within the year of 2011 (Table 1). Some marine actinomycetes were too difficult to be isolated from some other sites. Despite of many efforts of scientists to success the marine actinomycetes isolation, their abundance and diversity are still rare (Stach *et al*., 2003; Maldonado *et al*., 2005; Gontang *et al*., 2007; Bouvier and del Giorgio, 2007). Most of the marine actinomycetes live in sea water in the form of few colonies and cannot grow under laboratory

conditions (Manivasagan *et al*., 2014). The media containing macromolecules like casein and supplemented with sea water are suitable for promoting the growth of rare marine actinomycetes (Qiu *et al*. 2008; Bredholdt *et al*. 2008; Hong *et al*. 2009; Zhang and Zhang, 2011). All the purified isolates showed morphological characteristics of typical *Streptomyces* species, as their colonies possessed an earthy odor and were slow growing, aerobic, powdery, folded with aerial and substrate mycelia of different colors (Anderson and Wellington, 2001).

Morphological studies were carried out and the characteristics of the isolates were compared with the standard characteristics described in Bergey's manual of systematic Bacteriology (William *et al*., 1989). They formed colored, tough and leathery colonies that were hard to pick from the culture media. Microscopic studies also showed that the cell of isolates formed long branched network of mycelia which is characteristic of *Streptomyces* sp as previously described by Kieser *et al*. (2000). These isolates were categorized culturally and morphologically into two series according to the color of their mature sporulating aerial mycelium. Ten out of them were grouped in the grey color group and the other were belonging to the white color group as shown in Table (1). Estimation of the pH, total dissolved salts (TDS) and electric conductivity (EC) for the collection locations were useful to optimize the suitable growth conditions for the isolates. The measured pH, EC and TDS were about 7.0 ± 0.4, 54 ms/ cm and 34.560 g/l, respectively, for most sites.

Table 1. Collection sites of soil samples and color grouping of the isolates.

Color of series	Isolate No	Color of aerial mycelia	Color of substrate mycelia	Cover plant	Site of sampling	Color of series	Isolate No.	Color of aerial mycelia	Color of substrate mycelia	Cover plant	Site of sampling
Grey	1	Gray	Grey	Mucilaginous algal layer	Ras Bar	**White**	10	White	White	Sea shore	Gamasa
	2	Gray	Brown	Mucilaginous algal layer	Ras Bar		11	White	White	Sea shore	Gamasa
	3	Gray	Brown	Rhizosphere of *Salsola kali*	Ras Bar		12	White	White	Sea shore	Gamasa
	4	Gray	Brown	Rhizosphere of *Salsola kali*	Ras Bar		13	White	White	Sea shore	Gamasa
	5	Light gray	Light grey	Rhizosphere of *Spergularia marina*	Ras Bar		14	White	white	Rhizosphere of *Bassia indica*	Manzala lake bank
	6	Gray	Brown	Sediment around *Spergularia marina*	Ras Bar		15	White	White	Rhizosphere of *Bassia indica*	Manzala lake bank
	7	Gray	Light grey	Sediment around *Spergularia marina*	Ras Bar		17	White	white	Rizosphere of *Zygophyllum album*	El-Gamail beach
	8	Gray	Light grey	Sediment around *S. marina*	Ras Bar		18	White	White	Rhizosphere of *C. murale*	Damietta Gededa
	9	Gray	yellow	Mucilaginous algal layer	Ras Bar		19	White	White	Rhizosphere of *Ceratophyllum demersum*	Manzala lake bank
	16	Gray	Brown	Rhizosphere of *Halocnemum strobilaceum*	Manzala lake bank		20	White	White	Rhizosphere of *Ceratophyllum demersum*	Manzala lake bank

Bioactivity of actinomycete isolates in primary screening

The primary screening exhibited that all twenty actinomycete isolates tested did not have bioactive metabolites against *Aspergillus niger* and *Alternaria alternata*. The actinomycete isolates from different color groups displayed varying degree of inhibition of the tested bacteria and fungi. Isolates coded by: REB5, REB9, G12 and DEG 18 exhibited a significant and wide range of antimicrobial activities against *Enterobacter cloacae* (Gram-negative bacterium), *Bacillus cereus* and *Bacillus subtilis* (Gram-positive bacteria) followed closely by *Staphylococcus aureus* that was inhibited by three out of four (75%) isolates tested. *Klebsiella pneumoniae* and *Escherichia coli* (25%) were the less sensitive pathogens tested. In addition to the antifungal activities of three out four (75%), isolates were

active against *Fusarium oxysporum* as shown in Table (2). The antibacterial pattern exhibited by the strains in the present investigation, where the antagonism against Gram-positive bacteria was greater than Gram-negative one and was almost similar to those the ones reported by Tan *et al*. (2004) and Kavithambigai (2006). The reason for different sensitivity between Gram-positive and Gram-negative bacteria could be due to the morphological differences between these microorganisms; Gram-negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes. Gram-positive bacteria were more susceptible to having only an outer peptidoglycan layer, which was not an effective permeability barrier (Pandey *et al*., 2002). The most potent isolates were selected for more study and identification. Shams *et al.* (2015) isolated some marine actinomycete isolates from Lipar area of Oman Sea and showed a good antibacterial activity against *Staphylococcus* species than Gram-negative bacteria including *Escherichia coli*.

Table 2. Inhibition spectrum (mm) of four actinomycetes isolates against the test pathogens in primary screening method. Data represented as means of three replications with standard error.

Isolate code	Tested bacteria						Tested fungi			
	E. cloacae	*K. penumoniae*	*E. coli*	*S. aureus*	*B. cereus*	*B. subtilis*	*F. oxysporium*	*A. niger*	*A. Flavus*	*A. alternata*
REB5	6.27± 0.17	0.0	3.33±0.09	5.3±0.15	4.6± 0.21	2.37± 0.1	4.3±0.44	0.0	0.0	0.0
REB9	10.5± 0.29	0.0	0.0	0.73±0.7	2.8±0.17	2.75±0.14	4.5± 0.29	0.0	0.0	0.0
G12	5.0± 0.29	0.0	0.0	0.0	6.67± 0.44	6.1±0.1	7.3±0.44	0.0	1.5±0.29	0.0
DEG18	15.4± 1.45	4.1± 0.81	0.0	6.5±0.3	5.0± 0.29	1.3±0.17	0.0	0.0	0.0	0.0

Identification of the most potential actinomycetes

Examination of four grown isolates on starch casein medium at 28º± 2º C for 7 days under light microscope and JSM-5300, Jed Scanning electron microscope revealed that, only the isolate REB5 has a spiral sporophore and spiny spore surface, however, isolates REB9, G12 and DEG18 have spiral sporophore and smooth spore surface as shown in Figure (1). The substrate mycelium (SM) had no distinctive color. It varied depending on the type of the used media. Strains grew well to moderate on the tested organic and synthetic media. The color of aerial mycelium varied depending on the type of used media (Table 3).

The results of the chemotaxonomic and physiological experiments of selected strains are shown in Table 4. It can be seen that the presence of a chemotype I cell wall characterized by L-DAP and no characteristic sugars were detected. All isolates can coagulate the milk and cannot produce melanoid pigments. All selected isolates can grow on starch casein media with 60g/l of salt concentration, whereas strain G12 can grow up to 70g/l of salt concentration, which shows the salinity tolerance ability as the characteristics of marine microorganisms.

The best media for growth of all these actinomycete isolates were starch casein agar media It was found that starch casein media are quite suitable and used for isolation of marine actinomycetes, this result agreed with the results of Lin and Liu (2010); Ballav *et al*., (2015). The isolate REB5 was additionally preferred to grow on starch nitrate agar and Czapek-Dox agar, while isolate DEG18 was additionally able to grow on starch nitrate agar; this result was in agreement with Attimarad *et al.* (2012) who reported that starch nitrate agar media were suitable for production of bioactive metabolites from some marine *Streptomyces* species against

The color of aerial mycelium was grey for isolate REB9 with yellow diffusible pigments, and isolate REB5 without diffusible pigments. It was off white for the isolates G12 and DEG18 on the studied media (Table 3). All the pigmentations of the studied isolates were non-sensitive towards the HCl and NaOH except isolate REB9 which showed sensitivity with HCl. The isolates REB5, REB9, G12 and DEG18 were able to grow at a wide range of temperatures (8ºC to 45ºC). The optimum growth was at 28ºC ± 2 ºC. The pH 6, 7, and 8 were suitable for their growth with optimum pH at 7. According to Guimarães *et al*. (2004), the pH of the culture medium is one of the most important environmental factors, because it exerts a marked effect on the activity of several enzymes that catalyze metabolic reactions, as well as exerting significant influence on complex physiological phenomena such as membrane

permeability and cell morphology (Guimarães *et al*., 2004). Bundale *et al.* (2015) reported that pH 7 was found to be optimum for both growth as well as bioactive metabolite production from isolate R3 toward *Bacillus cereus*. The characteristics of four strains such as obtained cellular morphology, cultural properties, physiological and chemotaxonomic characterization were compared with those of known species of actinomycetes described in Bergey's Manual of Systematic Bacteriology (Williams *et al*., 1989), suggested strongly that these isolates belongs to genus *Streptomyces* and named as *Streptomyces* sp strain REB5, *Streptomyces canaries* strain REB9, *Streptomyces* sp strain G12 and *Streptomyces albus* strain DEG18. They were isolated from three sites (Ras El-Bar, Gamasa and Damietta El-Gededa).

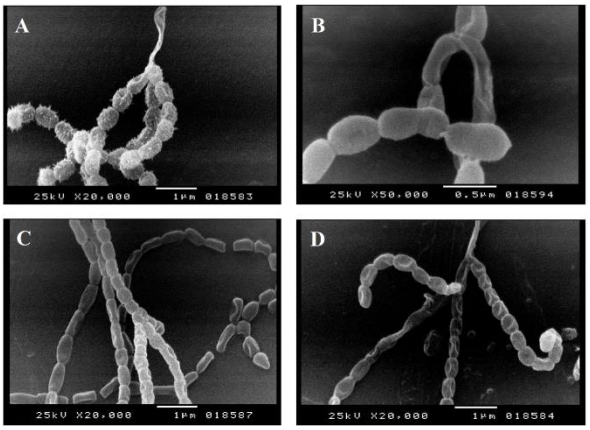

Figure 1. Spore and sporophore morphology of *Streptomyces* isolates using JSM-5300, Jed Scanning electron microscope. A: *Streptomyces* sp strain REB5, B: *Streptomyces albus* strain DEG18, C: *Streptomyces* sp strain G12, D: *Streptomyces canaries* strain REB9. *Staphylococcus aureus*, *Bacillus subtilis* and *Escherichia coli.*

Table 3. Cultural characteristics of the selected *Streptomyces* strains.

Characters		Media / Strain code	Starch casein	Starch nitrate	Starch amm. sulphate	Czapex-Dox	CM-1	Glycerol-Yeast	Glycerol-Asparagine
Appearance of aerial mycelium growth		REB5	Powdery, good	Powdery, good	Powdery, very weak	Powdery, good	Powdery, good	Powdery, weak	Powdery, very weak
		REB9	Powdery, good	Powdery, good	Powdery, very weak	Powdery, good	Powdery, good	Powdery, weak	Powdery, moderate
		G12	Powdery, good	Powdery, no aerial growth	Powdery, moderate aerial growth	Powdery, good	Powdery, good	Powdery, no aerial growth	Powdery, no aerial growth
		DEG18	Powdery, good	Powdery, no aerial growth	Powdery, moderate aerial growth	Powdery, good	Powdery, good	Powdery, no aerial growth	Powdery, no aerial growth
Color of	Aerial mycelium	REB5	Grey	Grey	Pale yellow	Whitish grey	Orange grey	Pale creamy	White
		REB9	Grey	White	Non	Grey	White	White	Whitish grey
		G12	Off white	Non	Off white	Pale yellow	White	Non	Non
		DEG18	Off white	White	Off white	Pale yellow	White	Non	Non
	Substrate mycelium	REB5	Pale grey	Dark grey	Yellow	White grey	Brown	Off white	Off white
		REB9	yellow	Off white	Yellow	Pale Yellowish grey	Yellowish grey	Yellow	Brown
		G12	Off white	Off white	Off white	Pale yellowish green	Pale yellow	Pale yellow	Pale yellow
		DEG18	Off white	Off white	Off white	Pale yellowish green	Pale yellow	Pale yellow	Pale yellow
	Pigments	REB5	Grey	Non	Non	Non	Non	Non	Non
		REB9	Yellow	Non	Non	Pale yellow	Dark yellow	Non	Brown
		G12	Non	Non	Non	Non	Non	Non	Non
		DEG18	Non	Non	Non	Non	Non	Non	Non
Growth intensity		REB5	++	+++	+	+	+	±	+
		REB9	++	+	±	+	+	±	+
		G12	+	±	+	+	+	±	±
		DEG18	-	-	-	-	-	-	-

Sensitivity towards	NaOH	REB5	-	-	-	-	-	-	-
		REB9	-	-	-	-	-	-	-
		G12	-	-	-	-	-	-	-
		DEG18	-	-	-	-	-	-	-
	HCl	REB5	-	-	-	-	-	-	-
		REB9	-	-	-	+	+	-	-
		G12	-	-	-	-	-	-	-
		DEG18	-	-	-	-	-	-	-

- no growth, ± doubt growth, + growth, ++ moderate growth, and +++ heavy growth.

Table 4. Physiological and chemo-type characterization for the selected *Streptomyces* isolates.

	Items	*Streptomyces sp*. strain REB5	*Streptomyces canaries* strain REB9	*Streptomyces sp*. strain G12	*Streptomyces albus* strain DEG18
Chemotaxonomic characteristics	Whole cell hydrolysate	LL-DAP	LL-DAP	LL-DAP	LL-DAP
	Whole cell sugar pattern	Non characteristic sugar (glucose)	Non characteristic sugar (glucose)	Non characteristic sugar	Galactose & glucose
Physiological characteristics	Liquefaction of gelatin	+	-	+	+
	Coagulation of milk	+	+	+	+
	Hydrolysis of starch	±	-	-	-
	Decomposition of cellulose	+	++	+	++
	Melanoid pigment	-	-	-	-
	Carbon utilization				
	Glucose	+	+	+++	++
	Fructose	+	-	+	+
	Galactose	++	+	+	-
	Xylose	+	+	++	+
	Rhamnose	++	+	++	+
	Lactose	+	+	+	+++
	Sucrose	+	+	++	+++
	Maltose	+	+	+	+
	Starch	++	+	++	+
	Cellulose	-	+++	+	+++
	Sodium acetate	+	++	+	-
	Sodium citrat	+	+	-	-
	Mannitol	+++	++	+	-
	Arabinose	++	++	+	+
	Raffinose	-	+++	-	-
	Inositol	+	+++	-	-
	Nitrogen utilization				
	L- proline	+++	+++	+++	++
	L-methionine	+	++	+	+
	DL-phenylalanine	+++	++	++	+
	L-histidine	+++	++	++	+
	Peptone	+++	+	+++	+
	Tryptone	++	+	+++	+
	L-tyrosine	+++	++	+++	++
	Sodium nitrate	++	++	±	+
	Casein	++	++	++	+
	Ammonium sulphate	++	++	+	++
	Salt tolerance	≤60 g/l	≤ 60 g/l	≤ 70 g/l	≤ 60 g/l

- no growth, ± doubt growth, + growth, ++ moderate growth, and +++ heavy growth.
≤60 g/l: growth occurred till concentration of salt ≤ 60 g/l
≤ 70 g/l: growth occurred till concentration of salt ≤ 70 g/l

Bioactivity of streptomycetes species in secondary screening

In secondary screening, all of these streptomycetes were detected to have the ability to inhibit the growth of one or another tested pathogens. The findings in the present study had exceeded the estimation of Ndonde and Semu (2000), where about 75% of *Streptomyces* species were estimated to produce antimicrobial substances of one type or another. This indicates that the potential of these marine environments was sampled to harbor antimicrobial-producing *Streptomyces* species. Preliminary data in Table 5 showed that all the streptomycetes species tested were inhibitory to at least one Gram-positive bacterium and one Gram-negative bacterium. All of them inhibited growth of *Enterobacter cloacae*. On the other hand, four strains inhibited at least one of the tested fungi and inhibited growth of *Fusarium oxysporum*. In secondary screening, growth inhibition of tested Gram-positive bacteria decreased by 25.8% of bioactivity in primary screening, while growth inhibition of tested Gram-negative bacteria decreased by 6.2%; no streptomycetes species showed any activity against *Klebsiella pneumoniae*. It could be suggested that a higher concentration of bioactive metabolites inhibitory towards Gram-positive and Gram-negative bacteria were produced in solid culture (Tan *et al*., 2004). On the other hand, growth inhibition of fungi was increased by 6%, growth inhibition of *Alternaria alternata* was evident in secondary screening, but none in primary screening. This indicated that the diffusible extracellular metabolites in solid medium did not induce this antifungal activity.

Extraction with ethyl acetate and acetone were important for the production of antifungal compounds from *Streptomyces albus* strain DEG18 against *Fusarium oxysporum* and *Alternaria alternata* in secondary screening, while it was none in primary screening. Extraction with ethyl acetate and acetone were not suitable for the same strain to produce antibacterial activities. In addition, these extractions were not suitable for all strains to produce potent activity against *Enterobacter cloacae*. The solvents were used for extraction may not be suitable for the strains (Pandey *et al*., 2002). This was contrary to the results reported by Farida *et al.*, (2007); Lin and Liu, (2010); Attimarad *et al.*, (2012); Jose *et al.*, (2013); Bundale *et al.*, (2015) who found that ethyl acetate is the most appropriate solvents for antibiotic extraction. This might be due to presence of greater amount of active antimicrobial components which are more soluble in organic solvent than water (Karima *et al.*, 2015). All selected strains of *Streptomyces* species displayed a broad spectrum activity against at least one of the fungi, Gram-positive and Gram-negative bacteria tested in secondary screening. Bioactivity of a single strain of *Streptomyces* against a variety of pathogenic microorganisms indicated that a single strain of *Streptomyces* could possibly produce a variety of antimicrobial substances. These strains could possibly produce the same bioactive metabolites inhibitory against the Gram-positive and Gram-negative bacteria tested. Many antibacterial compounds have the inhibitory effect against both of Gram-positive and Gram-negative bacteria and produced previously from *Streptomyces violaceus* and *Streptomyces coelicolor* (Hobbs *et al*., 1992); *Streptomyces tokumonensis* (Betina, 1994) and *Streptomyces tenebrarius* H6 (Du *et al.*, 2004).

Comparison of antibacterial bioactivity of marine-derived actinomycetes in primary and secondary screenings revealed that all strains were active against at least the same one tested bacterium in both primary and secondary screenings. The same pattern of activity indicated that the *Streptomyces* spp. produced extracellular and intracellular bioactive metabolites antagonistic towards the same microorganisms. Interestingly, *Streptomyces albus* strain DEG18 that was inactive against *F. oxysporum* and *Alternaria alternata* in primary screening, inhibited them in secondary screening (Table 5). There were a few factors that could lead to this pattern of improved activity. Probably, the low concentration of the bioactive metabolites or the intracellularly-bound bioactive metabolites within the *Streptomyces* species was the reason why no inhibition was detected in primary screening. In addition, the increased production of the intracellular or extracellular bioactive metabolites in liquid medium and subsequently in the crude extracts might have increased the antifungal potential of the strains in secondary screening (Tan, 2007). The extraction of the intracellular or membrane-bound bioactive metabolites needed to be performed on this strain. Fragmentation of mycelia in liquid medium during fermentation might cause inactivation of the bioactive metabolites in the extracts (Shomura *et al*., 1979; Tan, 2007). Thus, this could explain the non-inhibitory effect of some strains *Streptomyces* species against some tested bacteria and fungi in secondary screening, although they were

inhibitory towards them in primary screening. The insufficient bioactive metabolites in the crude extracts do not reach the effective dose could be another possible reason for the non-inhibitory effect (Tan, 2007). *Streptomyces albus* strain DEG18 and *Streptomyces* sp strain REB5 were promising strains for the production of bioactive metabolites with suitable solvents.

Table 5. Metabolites and biomass extraction antimicrobial activity for the selected *Streptomyces* strains against some bacteria and fungi as inhibition zone (mm). Data represented as means of three replications with standard error.

Strain name and fractions	Tested Bacteria						Tested fungi			
	E. cloacae	***K. penumoniae***	***E. coli***	***S. aureus***	***B. cereus***	***B. subtilis***	***F. oxysporium***	***A. niger***	***A. flavus***	***A. alternata***
***Streptomyces sp.* strain REB5**	0.6±0.0	0	3.3±0.0	3.18± 0.5	4.0 ±0.3	0	1.35± 0.35	0	0	0
	0	0	0	0	0	0	1.05± 0.05	0	0	0
	0	0	0	0	3.05± 0.05	0	0	0	0	0
***Streptomyces canaries* strain REB9**	2.55± 0.25	0	0	5.35±0.05	2.47± 0.37	0	4.35±0.05	0	0	0
	0	0	0	0	0	0	3	0	0	0
	0	0	0	0	0	0	0	0	0	0
***Streptomyces sp.* strain G12**	4.8	0	0	0	0	6.49±0.19	5.5±0.09	0	0	0
	0	0	0	0	0	0	4.9±0.09	0	0	0
	0	0	0	0	0	0	8.5± 0.5	0	0	0
***Streptomyces albus* strain DEG18**	1.19±0.015	0	0	0	4±1.01	1.15±0.15	0	0	0	0
	0	0	0	0	2.35±0.05	0	4.05± 0.04	0	0	0
	0	0	0	0	0	0	0	0	0	9.2± 0.2

Deepa *et al.* (2013) found that, all the sixteen actinomycete isolates comprised *Streptomyces albus* were highly active against *Staphylococcus aureus* and *Klebsiella pneumoniae.* Pandey *et al*. (2002) found that 27 out of 36 (75%) and 31 (86.1%) actinomycetes were active against *S. aureus* and *B. subtilis*, respectively in primary screening. In secondary screening, 23 out of 36 (63.9%) strains were inhibitory towards both *B. subtilis* and *S. aureus*. Zheng *et al*. (2000) reported that *B. subtilis* was inhibited by nine out of fifteen *Streptomyces* species with an inhibition zone diameter of less than 10 mm. *Enterobacter cloacae* was described as the most susceptible bacterial species because all *Streptomyces* species showed antibacterial activity against it in both primary and secondary screenings. These results showed that diffusible extracellular metabolites produced on agar plate and the intracellular or extracellular metabolites in liquid medium and subsequently in the crude extracts could greatly induce the antibacterial activity against *Enterobacter cloacae*. *Aspergillus niger* was the most insensitive microorganism, where all *Streptomyces* species were inactive toward it in both primary and secondary screenings, this indicated that these strains did not produce intracellular and extracellular bioactive metabolites inhibitory towards it (Tables 2 and 5). This was contrary to the results reported by Deepa *et al.* (2013) and Nandhini *et al.* (2015), who isolated different strains of *Streptomyces albus* from South East Coast of India and Tamil Nadu coastal areas, respectively, which showed the maximum level of inhibition zone towards the *Aspergillus niger*. According to Ndonde and Semu (2000), the sensitivity of the pathogens tested to the bioactive metabolites produced by the *Streptomyces* species might due to non-exposure of the pathogens tested to similar bioactive metabolites previously. As a result, they were still susceptible to such metabolites. Greater resistance of the pathogens tested might be due to previous exposure to antibiotics routinely used in disease control which might be similar to those produced by the present *Streptomyces* species. In addition, the sensitivity of the antimicrobial substances *ex-situ* towards light and temperature, the natural instability after prolonged storage, or low amount of the bioactive substances present in the crude extracts were the possible explanations for the low antimicrobial potential (Tan *et al*., 2004).

Conclusion

In this study, four marine *Streptomyces* strains (*S. albus* DEG18, *S. canaries* REB9, *S.* sp. REB5 and

S. sp. G12) were isolated and identified based on the morphological, chemotaxonomic and physiological characterizations. The primary screening for those strains showed a wide range of antimicrobial activities. Extractions from their metabolites using ethyl acetate and their biomass using acetone exhibited also antimicrobial activities toward some bacteria and fungi. The bioactive metabolites from those marine *Streptomyces* strains are promising for probable novel antimicrobial agents' production. This would need more structural characterizations in the future work.

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الملخص العربي

عنوان البحث: الأنشطة ضد الميكروبية لبض الأكتينومايستات البحرية

محمد أبودبارة وأحمد قاسم السيد وأميرة على الفلال وجيهان زهران
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تم عزل عشرون عزلة أكتينومايستات بحرية من ساحل شمال دلتا مصر، كما تم اختيار و تعريف أربعة عزلات لها نشاط ضد ميكروبى واسع المدى ضد بعض البكتيريا الموجبة والسالبة لصبغة جرام و بعض الفطريات، تم تعريف العزلات على أساس الصفات المورفولوجية و الفسيولوجية و الخواص التصنيفية الكيميائية وتم تسميتها كالتالي:

Streptomyces albus strain DEG18, *Streptomyces canaries* strain REB9, *Streptomyces* sp. strain REB5 و*Streptomyces* sp strain G12.

بعد المسح الأولى تم استخلاص المادة الضد ميكروبية بمذيب الإيثيل أسيتات لرشيح ناتج الأيض و من الكتلة الحيوية للخلايا بمذيب الأسيتون، تم عمل المسح الثانوى باختبار مستخلصات ناتج الأيض و الكتلة الحيوية و الذى أظهر أن جميع العزلات لها نشاط ضد ميكروبى ضد *Enterobacter cloaca* **و** *Fusarium oxysporum* **، بينما ثلاثة عزلات أظهروا نشاط ضدى ميكروبي ضد** *Bacillus cereus* **و إثنان ضد** *Bacillus subtilis* **و** *Staphylococcus aureus* **و البعض كان له نشاط ضد** *Klebsiella pneumoniae* **و** *Alternaria* alternata.