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Isolation, Examination and Characterization of Actinomycetes as a Source of Antimicrobial Agents from Libyan Soil

Galal S. Salem[1*], Saleh H. Baiu[2], Ali A. Ali[3]

[1]Department of Botany, Faculty of Science, University of Benghazi, Benghazi, Libya
[2]Food Science Department and MIRCEN Cairo, Faculty of Agriculture, Ain-Shams University, Cairo, Egypt
Email: *galalsalem@uob.edu.ly

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Abstract

The rapid increase of bacterial resistance strains to multiple antibiotics has become a global public health concern. In the present study, actinomycetes from different districts of Libyan soil were isolated and screened for their inhibitory activity against pathogenic bacteria and fungi. Three hundred soil samples were taken from 77 diverse ecosystems, including deserts, forests, pastureland, and cropland located in different climatic regions in Libya. A total of 164 actinomycetes were obtained. Of 164 isolates, 38 (23.2%) isolates were morphologically and microscopically characterized by spore chain and surface morphology, aerial and substrate mycelia and soluble pigments. The preliminary classification of the isolates illustrates that all isolates belong to the genus *Streptomyces*. These isolates were further examined for their antagonistic potential against nine pathogenic bacteria and fungi. Out of 38, 11 (28.9%) isolates showed their capability to produce inhibitory substances against at least two tested strains. Among bacterial strains, *Staphylococcus aureus* was susceptible to almost all eleven isolates (90.9%), while *Streptococcus pyogenes* was found to be resistant to most selected isolates (18.2%). The isolate, 063 (Wadan soil-desert zone) was the only isolate that exhibited broad spectrum antimicrobial activity against all tested pathogenic microbes, and hence was selected for further study. According to its cultural, morphological, physiological, and biochemical characteristics, the isolate 063 was identified as *Streptomyces rochei*. The results obtained indicate that the Libyan soil, particularly in extreme environments, could be a potent source of bioactive metabolites with antimicrobial potency against a wide variety of microbial pathogens. Thus, this investigation suggested that exploring new habitats in unexplored and untapped regions of Libya could provide a promising source of biologically active compounds for therapeutic applications.

Keywords

Actinomycetes, Antimicrobial Activity, Isolation, *Streptomyces*

1. Introduction

Soil is a highly exploited ecological niche in which the occupants create a variety of biologically active natural chemicals, including antibiotics that are clinically relevant. Most antimicrobial drugs have been derived from the natural products of actinomycetes and fungi [1] [2]. Among the genera, streptomycetes are particularly ubiquitous in soil. Members of the genus *Streptomyce* are gram-positive, aerobic soil inhabitants and are widely distributed in nature, constituting a significant component of the microbial community in most soils [3].

The most beneficial and important characteristic of actinomycetes is their ability to produce antibiotics and other secondary metabolites that exhibit a variety of biological activities like antibacterial, antifungal, antiviral, antitumoral, antimalarial, cytotoxic, cytostatic, immunosuppressive, anti-inflammatory, antiparasitic, antioxidant, anti-angiogenesis, pigments, and enzymes [4]-[8]. In addition, the substances produced by actinomycetes have a diverse array of chemical structures, including macrolides, tetracyclines, aminoglycosides, glycopeptides, and ansamicines, which are used in combating infectious diseases, while anthracyclines support chemotherapy for cancer [9].

In spite of the significance of these biological activities, a plethora of secondary metabolites derived from actinomycetes have become ineffective due to multidrug-resistant bacteria [10] [11] [12]. As a result, there is an urgent need for the discovery and development of unique bioactive substances from new species of actinomycetes that have not yet been studied or exploited for their medical, agricultural, and industrial applications. Furthermore, the bioactivity of actinomycetes might also be variable depending on the kind of soil and its composition [13] [14] [15].

Libyan soils may offer immense potential for discovering new actinomycetes due to their vast and largely unexplored regions. Therefore, exploring new areas and developing innovative techniques to reveal novel actinomycetes with potent antimicrobial activities has become increasingly urgent. Consequently, the present investigation was designed to isolate, examine, and characterize actinomycetes from Libyan soil as a pivotal source of bioactive metabolites.

2. Material and Methods

2.1. Sample Sites and Sample Collection

A total of 300 soil samples were randomly collected from 77 different ecosystems, including deserts, forests, croplands, and pasturelands, located in various climatic zones in Libya (**Figure 1**). In addition, densely and sparsely populated areas were taken into account when collecting soil. Soil samples were obtained

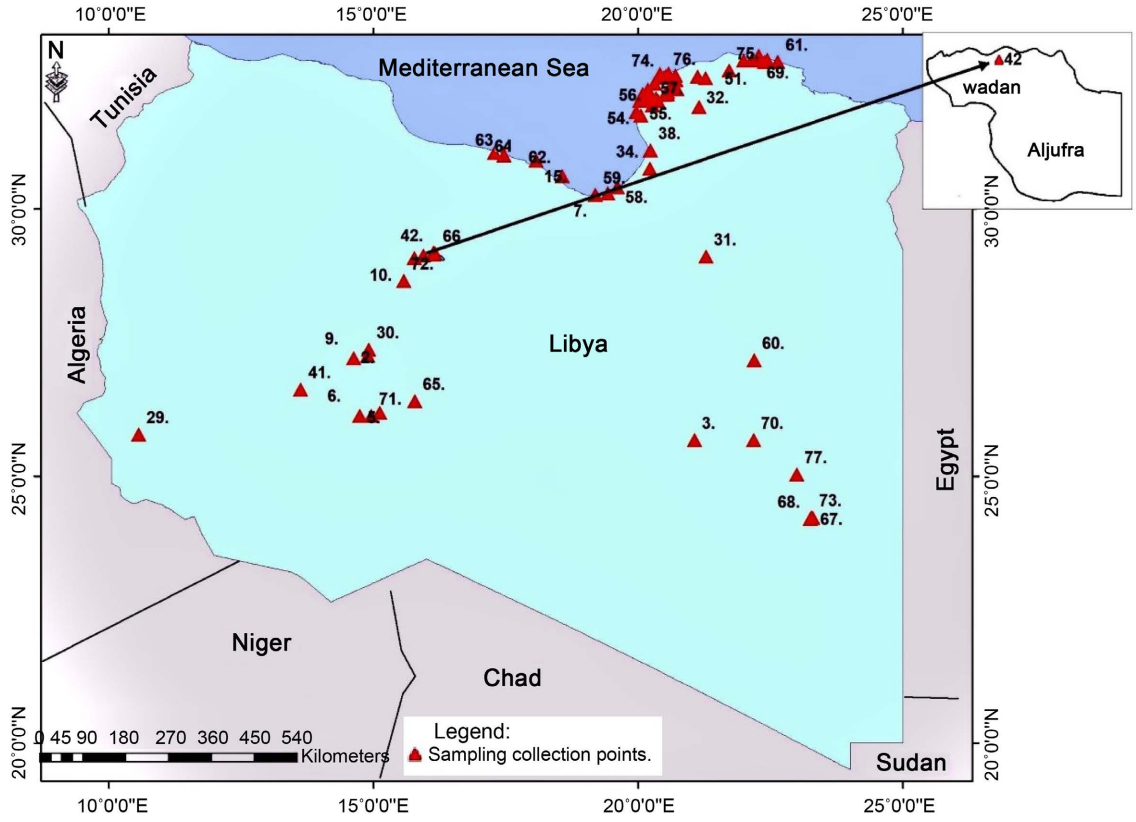


Figure 1. A map of the study area and soil sample locations.

during a six-month period (December 2006 - July 2007).

The soil samples were obtained from a depth of 10 cm after removing the loose surface litter layer and placed in sterile tubes, closed tightly, and then stored at 4˚C before screening was done.

2.2. Isolation and Cultivation of Actinomycetes

The following screening procedure was adopted for the isolation of actinomycetes [16]. The media used were those recommended by Shirling & Gottlieb [17]. Actinomycetes were isolated utilizing the soil dilution plate technique and yeast extract-malt extract agar (ISP2) for purification of actinomycetes isolates slants. One gram of dried soil was placed in 9 ml of distilled water, agitated for 15 minutes and then allowed the suspension to settle for 15 min. Test tubes containing a 10^{-4} - 10^{-6} dilution of soil samples were placed in a water bath at 45˚C for 16 h so that the spores would be separated from vegetative cells, and the dilutions were inoculated on the surface of actinomycete isolation agar plates. Selected actinomycetes colonies were transferred to agar plates and cultured at 28˚C for 7 - 14 days.

2.3. Test Microorganisms

The test microorganisms used were *Staphylococcus epidermidis* (*Staph. epidermidis*) ATCC 12228, *Staphylococcus aureus* (*Staph. aureus*) ATCC 29737, *Ba-*

cillus subtilis (*B. subtilis*) ATCC 6633, *Streptococcus pyogenes* (*Strep. pyogenes*) DSM 2072, *Mycobacterium phlei* (*Mycob. phlei*) EMCC 1113, *Escherichia coli* (*E. coli*) DSM 498, *Salmonella enterica* (*Sal. enterica*) ATCC 25566, *Candida albicans* (*C. albicans*) EMCC 105 and *Aspergillus niger* (*A. niger*) EMCC 132. All these organisms were obtained from MIRCEN Cairo, Faculty of Agriculture, Ain-Shams University, Egypt.

2.4. Screening of Actinomycetes for Antimicrobial Activity

Actinomycetes isolates were tested for their antimicrobial activity against the selected microorganisms by the agar diffusion method according to Bauer *et al*., 1966 [18]. The isolates were cultured on yeast extract-malt extract agar (ISP2) for 7 - 14 days of incubation at 28˚C. Agar discs with a diameter of 9 mm were cut off by a sterile cork borer and transferred to the surface of agar plates, previously inoculated with the test organism. A nutrient agar medium was used for the cultivation of *Staph. epidermidis*, *B. subtilis*, *E. coli*, *Staph. aureus* and *Sal. enterica*. Tryptone Soya Agar was used for the cultivation of *Streptococcus pyogenes*. Glycerol-soil agar was used for the cultivation of *Mycob. phlei*. Sabouraud's dextrose agar was used for cultivation of *C. albicans*. *A. niger* was grown on potato dextrose agar. The plates were incubated for 48 h, at 30˚C for *C. albicans* and *A. niger* of test microorganisms, while *B. subtilis*, *E. coli*, *Staph. aureus*, *Staph. epidermidis*, *Strep. pyogenes*, *Mycob. phlei* and *Sal. enterica* were incubated at 37˚C for 24 h.

2.5. Examination of Inhibition of Indicator Organisms

The presence of a clear halo around the colonies on each plate indicates the efficiency of the colony in producing antimicrobial compounds. All observations and data were recorded.

2.6. Culture Media for Morphological Studies

The standard culture media for morphological studies for all cultures were: yeast extract-malt extract agar (ISP-2); oatmeal agar (ISP-3); inorganic salts-starch agar (ISP-4); and glycerol-asparagine agar (ISP-5), as used by Shirling and Gottlieb [17]. The other media used were glucose-asparagine agar, czapek agar, tyrosine agar (ISP-7), nutrient agar and Bennett's agar (glucose-casein digest-yeast-beef agar), according to Higgens and Kastner, 1971 [19].

2.7. Characterization of Actinomycetes

The International *Streptomyces* Project (ISP) [17] and Bergey's Manual of Systematic Bacteriology were used to characterize actinomycete colonies morphologically and physiologically [20]. Cultural characteristics of pure isolates on various media were recorded after incubation for 7 - 14 days at 28˚C. Morphological observations were made microscopically by using the method of Shirling and Göttlieb [17]. The color of the aerial mycelium, the substrate mycelium and

the soluble pigment produced were determined using different agar media [21]. Light microscopy (Nikon Inc. NY, USA) and transmission electron microscopy (TEM) (Zeiss EM-10, Oberkochen, Germany) were used to examine the micro-morphological characteristics of 7th, 14th, and 21st day old cultures grown on 9 different media, including ISP 2, 3, 4, 5, and 7, Bennett's agar, Czapek's agar, glucose-asparagine agar, and nutrient agar.

2.8. Identification and Classification of Actinomycetes Isolates

The cultural, morphological, physiological, and biochemical characteristics described by Buchanan and Gibbons [22] and Nonomura [23] served as the basis for the identification of the chosen isolates to species level. Furthermore, a numerical taxonomy study of sterptomycetes was introduced to identify the selected isolates. Groups were obtained using similarities and differences in spore chain morphology, aerial and substrate mycelia, soluble pigments, the production of melanin pigment, and the utilization of a wide range of carbon sources [17]. The culture was assigned to various series (gray (GY), red (R), yellow (Y), blue (B), green (GN), violet (V), and white (W) as described by Trenser and Backus [24].

3. Results

3.1. Isolation of Actinomycetes

From the 300 soil specimens that were collected from 77 different locations in Libya, 164 isolates of actinomycetes were obtained. Of the 164 isolates, 38 (23.17%) were morphologically characterized. The isolates were then screened for antimicrobial activity against seven bacterial and two fungal strains. Preliminary antimicrobial screening showed that among the 38 isolates, the isolates namely 07, 010, 025a, 025b, 037, 057, 062, 063, 067, 069, and 071 exhibited antimicrobial potency against at least more than one of the tested microbial pathogens (**Figure 2** and **Table 1**). The isolate 063 from the city of Waddan (a desert region) exhibited a wide spectrum of antimicrobial activity against all the tested microorganisms, with zones of inhibition ranging from 10 to 15 mm in diameter. All isolates showed no antimicrobial activity against *Strep. pyogenes*, except isolates 25a and 063. Furthermore, the results showed that the isolates had antibacterial activity against almost all Gram-positive bacteria tested, particularly *Staph. epidermidis* (**Table 1**).

Based on the results of primary antimicrobial screening, the actinomycete isolate 063 was selected and examined microscopically, and its morphological, cultural, physiological and biochemical characteristics were identified.

3.2. Identification of the Isolate 063

3.2.1. Cultural and Morphological Characteristics of Actinomycete Isolate (063)

According to Nonomura's criteria in 1974, the selected isolate formed a defined

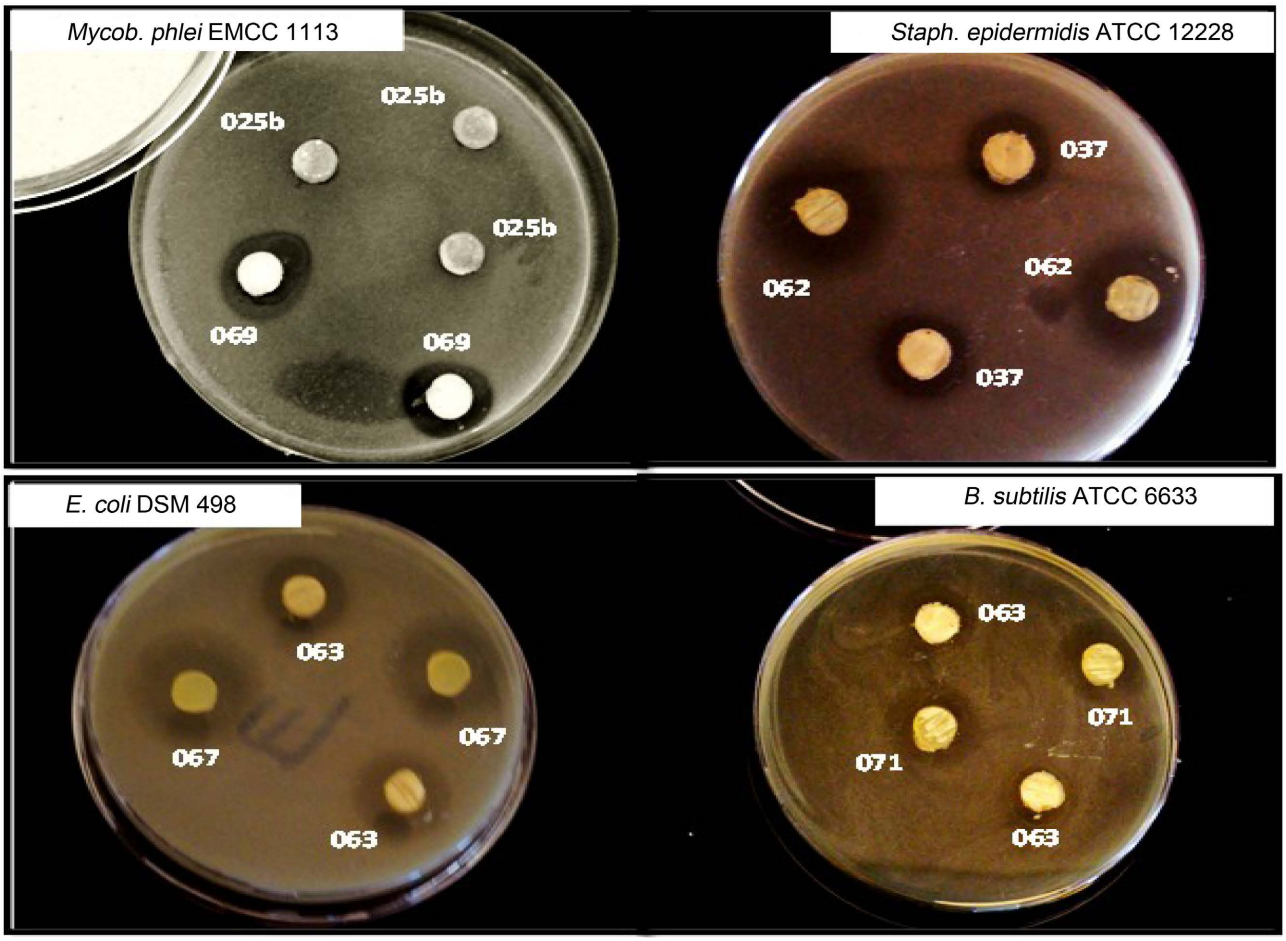


Figure 2. Antimicrobial activity against *Mycob. phlei* EMCC 1113, *Staph. epidermidis* ATCC 12228, *E. coli* DSM 498 and *B. subtilis* ATCC 6633 shown by actinomycetes isolates (Kirby-Bauer method).

Table 1. Diameter of clear zones and inhibition activity of actinomycetes against tested organisms.

Isolate symbol	Zone of inhibition (mm) against test microbes								
	Staph. epidermidis	*B. subtilis*	*E. coli*	*Staph. aureus*	*Strep. pyogenes*	*Mycob. phlei*	*Sal. enterica*	*A. niger*	*C. albicans*
07	-	-	-	-	-	11	-	15	-
010	12	15	-	-	-	11	-	11	-
025a	12	-	-	12	14	12	-	-	-
025b	13	-	-	14	-	-	12	-	11
037	14	18	15	14	-	10	10	11	11
057	12	11	14	12	-	12	13	11	12
062	16	10	16	15	-	11	11	-	11
063	14	12	14	13	15	11	10	11	12
067	13	12	16	13	-	11	10	-	11
069	19	10	13	18	-	13	14	11	11
071	12	15	14	15	-	-	10	11	-
	90.9%	72.7%	63.6%	81.8%	18.2%	81.8%	72.7%	63.6%	63.6%

-, No inhibition zones; *Staph. epidermis* = *Staphylococcus epidermis*; *B. subtilis* = *Bacillus* subtilis; *E. coli* = *Escherichia coli*; *Staph. aureus* = *Staphylococcus aureus*; *Strep. pyogenes* = *Streptococcus pyogenes*; *Mycob. phlei* = *Mycobacterium phlei*; *Sal. enterica* = *Salmonella enterica*; *A. niger* = *Aspergillus niger* and *C. albicans* = *Candida albicans*.

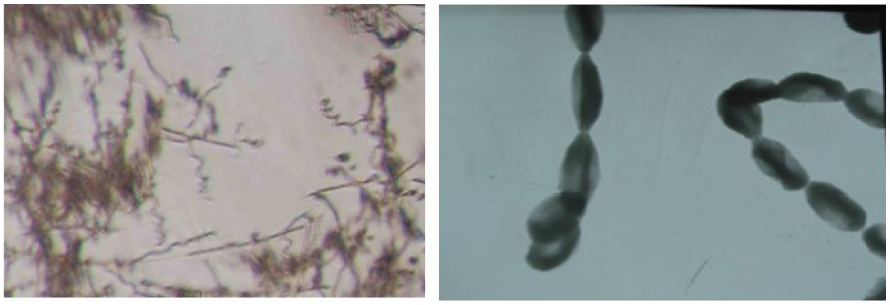
Table 2. Cultural and morphological characteristics of the isolate (063) on different media.

Characteristic	Results
Cultural	
Yeast extract-malt extract agar (ISP-2) medium	
Growth	+++
Aerial mycelium	gray
Substrate mycelium	gray yellow
Soluble pigment	-
Oatmeal agar (ISP-3) medium	
Growth	+++
Aerial mycelium	gray
Substrate mycelium	gray yellow
Soluble pigment	pale yellow
Inorganic salt starch agar (ISP-4) medium	
Growth	+++
Aerial mycelium	gray
Substrate mycelium	gray yellow
Soluble pigment	-
Glycerol-asparagine agar (ISP-5) medium	
Growth	+++
Aerial mycelium	gray
Substrate mycelium	grayish
Soluble pigment	-
Bennett's agar medium	
Growth	+++
Aerial mycelium	gray
Substrate mycelium	grayish
Soluble pigment	-
Czapek's agar medium	
Growth	+++
Aerial mycelium	gray
Substrate mycelium	grayish
Soluble pigment	-
Glucose-asparagine agar medium	
Growth	++
Aerial mycelium	gray

Continued

Substrate mycelium	velvety
Soluble pigment	-
Tyrosine agar (ISP-7) medium	
Growth	+
Aerial mycelium	white
Substrate mycelium	velvety
Soluble pigment	-
Nutrient agar medium	
Growth	++
Aerial mycelium	gray
Substrate mycelium	grayish
Soluble pigment	-
Morphological	
Spore chain	spiral
Spore surface	smooth

-, No growth; +, poor growth; ++, moderate growth; +++, good growth.



(a) (b)

Figure 3. Spore chain and surface; (a) spore chain morphology of the selected isolate under light microscopy and (b) spore surface morphology under transmission electron microscopy.

pigment only on ISP-3 medium with a pale-yellow color (Table 2). The selected isolate displayed different substrate and aerial mycelia colors. The color of the aerial mycelium in all tested media except ISP-7 was gray, while the color of the substrate mycelium varied from gray-yellow to grayish and velvety (Table 2). The spiral chain was observed in actinomycete isolate 063 at 1000× magnification using light microscopy (Figure 3(a)). The spore surface morphology of the selected isolate was observed to be smooth under 20,000× magnification by transmission electron microscopy (Figure 3(b)).

3.2.2. Physiological and Biochemical Characteristics

The actinomycete 063 was further identified using physiological and biochemical properties. The culture's growth on carbon compounds ranged from good to

moderate to weak for the selected isolate. The results further indicated that the isolate was unable to utilize raffinose as the sole carbon source. Furthermore, the nitrate and gelatine reduction tests were positive for the selected isolate, while the coagulation of milk test showed a negative result (**Table 3**).

Based on its culture, microscopic, and morphological properties, as well as its physiological and biochemical properties, our research showed that isolate 063 belongs to the genus *Streptomyces*. Comparisons with descriptions of *Streptomyces* species in Nonomura's key [23], as well as references from ISP [17] and *Bergey's Manual of Determinative Bacteriology* [20] exhibited that isolate 063 is closely related to *Streptomyces rochei* (**Table 4**).

Table 3. Physiological and biochemical characteristics of the actinomycete 063.

Characteristics	Results
Hydrolysis reaction	
Production of melanin pigment	negative
Nitrate reduction	positive
Gelatine reduction	positive
Coagulation of milk	negative
Carbon utilization of	
D-glucose	+++
D-xylose	+++
L-arabinose	+++
L-rhamnose	+++
D-fructose	++
D-galactose	+++
raffinose	negative
D-mannitol	+++
Meso-inositol	++
Salicin	+
Sucrose	+

+: Weak growth; ++: Moderate growth; +++: good growth.

Table 4. Basic taxonomical characteristics of the isolate (063) and related *Streptomyces* species.

Isolate and Name of species	Aerial mycelium color	Melanoid pigment	Spore chain	Spore surface	D-Glucose	D-Xylose	L-Arabinose	L-Rhamnose	D-Fructose	D-Galactose	Raffinose	D-Mannitol	Salicin	Sucrose
Isolate 063	GY	-	S	SM	+	+	+	+	+	+	-	+	+	+
**Streptomyces rochei*	GY	-	S	SM	+	+	+	+	+	+	-	+	+	-
**Streptomyces olivaceoviridis*	GY	-	S	SM	+	+	+	+	+	NA	-	+	NA	±
**Streptomyces recifensis*	GY	-	S	SM	+	+	+	-	+	+	+	+	+	+

GY: Gray; S: Spiral; SM: Smooth; +: Carbohydrate utilized; ±: very slight utilization; -: not utilized; NA: not available. *Data were taken from Bergey's Mannual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

4. Discussion

Actinomycetes represent a diverse group of bacteria that have been extensively studied due to their capacity to produce bioactive compounds with potent antimicrobial activities. However, with the rising numbers of multidrug-resistant microbes, the need for new and powerful antimicrobial agents has become increasingly urgent. These actinomycetes are primarily found in soil and can vary depending on the kind of soil, making it essential to examine a wide range of areas for the discovery of valuable antimicrobial compounds. Isolation of actinomycetes from unexplored and underexploited habitats could provide a promising avenue for discovering novel and effective chemicals and bioactive compounds [13] [14]. Libya has vast and largely unexplored regions that could potentially produce unique and novel bioactive secondary compounds.

Hence, research on actinomycetes in Libya may uncover antimicrobial substances that have not yet been investigated as potential sources of therapeutic metabolites for their applications in pharmaceutical and agricultural techniques. By exploring these uncharted and untapped habitats, we may reveal new species of actinomycetes that have not yet been studied or exploited for their medical, agricultural, and industrial applications.

Our findings indicated that 164 actinomycete isolates were obtained from 300 soil specimens collected from 77 different locations in Libya. Out of 164 actinomycete isolates, 38 isolates (23.17%) were morphologically distinct. Our screening results reported that out of 38 isolates, 11 isolates (28.95%) showed antimicrobial activity against tested microorganisms. The antibacterial activity of the isolates was found to be effective against *Staph. epidermides* (90.9%), *Staph. aureus* and *Mycob. Phlei* (81.2%), *B. subtilis* and *Sal. enterica* (72.7%), *E. coli* (63.6%) and *Strept. Pyogenes* (18.2%), while (63.6%) of the isolates were active against *A. niger* and *C. albicans*. The lowest activity or the resistance of *Strept. pyogenes* to the most of the isolates may attributed to produce an enzyme, which inactivated any substance produced.

In addition, our results demonstrated that more isolates were active against Gram positive bacteria than Gram negative bacteria, as reported by Thakur *et al.* [25] and Gurung *et al.* [26]. This could be due to morphological differences in the cell walls of these microbes. The outer membrane of Gram-negative bacteria provides a formidable barrier that is impermeable to lipophilic compounds. Moreover, the lipid and protein compositions of the outer membrane have a major role in providing a barrier to hydrophobic antibiotics and other compounds, while porin acts as a selective permeability barrier to the hydrophilic solutes [26] [27]. Gram positive bacteria, on the other hand, have only a peptidoglycan layer, which is ineffective as an antibacterial agent permeability barrier [26].

Of all the isolates assayed in the present study, strain 063, which was isolated from Wadan soil (a desert region), showed highly broad-spectrum antimicrobial activity and inhibited the growth of all the test organisms used in this investiga-

tion. It is therefore possible that an isolate might produce various metabolically active compounds. El-Barasi *et al*. [28] revealed that the chemical structure of Wadan soil contained a high concentration of calcium carbonate ($CaCO_3$). Soil samples treated with calcium carbonate were found to be most effective for the isolation of bioactive actinomycetes, which provide antimicrobial products [29] [30] [31]. Uzcátegui *et al*. [32] demonstrated that calcium carbonate was one of the most effective additive substances in promoting Actinomyces antimicrobial activity against pathogens. As a result, Wadan soil could be a significant source of new natural products derived from microbes.

Parameters such as spore chain morphology and ornamentation of the spore surface are fundamental for the classification and identification of the Actinomycetes species [17] [20] [23] [33]-[38]. Microscopic examination demonstrated that the isolates belong to the genus *Streptomyces*. The frequency and dominance of *Streptomyces* isolates among actinomycetes in various soil types were also reported by several investigators [39]-[45]. Based on the color of aerial mycelium, the selected isolate was closely related to the gray series. These results were in agreement with other previously obtained results, which showed that the gray and white color series of Actinomycetes were the predominant patterns in the soil [25] [46]. Microscopically, it was observed that the spore chains of the aerial mycelium of the selected isolate showed a spiral chain. Similarly, Thakur *et al*. [25] stated that the majority of the isolates were considered to be spiral and rectus-flexibilis sporophores.

In the current research, the morphological evaluation of the spore surface indicated that the tested isolate belonged to the smooth spore surface. Similar results were also reported by Okudoh and Wallis [47]. According to Pridham and Gottlieb [48], the use of carbon compounds is crucial for the taxonomic classification of actinomycete species. Carbon sources such as glucose, xylose, arabinose, rhamnose, galactose, mannitol, and meso-inositol were all successfully metabolized by isolate 063. However, the chosen isolate was unable to use raffinose as a carbon source.

5. Conclusion

This study aimed to isolate actinomycetes from Libyan soils and assess their antimicrobial activities against a wide variety of pathogenic microorganisms. Our findings indicated that all isolates belong to the genus *Streptomyces*. The selected isolate 063 from an extreme geographic and climatic area (the Wadan soil of the Aljufra area) exhibited promising antimicrobial activity against all the test microbes, which might be related to the presence of several bioactive compounds. In general, the exploration of Libyan soil for novel actinomycetes is a promising avenue for discovering unique antimicrobial agents. Additionally, studying these actinomycetes using molecular techniques could lead to a better understanding of the mechanisms behind their ability to produce these valuable substances.

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Conflicts of Interest

The authors declare no conflict of interest.

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