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Identification and preliminary characterization of non-polyene antibiotics secreted by new strain of actinomycete isolated from sebkha of Kenadsa, Algeria

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ABSTRACT

Objective: To study the antimicrobial activity of actinomycetes isolated from sabkha of Kenadsa and identification of the isolate interesting.
Methods: Eighteen strains were isolated, using four culture media from sebkha of Kenadsa (Bechar, Southwestern Algeria). Screening of antimicrobial activity consisted of two steps: in primary screening, antibacterial activity was determined by using the agar plug method against test strains; in secondary screening, better isolate which showed a good activity in the first screening was selected to extract antimicrobial substances. The antimicrobial activities of extracts were evaluated by using Kirby–Bauer disc diffusion method. Partial characterization of antimicrobial products was performed on the basis of chemical revelations, UV–vis spectrometry and infrared spectroscopy. The identification of isolate interesting was performed through morphological, chemical, biochemical and physiological characteristics.
Results: All isolates showed antimicrobial activity against at least one microorganism test. One isolate, LAM143cG3, was selected for its broad spectrum and high antimicrobial activity. The isolate LAM143cG3 was identified as *Spirillospora* sp. The comparison between the species of this genus (*Spirillospora rubra* and *Spirillospora albida*) and our isolate indicated the existence of several physiological and biochemical differences which led us to suppose that this was a new member of this genus. Primary characterization of antimicrobial substances produced by the isolate LAM143cG3 indicated the presence of amines and phenols. The UV–vis spectrum suggested a non-polyenic nature of substances secreted by our isolate, while infrared confirmed the presence of amine groups.
Conclusions: The result of the present study revealed that sebkha of Kenadsa was rich in rare actinomycetes, that secreted interesting antimicrobial substance.

1. Introduction

The rapid emergence of drug resistance among pathogenic bacteria, especially multi-drug resistant bacteria, underlines the need to look for new antibiotics [1,2]. Filamentous actinomycetes are known to have the ability to produce a wide variety of secondary metabolites [3,4], many of which have useful applications in human and veterinary medicine and agriculture [5], such as enzyme immuno-modulators, antibiotics, insecticides, herbicides and anticancer agents [6]. Among actinomycetes, *Streptomyces* which has long been recognized as a major source of bioactive molecules covers around 70% of the total antibiotic products [7,8]. Actinomycetes are widely distributed and are next to bacteria in the order of abundance in soil [9]. In the past two decades, there has been a decline in the discovery of new lead compounds from common soil-derived actinomycetes [10], hence the scientists have tried to investigate unexplored habitats for novel actinomycetes as possible candidates of new antimicrobials substances [11,12]. Among them, sebkha is

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an extreme environment, which inhabits organisms that survive in very high salinities, high temperatures and withstand severe solar radiations. The aims of the present work were to study the biodiversity of halophilic actinomycetes in sebkha of Kenadsa and to highlight their potential to produce bioactive substances.

2. Materials and methods

2.1. Samples collection

Soil samples were collected at a depth of 15–20 cm below the surface from sebkha of Kenadsa (Bechar, Southwestern Algeria), according to Pochon and Tardieu technic [13]. The samples were packed in sterile polyethylene bags and aseptically transported to the laboratory as quickly as possible for further analysis.

2.2. Samples treatment

The samples were subjected to a pretreatment method in order to facilitate isolation of actinomycetes. The collected samples were air dried for 7 days, and then they were pre-treated with 1% calcium carbonate ($CaCO_3$) and incubated at 25 °C for 2 weeks [14].

2.3. Isolation of the actinomycetes

Actinomycetes were isolated by the serial dilution method [15,16]. Stock solution was prepared by diluting 1 g of soil sample in 9 mL of sterile saline water and shaked well by using a vortex mixer. From the stock solution, 1 mL was used to prepare the final volume of 10^{-2} and 10^{-3} by serial dilution method. About 1 mL of each dilution was sown on the surface of four different mediums: ISP2 (yeast extracts: 4 g/L; malt extract: 10 g/L; glucose: 4 g/L; agar: 20 g) [17]; Bennett (D-glucose anhydre: 10 g/L; casaminoacides: 2 g/L; yeast extract: 1 g/L; meat extract: 1 g/L; agar: 15 g) [18]; Starch casein (starch: 10 g/L; casein: 0.3 g/L; KNO_3: 2 g/L; NaCl: 2 g/L; K_2HPO_4: 2 g/L; $MgSO_4 \cdot 7H_2O$: 0.05 g/L; $CaCO_3$: 0.02 g/L; $FeSO_4 \cdot 7H_2O$: 0.01 g/L and agar: 18.0 g) [19]; Starch yeast extract peptone (starch: 10 g/L; yeast extract: 4 g/L; peptone: 2 g/L; agar: 18 g/L) [20]. About 7% NaCl was added to each culture medium. Amphotericin B (25 µg/mL) and oxytetracycline (10 µg/mL) were added in each medium to inhibit fungal and bacterial contamination respectively. Plates were incubated for 7–20 days at 28 °C, and observed intermittently during incubation [21].

2.4. Preliminary screening of antimicrobial activity using agar piece method

Eight bacteria and one yeast were used as tests organisms: *Starphylococcus aureus* (ATCC 25923) (*S. aureus*), *Enterococcus faecalis* (ATCC 25212), *Bacillus cereus* (ATCC 11778) (*B. cereus*), *Bacillus subtilis* (ATCC 6633) (*B. subtilis*), *Klebsiella pneumonia* (ATCC 70603) (*K. pneumonia*), *Pseudomonas aeruginosa* (ATCC 27853) (*K. pneumonia*), *Escherichia coli* (ATCC 25522) (*E. coli*), *Bacillus stearothermophilus* (ATCC 12980) (*B. stearothermophilus*) and *Candida albicans* (ATCC 10231) (*C. albicans*). The strains of actinomycetes were inoculated on Bennett medium then incubated at 28 °C for 14 days. Cylinders agar (6 mm in diameter) of well grown cultures were cut and placed on plates already seeded with the test strains. Plates were kept at 4 °C for 4 h for a good diffusion of the antimicrobial metabolite, and then incubated bacteria at 37 °C for 24 h and *C. albicans* at 30 °C for 48 h. The best isolate which showed a good antimicrobial activity was selected [22].

2.5. Optimization of secretion of secondary metabolites

In the investigation of the culture medium that would enable optimal production of antimicrobial molecules, the selected isolate was inoculated in five culture media: ISP1 (tryptone: 5 g/L; yeast extract: 3 g/L; agar: 18 g) [17]; GYEA (yeast extract: 10 g/L; glucose: 10 g/L; agar: 18 g) [23]; PELG (peptone: 5 g/L; glucose: 10 g; yeast extract 2 g/L; agar: 20 g); Bennett and ISP2 (compositions are mentioned above). The plates were incubated at 30 °C for 14 days. The antimicrobial activity against test organisms was determined by the agar piece method [22].

2.6. Extraction of antimicrobial compounds

The selected antagonistic actinomycetes were inoculated into 250 mL of the best medium for production of antimicrobial substances and incubated at 30 °C in a shaker (150–180 r/min) for 14 days. After incubation the broths were centrifuged at 3000 r/min for 15 min, and the cell-free supernatant was extracted whit equal volume of three solvents including: *n*-butanol, chloroform and ethyl acetate [24]. Each organic extract was concentrated at 45 °C for dryness by using a rotary evaporator, and then recuperated in 1 mL of methanol and tested for their antimicrobial activities by using Kirby–Bauer disc diffusion method. The disks were deposited on the surface of Muller–Hinton medium, already sown by the test germs. The plates were incubated at 4 °C for 2 h and later at 37 °C. The diameter of the aureoles of inhibition was measured after 24 h [25].

2.7. Thin layer chromatography (TLC) and bioautography

TLC with silica gel plates was used for primary analysis of the antibacterial substances. A dry crude extract, dissolved in methanol, was spotted and developed in the solvent system: ethanol-ammonia-water (8:1:1, v/v). The developed TLC plates were air dried to remove all traces of solvents. The separated compounds were visualized under UV at 254 nm and at 365 nm and the active spots were detected by bioautography [26].

Chromatogram was placed in a sterile bioassay Petri dish containing Muller–Hinton agar medium inoculated with *B. stearothermophilus* as target organisms and incubated for 24 h at 30 °C. Clear areas due to the inhibition of the growth of target organisms indicated the location of antibiotic compounds, the retention factor (R_f) of the active spots was measured [27].

2.8. Partial characterization of products

The active substances were revealed on silica gel TLC plates with several chemical agents including ninhydrin, ferrous iron chloride, formaldehyde-H_2SO_4 and Molish's reagent. UV–visable

absorption spectra were recorded on an SPECTRONIC UNICAM UV 500, while the infrared was determined by Agilent technologie cary 600 series Fourier transform infrared spectroscopy spectrometer.

2.9. Taxonomy of potential actinomycete strain

Taxonomic studies were performed based on morphological, chemical, biochemical and physiological analyses.

2.9.1. Chemotaxonomic analysis

Standard analytical procedures were used to extract and analyse the isomeric forms of diaminopimelic acid [28] and whole-organism sugars [29].

2.9.2. Morphological and cultural characters

Morphology studies were performed by using the methods described by Shirling and Gottlieb [17]. The microscopic characterization was done by cover slip culture method. The cultural characteristics were examined by culturing isolates on different ISP media: yeast extract-malt extract-agar (ISP2), oatmeal-agar (ISP3), inorganic salts-starch-agar (ISP4), and glycerol-asparagines-agar (ISP5). In each medium, color and growth of aerial and substrate mycelium as well as the formation of soluble pigments were noted after incubation at 30 °C for 14 days.

2.9.3. Biochemical and physiological characterization

The ability of the selected isolate to utilize 10 different carbon sources were determined on plates containing basal medium (ISP9) to which carbon sources were added to a final concentration of 1%. The plates were incubated at 28 °C for 14 days [17]. The research of melanoides pigments was carried out by using ISP6 and ISP7. The actinomycete isolate was tested for its ability to grow in different concentrations of NaCl range from 0% to 15%, and temperature range from 25 to 50 °C, on Bennett [17].

3. Results

3.1. Isolation of the actinomycetes

In the present study, 18 isolates of actinomycetes were isolated from soil samples collected in sebkha of Kenadsa by using four culture media. The best medium which allowed the isolation of actinomycetes from extreme area was starch casein agar (9 colonies) (Figure 1).

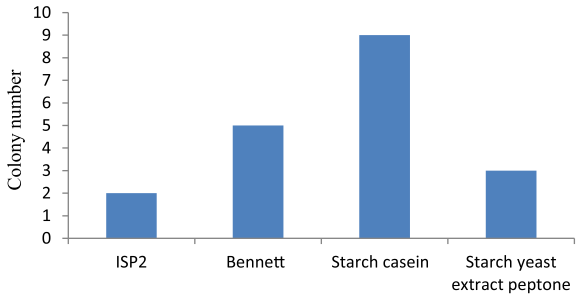

Figure 1. Number of actinomycetes colony isolated from each culture medium.

3.2. Screening of antibacterial activity

All isolates showed antimicrobial activity against at least one microorganism test (Table 1). Two isolates (LAM143dL and LAM142cE) were actives against only Gram-negative bacteria; three isolates (LAM142bH1, LAM142bH3 and LAM153aI2) were actives against Gram-positive bacteria, and twelve isolates were actives against both Gram-positive and Gram-negative bacteria. Seven isolates were actives against *C. albican*. The isolate LAM143cG3 was selected for its broad spectrum and high antimicrobial activity, as well as its morphology interesting.

Table 1
Antimicrobial activity of actinomycete isolates.

Isolates	*S. aureus*	*E. faecalis*	*B. cereus*	*B. subtilis*	*K. pneumoniae*	*P. aeruginosa*	*E. coli*	*C. albicans*
LAM142cE1	9.33	8.33		15.66	–	13.00	10.00	11.00
LAM143dF	–	–	–	–	–	–	–	23.66
LAM142cE3	–	–	–	–	–	–	7.00	–
LAM143dL	–	–	–	–	12.00	19.66	–	–
LAM142bH1	8.33	16.00	12.00	10.00	–	–	–	–
LAM142bH3	11.66	10.00	9.00	14.00	–		–	17.33
LAM142bH2	8.50	8.00	9.16	11.00	–	–	8.50	11.00
LAM143cG2	9.00	8.16	10.50	–	–	–	8.50	11.00
LAM153aI2	8.16	8.00	10.50	–	–	–	–	10.00
LAM192aM	15.00	9.66	–	–	8.00	–	8.33	–
LAM142aK2	9.33	9.33	15.00	–	–	16.00	–	12.00
LAM142cE5	9.00	9.66	9.66	12.33	12.16	11.33	9.33	–
LAM142cE4	8.00	–	8.00	–	9.00	–	–	–
LAM142aK1	8.83	9.66	8.67	10.33	–	14.33	9.33	
LAM143cG3	12.67	–	12.67	11.67	–	15.00	16.00	–
LAM142cE2	9.83	–	10.66	10.00	–	–	9.33	
LAM143cG1	9.33	8.00	–	–	–	–	9.33	
LAM153aI1	9.33	10.33	11.66	–	8.00	10.66	–	–

E. faecalis: Enterococcus faecalis (ATCC 25212).

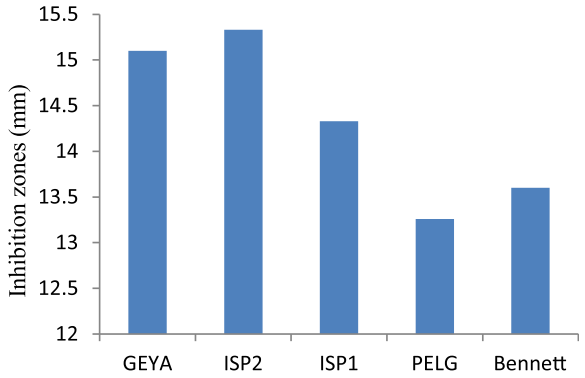

Figure 2. Antibiotic production by the isolate LAM143cG3 on different solid media.

3.3. Optimization of secretion of secondary metabolites secreted by the isolate LAM143cG3

Antibacterial activities were obtained on all media tested (Figure 2). Among the media, which allowed a good antimicrobial production, ISP2 medium was found to be the best for antibacterial activities.

Table 2
Antimicrobial activity of organic extracts of the isolate LAM143cG3 against tests strains.

Souches tests	Ethyl acetate extract	Butanol extract	Chloroform extract
S. aureus	15.00	14.33	–
B. cereus	8.00	9.00	–
B. subtilis	16.33	15.33	–
P. aeruginosa	–	–	–
K. pneumoniae	15.00	12.00	–
E. coli	–	–	–
B. stearothermophilus	30.00	25.00	–

3.4. Extraction of antimicrobial compounds

Three organic solvents were tested for extraction of antimicrobial products. The results indicated that ethyl acetate and butanol were the most appropriates for antibiotics extractions (Table 2).

3.5. TLC and bioautography

Microbiological revelation, by bioautography, indicated that the ethyl acetate extract allowed us to identify a single active spot on the solvent systems (ethanol-ammonia-water), its $R_f = 0.74$. This spot showed strong antibacterial activities against *B. stearothermophilus*, and it had a brown color to the naked eye, light blue at 256 nm while it was dark at 355 nm.

3.6. Partial characterization of products

Chromogenic reactions were positive whit ninhydrin and ferric chloride, while Molish and formaldehyde-H_2SO_4 tests were negative.

The UV–vis spectrum of the butanol extract showed maxima absorption at 240 and 276 nm (Figure 3).

The infrared spectrum of the butanol extract indicated presence of a characteristic band at 3345.562 cm^{-1} (Figure 4).

3.7. Identification of isolate LAM143cG3

The isolate LAM143cG3 was characterized by unfragmented vegetative mycelium (Figure 5A). The aerial mycelium was stable and carried globular sporangia containing spores arranged in a spiral (Figure 5B). Spores were mobiles and had different forms (slightly curved or spiral rods).

The chemotaxonomic study showed the presence of meso-diaminopimelic acid isomer in the cell-wall but not glycin. Revelation sugars indicated the presence of two spots (Figure 6), one

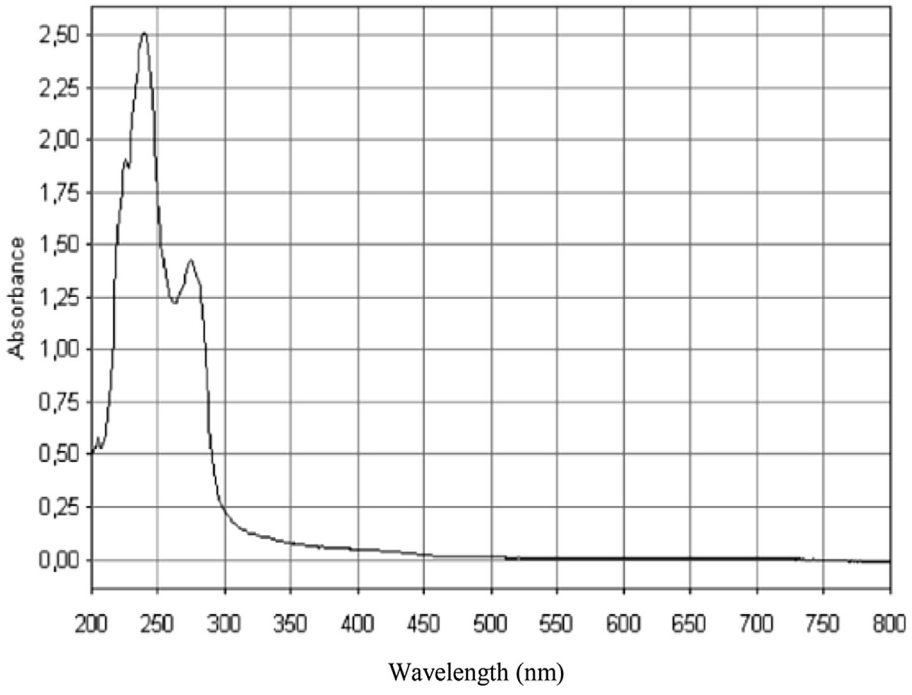

Figure 3. UV–visable spectrum of butanol extract of the isolate LAM143cG3.

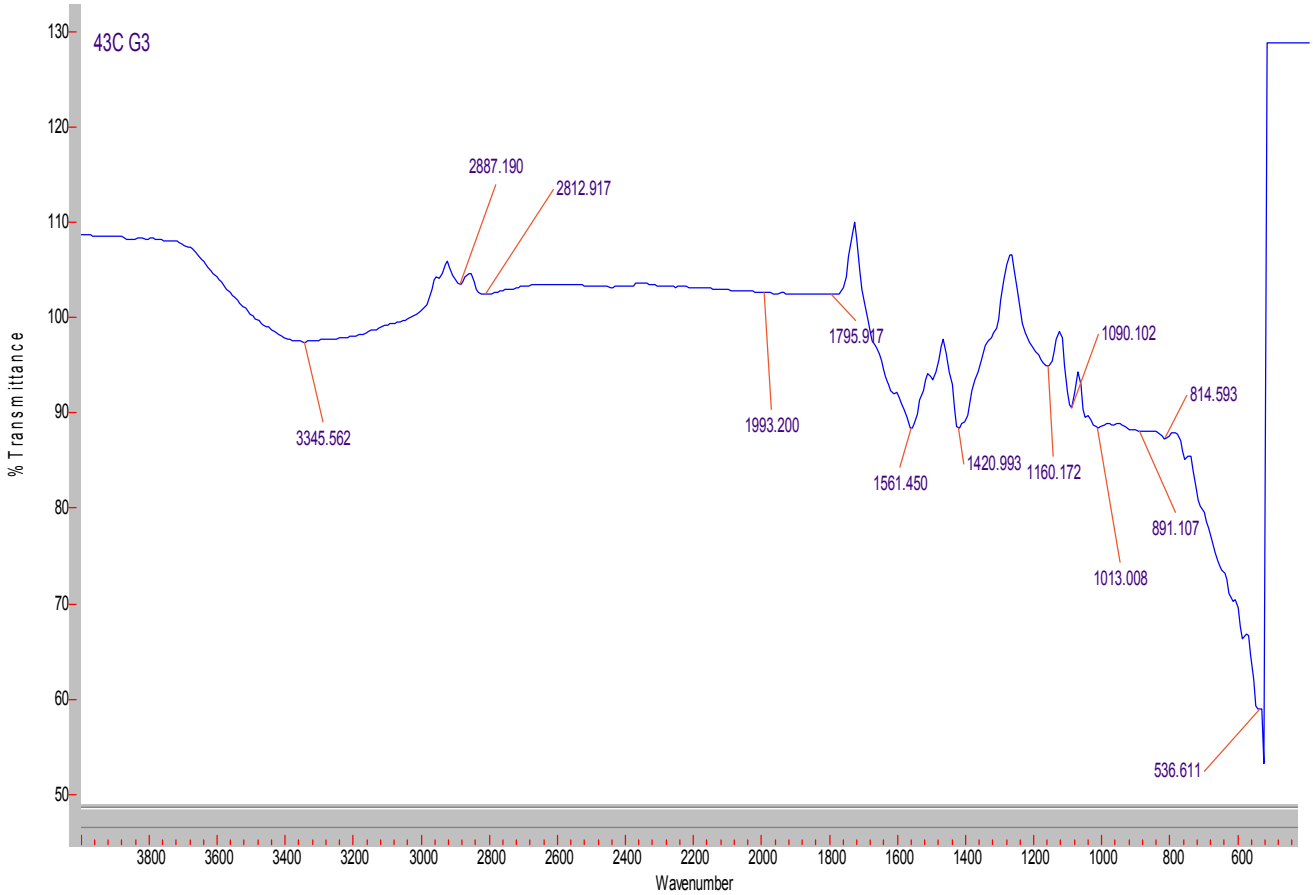

Figure 4. Infrared spectrum of butanol extract of the isolate LAM143cG3.

down, corresponded to glucose, and the other at the top, which did not correspond to any sugar found in the walls of actinomycetes.

3.8. Difference between isolate LAM143cG3 and species of the genus Spirillospora

The comparison between the species of *Spirillospora* [*Spirillospora rubra* (*S. rubra*) and *Spirillospora albida* (*S. albida*)] and our isolate was showed in Table 3.

For physiological characteristics, our isolate tolerated up to 15% NaCl and had an optimum growth temperature between 42 and 45 °C, while the species *S. albida* and *S. rubra* tolerated 2.5% NaCl and had optimum temperature growth at 30 °C [30].

For biochemical characteristics, the species *S. rubra* did not use xylose, lactose, galactose and rhamnose [30], while the isolate LAM143cG3 used all carbon sources for growth.

For cultural characteristics, the substrate mycelium of the species *S. albida* was beige while it was red-brown for the species *S. rubra* [30], whereas for the isolate LAM143cG3 it was yellow imperial on ISP2, off white on ISP3, lemon yellow on ISP4 and colorless on ISP5.

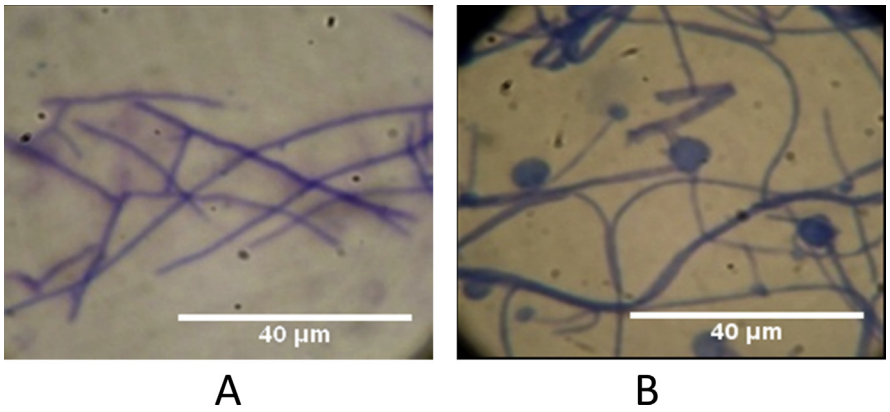

Figure 5. Microscopic observation of the isolate LAM143cG3. A: Vegetative mycelium; B: Aerial mycelium.

Figure 6. Separation of whole-cell sugars from the cell hydrolyzate of strain LAM143cG3 by TLC.

4. Discussion

Recent research efforts have focused on the exploration of underexplored habitats to discover novel bioactive secondary metabolites from the autochthonous microbiota.

Sebkha is among the extreme environments underexplored. In fact, studies on actinomycetes isolated from the Algeria sebkha are very few [20,31,32].

Among the four used culture media, the best for the isolation of actinomycetes from sebkha of Kenadsa is the medium starch casein agar. This performance can be explained by the presence of starch and casein in the media which stimulate the growth of actinomycetes in preference to other bacteria [33].

The bioactivity of the isolates was dissimilar between Gram-positive and Gram-negative bacterial strains. The results clearly demonstrate that a Gram-positive bacterium was highly susceptible to the tested crude extracts compared to Gram-negative bacteria. This different sensitivity between Gram-positive and Gram-negative bacteria could be ascribed to morphological differences such as outer membrane of Gram-negative bacteria having lipopolysaccharide which makes the cell wall impermeable to lipophilic extracts. However, a Gram-positive bacterium was more susceptible because of lacking of outer membrane [25,34].

Chromogenic reactions of bioactive substances secreted by the isolate LAM143cG3 have revealed the presence of phenol and amine. In contrast, carbohydrate residues are absent. The presence of amine groups is confirmed by the band 3345.562 cm^{-1} of

Table 3
Characteristics differentiating strain LAM143cG3 from other *Spirillospora* species.

Characteristics	Isolate LAM143cG3	*S. albida*	*S. rubra*
Morphologys			
Aerial mycelium	Stable	Stable	Usually absent
Substrate mycelium	Unfragmented	Unfragmented	Unfragmented
Sporangium	Spherical	Spherical to vermiform	Spherical
Physiological characteristics			
NaCl tolerance	15%	2.5%	2.5%
Optimum temperature for growth	42 °C	30 °C	30 °C
Utilization of			
Fructose	+	+	ND
Arabinose	+	+	ND
Xylose	+	–	ND
Lactose	+	–	ND
Glucose	+	+	
Saccharose	+	+	ND
Rhamnose	+	–	ND
Galactose	+	–	ND
Mannitol	+	+	ND
Color of aerial mycelium	Blanc	Blanc	Blanc
Color of substrate mycelium			
ISP2	Yellow imperial	Beige	Red to red-brown
ISP3	Off white		
ISP4	Lemon yellow		
ISP5	Colorless		
Production of melanoid pigments			
ISP6	–	–	–
ISP7	–	–	–

the infrared spectrum which corresponds to the secondary amines (NH).

Analysis of the crude butanol extract by UV–visible spectrophotometry indicates that this sample is not polyenic in nature, which is characterized by the presence of three highly characteristic maxima between 291 and 405 nm [35].

According to the results of the chemotaxonomic study, the isolate LAM143cG3 is affiliated to chimiotype III according to classification of Lechevallier and Lechevallier 1970 [36].

Based on the chemical and morphological characteristics, this isolate was identified as a member of the genus *Spirillospora* according to Bergy's Manual of Systematic (2012) [37]. But this genus is characterized by presence of madurose in cell wall, which is absent in wall of our isolate. This can be explained by the effect of optimum temperature required by this isolate (42–45) °C which can inhibit the synthesis of madurose. This point has already been made for thermophilic *Actinomadura* (madurose synthesized in trace amount) and *Thermomonospora* (lack madurose) [30].

The comparison between our isolate (LAM143cG3) and species of *Spirillospora* suggested a lot of biochemical, physiological and culturals differences, allowing us to suppose that our isolate may be a new member in the genus *Spirillospora*. These results should be confirmed later by sequencing ADN16s.

Conflict of interest statement

We declare that we have no conflict of interest.

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