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Actinobacteria isolated from Algerian hot spring waters: A potential source of important enzymes

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ABSTRACT

The presence of Actinobacteria in Algerian hot springs was proved for the first time in this work. From water samples, 23 morphologically distinct strains were isolated and successfully identified. 16S rRNA sequence analysis showed that the isolates were represented in the families *Streptomycetaceae*, *Nocardiaceae* and *Microbacteriaceae*, the majority being affiliated to the genus *Streptomyces*. Four isolates were characterized in depth (chemotaxonomy, spore morphology, pH and temperature tolerance, G + C content) and proved to produce enzymes with biotechnological interest (cellulase, xylanase, lipase and protease). The success of isolation in relation to the temperature and the chemical characteristics of these waters is also discussed, where the highest temperature hot spring yielded no Actinobacteria, whereas ferruginous calcic carbonate hot springs yielded the most.

Key words : *Hot springs, Actinobacteria, Streptomyces, Enzymatic activities,*

Introduction

In recent years, research has shown great interest in thermophilic and thermotolerant bacteria mainly for reasons related to the production of primary and secondary metabolites that are used in different industries, in medicine, agriculture and biotechnology (Tang *et al.,* 2006; Elbendary *et al.,* 2018). The main reason is the possibility of carrying out fermentation processes at significantly higher temperatures, which alleviates the problems of contamination of fermentors by mesophiles, in addition to several other economic and technical advantages in the fermentation processes.

Actinomycetes, now called Actinobacteria, are Gram positive bacteria highly sought for their ability to produce different biomolecules with multiple interests. These bacteria have been for a very long time mainly isolated from soils (McVeigh *et al.,* 1996; Harir *et al.,* 2017), and waters (Goodfellow *et al.,* 1990; Hayakawa *et al.,* 2000; Radhika *et al.,* 2011). Several researchers have oriented their investigations towards the harshest and least explored ecosystems in order to discover molecules with new potentialities. Thus, these bacteria were found in marine sediments (Goodfellow and Haynes, 1984; Pathom-aree *et al.,* 2006), in salt lakes (Oren, 2002; Tang *et al.,* 2010), in arid soils (Sabaou *et al.,* 1998; Boudemagh *et al.,* 2005) and in many other ecosystems (Kitouni *et al.,* 2005). Studies on the biodiversity of Actinobacteria in thermal waters are very rare and have shown diverse results. Indeed, some researchers have found that Actinobacteria seem to be absent in highly acidic waters and hot

springs of volcanic origin (Goodfellow and Williams, 1983). Others observed that hot springs are very poor in Actinobacteria (Lacey, 1988). Nevertheless, the presence of Actinobacteria in some of these ecosystems has been reported (Xu *et al.*, 1998). In the same work, a new species named *Streptomyces thermogriseus* was isolated from hot springs, lakes and soils of Yunnan, China (Xu *et al.*, 1998). In a relatively recent research by Zhaoqi Song *et al.*, 2009, on three hot springs located in Tengchong (China), Kamchatka (Russia) and Nevada (U.S.A), several Actinobacteria belonging to known or unknown genera have been isolated and identified (Song *et al.*, 2009). To this day and according to our knowledge, the isolations of Actinobacteria from thermal waters are still very scarce and no work of this kind has been carried out in North Africa or even in the Arab world.

Hot springs are low in organic matter, but rich in cations (such as sodium and calcium) and anions (such as chlorides, bicarbonates and sulphates). This chemical composition combined with the high temperature of these waters, gives the microorganisms an ecosystem of the most extreme and unique. Therefore, oligotrophic bacteria and especially Actinobacteria, which exist in these places, can show exceptional adaptability that may have interesting metabolic features.

The purpose of this work is to isolate and identify the Actinobacteria from some hot springs located in the eastern region of Algeria. We also determine the physiological and metabolic biodiversity of these bacteria, and their biotechnological potential as enzymes producers.

Materials and Methods

Sampling, isolation and enumeration of Actinobacteria

Four hot springs located in northeastern Algeria have been studied Figure 1. Water samples from each site were collected, and temperature and pH were determined in Table 1. Samples were collected aseptically from the following thermal water stations: Hammam Béniharoun (Mila town); Hammam des Frères Chaouch (Mila town); Hammam Essalihine (Khenchela town); Hammam Debagh (Guelma town) (Table 1, Figure 1). The hot-springs samples were transported to the laboratory in a cooler and stored at 4 °C for later use. Afterwards,

Table 1. Description of sampling sites and percentage of isolates by sampling site and incubation temperatures.

Sampling sites	GPS location	Water pH	Water temperature	Number of isolates by isolation medium			Percentage of isolates by incubation temperature			Percentage of total isolated *Actinobacteria*
				AIA	SCA	ISP2	28 °C	37 °C	55 °C	
Hammam des Frères chaouch (Mila town)	36°06′55′′ North 6°21′51′′ East	7.6	86 °C	6	16	0	26%	26%	20%	75.86%
Hammam Debagh (Guelma town)	36°27′41′′ North 7°15′49′′ East	7.5	98 °C	0	0	0	-	-	-	-
Hammam Béniharoun (Mila town)	36°33′19′′ North 6°16′11′′ East	7.6	45 °C	4	0	0	6%	3%	3%	13.79%
Hammam Essalihine (Khenchela town)	35°26′25′′ North 7°05′04′′ East	7.4	70 °C	2	1	0	6%	3%	0%	10.34%

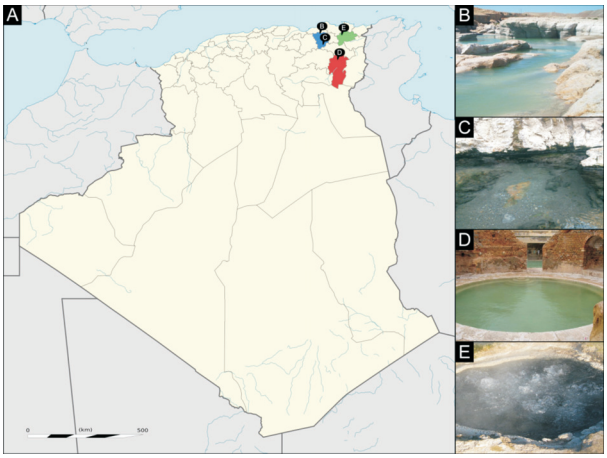

Fig. 1. (**A**) Geographical distribution of the algerian hot springs studied in this work. In color, the provinces where the sampling sites are located: Mila (blue), Khenchela (red) and Guelma (green). (**B**) Béniharoun hot spring (Mila, 36° 332 193 N, 6° 162 113 E). (**C**) Frères Chaouch hot spring (Teleghma, Mila, 36° 062 553 N, 6° 212 513 E). (**D**) Essalihine hot spring (Khenchela, 35° 262 253 N, 7° 052 043 E). (**E**) Debagh (Maskoutine) hot spring (Guelma, 36° 272 353 N, 7° 162 103 E).

they were diluted with N-saline solution (0.85% NaCl in sterile demineralized water) (Pednekar *et al.*, 2011), by the serial dilution method. An amount of 100 µL of each dilution was plated on different selective culture media: Actinomycete Isolation Agar (AIA) (Jayashree *et al.*, 1991; Uzel *et al.*, 2011), Starch Casein Agar (SCA) (Uzel *et al.*, 2011; Mohseni *et al.*, 2013) and ISP2 medium (Mohseni *et al.*, 2013). Each medium was supplemented with 25 µg/mL of nystatin (to minimize fungal contamination) and 10 µg/mL of nalidixic acid (to inhibit growth of Gram-negative bacteria) (Takizawa *et al.*, 1993; Ravel *et al.*, 1998). The plates were protected in sealed plastic boxes and incubated for 7 to 21 days at 28 °C, 37 °C and 55 °C. The colonies obtained were subcultured in order to isolate them. Then, they were stored in the presence of 20% (V/V) glycerol at -20 °C (Isik *et al.*, 1999).

Identification of actinobacterial isolates

Macroscopic and microscopic characterization. The colonies were observed under binocular, the presence or absence of filaments, color, texture and appearance were noted. Gram staining was performed for each isolate; colony observation was performed under objective ×100 by an optical microscope (Leitz Ortholux II).

16S rRNA gene sequence. The isolates obtained were identified by a molecular method based on 16S rRNA gene sequencing using specific Actinobacteria primers, following these steps:

Cell lysis: One colony from each fresh culture was put in an Eppendorf tube containing 1 mL of sterile distilled water; these tubes were heated for 10 min at 98 °C and centrifuged for 10 minutes at 13,000 rpm. The resulting supernatant was directly used for PCR (colony PCR).

Amplification: Bacterial 16S rRNA gene fragments were amplified using the following Actinobacteria specific primers: -S-C-ACT-235-a-S-20 (5′-CGC GGCCTATCAGCTTGTTG-3′) (Forward) and -S-C-ACT-878-A-a-19 (5′-CCGTACTCCCCAGG CGG GG-3′) (Reverse) (Song *et al.*, 2009). The PCR was carried out in a thermocycler (BIO-RAD T100), in a total volume of 50 µL containing: 1 µL of cells lysate, 10 µL of Phusion HF buffer (× 5), 1 µL of dNTPs (10 mM), 1 µL of each primer (10 µM), 1.5 µL of DMSO (5%), and 0.5 µL of Phusion DNA polymerase (0.02 U/µL). The amplification was carried out according to the following protocol: an initial denaturation cycle at 98 °C for 30 seconds, 35 cycles comprising denaturation at 98 °C for 10 seconds, hybridization at 59 °C for 30 seconds, extension at 72 °C for 15 seconds, and a final extension step at 72 °C for 7 minutes.

The PCR products were examined on 1% agarose gel electrophoresis, including a negative genomic DNA-free control substituting it with distilled water. The PCR products were purified according to the protocol recommended by the manufacturer from "Thermo Scientific GeneJET Gel Extraction Kit".

Sequencing of the 16S rRNA fragment and phylogenetic analysis: sequencing of the purified PCR products was performed in the SAI (Servizos de Apoio á Investigación) at the Universidade da Coruña, Spain. All sequences obtained were analyzed and edited using the Bioedit software version 7.2.5 (Hall, 1999) and Sequencher 5.4.6 software (Gene Codes Corporation). The 16S rDNA sequences obtained were compared to the sequences deposited on the NCBI GenBank and EzBioCloud Databases by the Basic Search Tool Alignment (BLAST). The phylogenetic tree was constructed using the Molecular Evolutionary Genetics Analysis (MEGA 5) (Tamura *et al.*, 2011), by the Neighbor-Joining method (Saitou and Nei, 1987).

In-depth characterization of selected isolates

Four isolates were studied further using the following techniques:

Morphological properties of spore chains: The scanning electron microscopy (SEM) of 14-day cultures grown on ISP medium 2 agar was used to examine the morphology of spore chains of the selected isolates H, J, K and N4, it was performed in the SAI (Servizos de Apoio á Investigación) at the Universidade da Coruña, Spain. The cover slip technique was used to observe hyphae and spore chains (Williams and davies, 1967).

Chemotaxonomy: Biomass for chemotaxonomic studies was prepared by growing organisms in 250 ml flasks containing 80 mL ISP medium 2 broth, with shaking for 4 days at 28 °C. The cells for chemical studies were washed in distilled water and freeze dried and stored at 20 °C until required. The diagnostic isomers of diaminopimelic acid (DAP) and whole-organism sugars were analyzed following well-stablished procedures (Staneck et Roberts, 1974; Hasegawa *et al.*, 1983). Analysis of polar lipids and fatty acids were carried out by the Identification Service of the DSMZ, Braunschweig, Germany. Analyses of respiratory quinones were carried out by the Identification Service and Fr. Brian Tindall, DSMZ, Braunschweig, Germany.

Growth at different temperatures and pH: the growth of Actinobacteria isolates at different temperatures was tested on ISP2 medium. Growth at 30 and 37 °C was observed after 7 and 14 days and at 4 and 10 °C after 6 weeks (Williams *et al.*, 1983). Regarding the survival at 50 °C we followed a modification of the protocol described by Gordon *et al.*, 1974 (Gordon *et al.*, 1974). The actinobacterial cultures were inoculated in tubes containing liquid ISP2 medium and then heated at 50 °C in a water bath, then the cultures were transferred to an incubator at the same temperature and incubated for 8 hours (Gordon *et al.*, 1974). The tubes were rapidly cooled, then incubated at 28 °C for 3 weeks, growth was determined using a spectrophotometer. To break up the filamentous appearance of Actinobacteria and facilitate reading in the spectrophotometer, sterile glass beads were added to the cultures followed by a vigorous agitation. The growth at different pH was tested using ISP medium 2. Growth at pH 4-3, pH 7.3 and pH 10.3 was observed after 7 and 14 days (Williams *et al.*, 1983).
DNA G+C content: the genomic DNA of the bacteria was specifically stained with SYBR GREEN I (Thermo Scientific Luminaris Color HiGreen qPCR Master Mix). The thermal denaturation of the DNA was monitored by measuring the decrease in fluorescence using a real-time PCR (Eco ™ Real-Time PCR System) thermal cycler according to the method proposed by Loveland-Curtze *et al.*, (2011) (Loveland-Curtez *et al.*, 2011). *E. coli* DH10B strain was used as a control.

Screening of enzymes with potential biotechnological interest

The four selected actinobacterial isolates were subjected to screening for lipase, cellulase, xylanase and protease activities. For screening of lipase, the isolates were plated onto modified Luria-Bertani (LB) agar plates (5 g/L peptone, 3 g/L yeast extract, 13 g/L bacteriological agar, 10 g/L gum arabic) containing 1% (v/v) emulsified tributyrin as substrate. Cells were grown at 37 °C for seven days. The appearance of clear halos around individual colonies indicated the hydrolysis of tributyrin and they were considered as possible lipase producing isolates.

For screening of cellulase and xylanase, the isolates were plated onto Luria-Bertani (LB) agar plates containing individually the insoluble chromogenic substrates AZCL-HE Cellulose and AZCL-Xylan powder, respectively, transferred in 96% ethanol and added into the LB media at the concentration of 0.1% w/v. Cells were grown at 37 °C for seven days. The appearance of a blue colored zone around colonies indicated the hydrolysis of AZCL-HE Cellulose or AZCL-Xylan, and they were considered as possible cellulase or xylanase producing isolates.

The screening of protease activity of the isolates was performed by detecting the fluorescence emitted when the culture was incubated in the presence of the fluorescent substrates contained in the commercial kit EnzChek® Protease Assay Kits E6638 Molecular Probes (Invitrogen detection technologies from Thermo Fisher Scientific®). The isolates were cultured in Luria-Bertani (LB) liquid medium. 500µL of each isolate were transferred into an Eppendorf tube and centrifuged at 3000 rpm for 5 minutes, thereafter 10 µL of the supernatant of each suspension was taken and loaded into a 384-well round bottom black plate (Corning) to detect fluorescence, which contained 10 µL of working solution of the flurorescent substrate (EnzChek® Protease Assay Kit [green fluorescence]). The microplate was incubated for 30 minutes at room

temperature (20-25 °C) in the dark. The fluorescence was measured in the Synergy H1 hybrid Reader (Biotek®) spectrofluorimeter, which has a microplate reader, and is proportional to the protease activity present (https://assets.thermofisher.com/TFS-Assets/LSG/manuals/mp06638.pdf). In order to select positive wells, a criterion was adopted that consisted in taking as positive values the fluorescence measurements higher than a threshold value. The threshold value is twice the standard deviation over the average value of all wells on the same plate (Nyyssönen *et al.*, 2013).

Results

Isolation

The isolates obtained from the thermal springs studied revealed the presence of Actinobacteria in this kind of extreme ecosystem. In total, 29 isolates of Actinobacteria with different morphological aspects were cultured, at a temperature of 28 °C, 37 °C and 55 °C (Table 1): twenty-two isolates derived from the water of Hammam des Fréres Chaouch, four isolates from Hammam Béniharoun, and three isolates from Hammam Essalihine, whereas no isolate was recovered from Hammam Debagh, the hot spring with the highest temperature (98 °C).

The AIA media allowed the isolation of 41% of Actinobacteria from the four ecosystems tested, the SCA favored the isolation of the remaining 59%, as the ISP2 medium did not yield any actinobacterial isolates (Table 1).

As expected, and from the results obtained, the isolation of these bacteria varied depending on several factors, namely the culture medium, the incubation temperature and the source hot spring.

Preliminary selection of Actinobacteria isolates was performed on the basis of macroscopic and microscopic features. They are dry-colored colonies with a hard texture that adheres strongly to the agar; the colonies are often powdery and may have a filamentous appearance (Figure S1). Microscopic observation of the isolates revealed filamentous or non-filamentous features with positive Gram stain (Figure S2).

Molecular analysis based on the partial sequence of the 16S rRNA gene

Among the 29 isolates obtained, 23 of them were analyzed with their 16S rRNA gene amplified and sequenced, whereas for the remaining 6 isolates the amplification could not be successfully achieved despite several attempts.

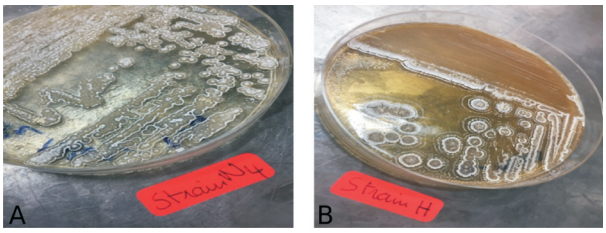

Fig. S1. (**A**) Macroscopic appearance of strain N4. (**B**) Macroscopic appearance of strain H.

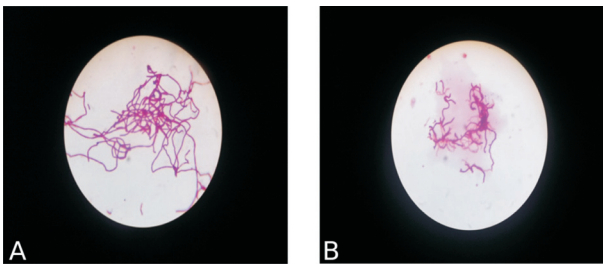

Fig. S2. (**A**) Microscopic appearance of strain N4. (**B**) Microscopic appearance of strain H.

BLAST alignment revealed that the 23 strains were assigned to three genera of Actinobacteria: *Streptomyces*, *Rhodococcus* and *Rathayibacter* (Table 2). The isolates showed more than 98% similarity with the 16S rRNA gene sequences of species listed in EzBioCloud Database. Only one of them reached 100% similarity. Their 16S rRNA sequences were uploaded to the NCBI GenBank Database with the accession numbers shown in Table 2.

Among the four different sites tested, the hot spring of Frères Chaouch (Teleghma, 86 °C) showed the greatest diversity of Actinobacteria (Table 2).

The phylogenetic results (Figure 2) showed that three families represent the isolates. Those are: *Streptomycetaceae*, *Nocardiaceae*, *Microbacteriaceae*. *Streptomyces* was the predominant genus among all strains isolated from the four hot springs, with a number of 20 out of 23 identified Actinobacteria.

Four isolates referred to as H, J, K and N4 (Table 2) were selected for an in-depth study as described below. These strains, which have been assigned respectively to *Streptomyces flavoviridis*, *Streptomyces pseudogriseolus*, *Streptomyces olivaceus*, and *Streptomyces griseoflavus*, are here described for the first time as being isolated from hot springs.

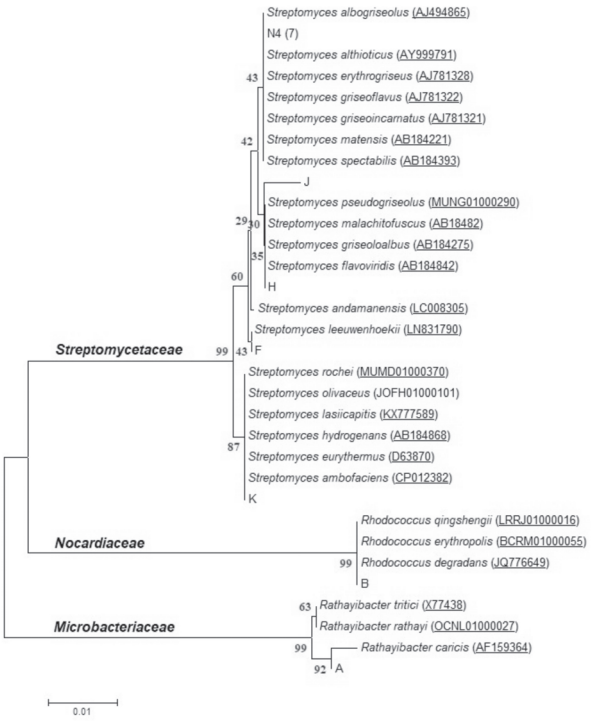

Fig. 2. Neighbor-joining tree showing the phylogenetic relationships of 16S rRNA gene sequences amplified from the hot spring isolates to closely related sequences from the EzBioCloud database. Representative Sequences identified in this study are A, B, F, J, K and N4, followed by the number of clones with identical nucleotide sequences. The EzBioCloud accession numbers of reference sequences are shown in parentheses. The optimal tree with the sum of branch length = 0.14624020 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). Only bootstrap (1000 replicates) values of >50% are shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. The analysis involved 32 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 564 positions in the final dataset.

Spore morphology

Scanning electron microscopy revealed variations in spore chains morphology, between the 4 selected isolates. The spore chains are composed of hairy, smooth or spiny spores in Retinaculiaperti-type, Rectiflexibile-type, Spira-type or Verticillati-type spore chains (Figure 3). The typical polysporous long chains morphology of the members of the genus *Streptomyces* (Li *et al*., 2016) is shown.

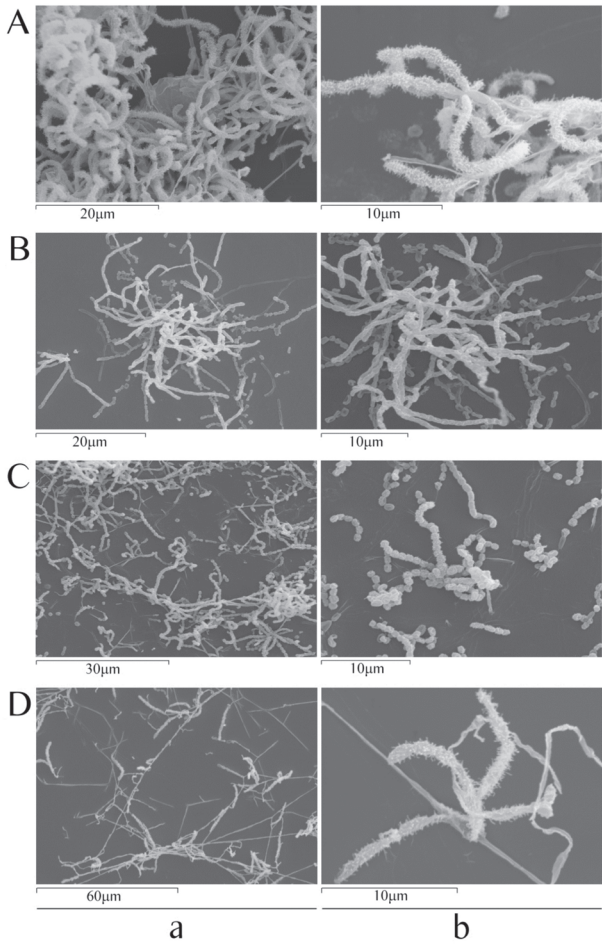

Fig. 3. Scanning electron microscopy (SEM) of four isolates. (A) Isolate H: Retinaculiaperti type spore chains with hairy rod-shaped spores. Bars, 20 µm (a) and 10 µm (b). (B) Isolate K: Rectiflexibile type spore chains with smooth globose spores. Bars, 20 µm (a) and 10 µm (b). (C) Isolate N4: Simple Retinaculiaperti type spore chains with spiny globose spores. Bars, 30 µm (a) and 10 µm (b). (D) Isolate J: Verticillati spore chains with hairy spores. Bars, 60 µm (a) and 10 µm (b).

Chemotaxonomy

Chemotaxonomic tests showed that the cell wall of the isolates H, J, K, and N4 contained LL-DAP and the absence of characteristics sugars, indicating that it has cell-wall type I (Lechevalier and Lechevalier, 1970). The phospholipids pattern is type PII

(Lechevalier *et al.,* 1977), containing predominantly Phosphatidylethanolamine (PE) and Diphosphatidylglycerol (DPG) in the cell walls of both isolates H and N4. The isolates H and N4 also contained the hexahydrogenated menaquinones with nine isoprene units as the predominant isoprenologue (MK9-H6). Fatty acid analysis showed that the isolates H and N4 respectively contained a high proportion of saturated straight-chain and iso- and anteiso-branched fatty acids:15:0-anteiso (15.71%), 16:0-iso (23.49%), 16:0 (11.26%), 17:0-anteiso (15.42%) and 15:0- iso (12.64%), 15:0-anteiso (12.23%), 16:0-iso (28.27%), 17:0-anteiso (10.25%) (Kroppenstedt, 1985). These results support the affiliation of the isolates to *Streptomyces*.

The morphological and chemotaxonomic characteristics of the selected isolates, as well as their cell-wall type, whole-cell sugar pattern, fatty acid profile and the sequence of their 16S rRNA gene, are consistent with the characteristics of members of the genus *Streptomyces*.

DNA G+C content

The results of the G+C analysis of the four Actinobacteria referred to as H, J, K and N4 are shown in Table 3. These species have percentages of G+C from 41.5 to 66%. We note that the G+C values of these isolates are relatively low compared to the usual values of Actinobacteria (on average about 73% G+C (Ventura *et al.,* 2007).

Table 3. Values of melting temperatures and G+C content of the genomic DNA of the four species selected.

Strains	G+C mol%* / standard deviation
H	56.77 ± 0.13
J	41.46 ± 0.44
K	66.13 ± 0.46
N4	59.24 ± 1.48
E.coli DH10B	48.02 ± 0.26

* The G+C% was obtained by applying the equation: G + C% = Tm-64.9 / 0.41 (Tm values were obtained from melting curves). http://www.biology.arizona.edu/biomath/tutorials/Linear/LinearFunctionApplication/DNAmelt.html

Growth of selected species at different temperatures and pH

Growth profile at different temperatures and survival at 50 ºC of strains H, J, K and N4 are shown in

Table 2. Molecular identification of hot springs Actinobacteria by 16S rRNA gene sequences.

Isolate codes	Source	Closest sequence match with Ez Bio Cloud (accession number)	Percentage of similarity	Isolates accession numbers
A	Teleghma	*Rathayibacter tritici* (X77438)	99.68%	MK929471
B	Béniharoune	*Rhodococcus erythropolis* (BCRM01000055)	99.69%	MK929472
C	Teleghma	*Streptomyces griseoflavus* (AJ781322)	99.84%	MK929473
C1	Teleghma	*Streptomyces griseoflavus* (AJ781322)	99.84%	MK929474
C3	Teleghma	*Streptomyces griseoflavus* (AJ781322)	99.69%	MK929476
C2	Teleghma	*Streptomyces olivaceus* (JOFH01000101)	99.83%	MK929475
K	Teleghma	*Streptomyces olivaceus* (JOFH01000101)	99.65%	MK908110
D	Teleghma	*Streptomyces ambofaciens* (CP012382)	99.84%	MK929477
H1	Teleghma	*Streptomyces ambofaciens* (CP012382)	99.84%	MK929480
L	Béniharoune	*Streptomyces ambofaciens* (CP012382)	99.84%	MK929482
M	Béniharoune	*Streptomyces ambofaciens* (CP012382)	99.84%	MK929483
E1	Khenchela	*Streptomyces ambofaciens*(CP012382)	98.90%	MK929489
I	Teleghma	*Streptomyces ambofaciens* (CP012382)	99.69%	MK929479
K1	Teleghma	*Streptomyces ambofaciens* (CP012382)	100%	MK929481
F	Khenchela	*Streptomyces leeuwenhoekii* (LN831790)	99.84%	MK929478
H	Teleghma	*Streptomyces flavoviridis* (AB184842)	99.69%	MK908107
J	Teleghma	*Streptomycespseudogriseolus* (MUNG01000290)	99.21%	MK908108
N4	Teleghma	*Streptomyces griseoflavus* (AJ781322)	99.84%	MK908109
N5	Teleghma	*Streptomyces griseoflavus* (AJ781322)	99.84%	MK929484
N7	Teleghma	*Streptomyces griseoflavus* (AJ781322)	99.53%	MK929485
N8.3	Teleghma	*Streptomyces griseoflavus* (AJ781322)	99.36%	MK929486
N3	Teleghma	*Streptomyces griseoflavus* (AJ781322)	99.47%	MK929488
N9	Teleghma	*Streptomyces althioticus* (AY999791)	99.68%	MK929487

Table 4. The four strains grow better at the higher temperatures (30-37 ºC) than at the lower one (14 ºC). At 4 ºC they do not grow at all. Only in the case of strain N4 the OD value is reduced at 50 °C compared to the control (Table 4). This was expected since these isolates come from extreme ecosystems whose temperature range is between 45 °C and 86 °C. The results of the temperature test show that the four selected isolates have a good growth at 37 °C and tolerate the temperature of 50 °C to survive. This finding categorizes our isolates as facultative thermophiles (Singh *et al.*, 2013). The selected isolates developed and sporulated at neutral and basic pH, with the exception of isolate H. Its growth was very low at basic pH, whereas the four isolates did not tolerate the acidic pH (Table 4).

Screening of enzymes with potential biotechnological interest

The performed enzymatic screening revealed that the isolates H, J, K and N4 have the ability to biosynthesize active enzymes of industrial use such as cellulase, xylanase, lipase and protease (Figure 4). Namely, isolates H and N4 both produce lipase, cellulase and xylanase. Isolate J is capable of producing lipase and protease. Isolate K is capable of producing lipase and xylanase.

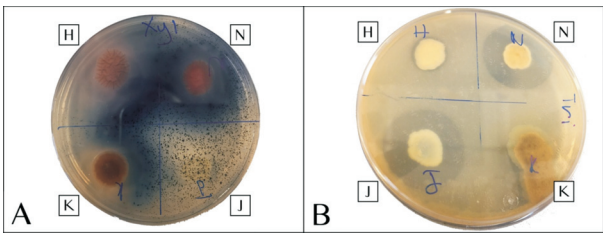

Fig. 4. Enzymatic substrates hydrolysis zones by the isolates H, J, K and N4 with (**A**)insoluble chromogenic xylan and (**B**) tributyrin as substrates.

Discussion

Actinobacteria is a group of microorganisms with known biotechnological application because they are producers of antibiotics, enzymes and other useful metabolites. Species living in thermophilic habitats are particularly interesting due to the high stability of their bioproducts.

In this work we have proved for the first time the presence of Actinobacteria in the waters of eastern Algeria hot springs. The number of isolates was however limited, which is in agreement with the work of Chaudhary and Prabhu (2016) who found that the sediments of the hot springs tested offer a higher quantity and a larger diversity of Actinobacteria compared to the water samples (Chaudhary and Prabhu, 2016). It should be noted that the majority of recent work on the isolation of Actinobacteria has been carried out on sediments (Song *et al.*, 2009; Uzel *et al.*, 2011; Thawai, 2012; Liu *et al.*, 2016).

The diversity of isolates obtained was dependent on the source (one hot spring, out of four, yielded the 76% of the isolates) and also on the culture medium used. Thus, both SCA and AIA media, but not ISP2, allowed the isolation of Actinobacteria.

The SCA medium contains starch and casein which are known to be favorable for the growth of Actinobacteria, preferentially to other bacteria (Cavalla and Eberlin, 1994; Lee *et al.*, 2014). The performance of the AIA medium can be explained by the presence of glycerol in the medium, which is used by most Actinobacteria as a carbon source (Oskay *et al.*, 2004). It also contains sodium propionate which acts as an additional antifungal agent to the nystatin added to the medium; therefore, eliminating invasive and competitive fungi for Actinobacteria (Cavalla and Eberlin, 1994). Furthermore, transpar-

Table 4. Growth of the selected Actinobacteria at different pH, temperatures.

Isolates	Temperature						pH		
	4°C	14 °C	30 °C	37 °C	50 °C (OD)	30 °C (OD) (Control)	4.3	7.3	10.3
H	-	+	+++	+++	1.4	0.4	-	+++	+/-
J	-	+	+++	+++	12	2.7	-	+++	+++
K	-	+	++	+++	1.4	1.2	-	+++	+++
N4	-	+	+++	+++	5	8	-	+++	+++

Keys: (-) = No growth; (+/-)= very weak growth; (+)= weak growth; (++)= Good growth; (+++)= good growth and sporulation; (OD)= optical density.

ency facilitates the observation of actinobacterial colonies.

The ISP2 (Yeast extract-malt extract agar) culture medium (Shirling and Gottlieb, 1966) is widely used for isolating Actinobacteria from different soil and water ecosystems (Xu *et al*., 1998; Mohseni *et al*., 2013; Ronoh *et al.,* 2013; Benhadj *et al.,* 2019). However, the performance of this medium in the selective isolation of these bacteria is not always optimal. In our study, the use of this medium for the isolation of Actinobacteria from thermal waters has not yielded results. According to some researchers, this medium is more suitable for studying the cultural characteristics of Actinobacteria (Shirling and Gottlieb, 1966; Baskaran *et al.,* 2011), and also for the purification and conservation of these bacteria (Devi *et al.,* 2013; Azimi *et al.,* 2017).

The most efficient culture media reported in the literature for the isolation of Actinobacteria mainly contains glycerol, starch, arginine, casein and nitrate (Jihani *et al.,* 2012; Siddique *et al.,* 2014). Our study supports this finding, as we isolated successfully actinomycetales from thermal waters using SCA and AIA media.

The success in the isolation of Actinobacteria was also variable depending on the source hot spring. Indeed, the thermal waters that allowed the isolation of a large number of actinobacterial isolates come from Fréres Chaouch hot spring and Béniharoun hot spring with respectively 76% and 14% of the total isolates. These waters are of ferruginous calcic carbonate nature with a slightly alkaline pH of 7.6. The hot spring Essalihine has a sulphurous nature with a pH of 7.4 and it offered 10% of actinobacterial isolates. The results, however, show an absence of Actinobacteria in the water of the hot spring Debagh (formerly called Meskoutine). This water is rich in sodium chloride, lime sulphate and lime carbonate with a pH of 7.3 (Bonnemain, 2009).

We suggest that the chemical nature is a parameter that plays an important role in the presence of these bacteria in hot springs. Additional studies based on the effect of the chemical composition of these thermal waters on the distribution of Actinobacteria might, in our opinion, confirm or refute this finding.

16S rRNA gene sequence and phylogenetic analysis allowed the classification of the isolates into three genera of Actinobacteria: *Streptomyces*, *Rhodococcus* and *Rathayibacter*, and to the families *Streptomycetaceae*, *Nocardiaceae* and *Microbacteriaceae*, respectively. *Streptomyces* was the most abundant genus. This is consistent with other similar work in a recent study by Lan Liu *et al*., (2016) in China on the biodiversity of culturable Actinobacteria from some Tengchong geothermal sites, 58 strains of Actinobacteria were isolated, the majority of which were affiliated with the genus *Streptomyces* (Liu *et al*., 2016). The investigations of Chitti Thawai (2012) in Thailand, based on the same culture techniques, showed that 32 Actinobacteria were isolated from hot spring sediments. The identification of these bacteria revealed that 18 were *Streptomyces* (Thawai, 2012).

In our and the above cited studies, culture-dependent techniques were adopted for the isolation of Actinobacteria from hot spring waters. However, *Streptomyces* have not been identified in such habitat by culture-independent techniques like metagenomics. According to our knowledge, the few studies recorded in the literature show that other genera have been found. In a study carried on four hot springs in China, Russia and the USA, 346 actinobacterial clones were assigned to the suborder *Frankineae*, Unclassified Actinobacteria and Uncultured Candidatus *Microthrix* (Song *et al.,* 2009). In another study performed on four geographically separate sites in Zambia, China, New Zealand and Kenya, a great phylogenetic diversity of actinobacterial OTUs was revealed. In these samples, the majority of the sequences belonged to unclassified taxa. Four of the 28 OTUs showed 79% homologies with *Mycobacterium*, *Glycomyces*, *Aeromicobacterium* and *Couchiplane* (Valverde *et al.*, 2012).

The use of these two techniques in the study of microbial biodiversity hot springs had various degrees of success. Regarding culture-dependent techniques, the success of isolation depends mainly on the strategies; techniques and selective media used. Researchers have made considerable and laborious efforts to optimize these cultural techniques (Alain and Querellou, 2009). However, despite these efforts, some extremely rare or demanding bacterial genera remain unable to grow in vitro, using conventional culture media (Dauga *et al.,* 2005). According to some researchers, molecular methods that are independent of culture are more suitable for studying microbial diversity in these ecosystems (Øvreås and Torsvik, 1998; Yang *et al.,* 2001; Singh *et al.,* 2013). Several studies have shown that the metagenomic approach has revealed the hidden di-

versity of these natural ecosystems (López-López *et al*., 2013), unlocking the proportion of non-culturable microorganisms (Dauga *et al.,* 2005). However, these techniques have also shown some limitations in the detection of rare species in a microbial community (López-López *et al*., 2013). It is well known that biogeographic and environmental factors directly influence the diversity of microorganisms in different ecosystems (Martiny *et al.,* 2006; Ramette and Tiedje, 2007; Song *et al.,* 2009; Valverde *et al*., 2012). Studies devoted to Actinobacteria clearly show that pH and especially temperature are the most important factors controlling the microbial community (Valverde *et al*., 2012; López-López *et al*., 2013).

Research on Actinobacteria in hot spring waters has been developed in recent years resulting in the discovery of several new genera, species and strains. A strain called "RA strain" was isolated from a hot spring water sample in Langkawi Island in Malaysia, the molecular analysis assigns this strain to the family *Rhodothermaceae* and constitutes a new genus of this family (Goh *et al.,* 2016). The "LA5T strain" was isolated from a hot spring in Yunnan Province, the phylogenetic analyses indicated that this strain belonged to the genus *Planifilum* and represents a new species of this genus proposed "*Planifilum yunnanense*"(Zhang *et al*., 2007). A relatively old study in Yunnan, China, shows the presence of a new strain affiliated to the genus *Streptomyces*, and proposed as a new species called "*Streptomyces thermogriseus*" (Xu *et al.,* 1998).

Four selected isolates were subjected to additional analysis. Spore morphology and chemotaxonomy supported their taxonomic affiliation to the genus *Streptomyces*, while the results of the assays of tolerance to temperature and pH were according to their extremophilic origin. Their low G+C values are in agreement with Ghai *et al.,* who described a group of Actinobacteria with a very low G+C content (40–50%) (Ghai *et al*., 2012; Ghai *et al.,* 2013). Lastly, these isolates are promising because they showed the potential to produce several enzymes that are of great biotechnological interest.

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