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Isolation and characterization of moderately thermophilic aerobic cultivable bacteria from Hammam Righa Hot Spring (Algeria): Description of their hydrolytic capacities

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ABSTRACT/RESUME

Abstract: *Microbial studies of hot-spring communities may provide a unique and wide-ranging source of novel microorganisms, containing a catalog of enzymes and other bioproducts of highly valuable interest for biotechnological developments and applications. Biodiversity in geothermal springs in Algeria appears scanty and has not been thoroughly investigated. In this country, geothermal springs are scattered in several areas. In the present study, thermophilic microorganisms were isolated from Hammam Righa hot spring and were studied for their ability to produce enzymes to be possibly used in biotechnological processes such as amylases, proteases, cellulases and xylanases. 14 bacterial aerobic strains were selected for this investigation and phenotypically characterized. The optimum temperature for growth of these isolates was 60 °C. 16S rDNA gene sequence analysis revealed them to be phylogenetically related to members of the genera Anoxybacillus, Geobacillus, Bacillus, Meiothermus, Tepidimonas, Albidovulum, and Hydrogenophilus. The presence of amylase, protease, cellulase and xylanase activities in these isolates are indicative of potential applications of them in biotechnological processes.*

I. Introduction

Extremophiles microorganisms are adapted to survive in environments characterized by difficult conditions such as high salt concentration, extremes pH, high pressure and high or low temperatures. Among them, thermophiles were the first extremophiles to be discovered [1, 2].
Thermophilic prokaryotes grow optimally at temperatures higher than 60 °C with hyperthermophiles possibly growing above 80 °C [3]. They have been isolated from hot terrestrial, subterrestrial and marine habitats including volcanically and geothermally heated hydrothermal vent systems such as hot springs and deep sea hydrothermal vents [4]. Many terrestrial hot springs exist on Earth. Thermophilic microorganisms associated with these ecosystems have received considerable interest in recent years [5-7] as they are of peculiar interest for regarding the production of thermostable enzymes like protease, cellulases, xylanases, and amylases to be possibly used in the detergent, leather, pulp and paper industries [7-10]. These enzymes are still active at temperatures which are even higher than the optimum

temperatures for the growth of the microorganisms themselves [11].

According to the freedonia group (http://www.freedoniagroup.com/World-Enzymes.html), the global demand for enzymes is forecast to grow on average 4.6 percent through 2020 to $7.2 billion. This market includes enzymes used in industrial applications. In this respect, concerted efforts and interdisciplinary approaches from academia and industry are required in order to meet the future challenges, modern, and innovative technologies for the production of new generation of enzymes and bioprocesses [12].

Nowadays, more than 282 thermal springs have been identified in Algeria [13].They are distributed in a heterogeneous way and multiply by going towards the northeast of the country. In addition to their therapeutic effects, these sources are among the most extreme ecosystems on the earth and promote the development of thermophilic microflora [14-17].

While experiments have been undertaken in these springs to isolate novel anaerobic thermophiles possessing thermostable enzymes of industrial interest [8, 18, 19], there is no information with regard to indigenous thermophilic aerobic microorganisms inhabiting these extreme environments so far. The main purpose of this study was to characterize thermophilic aerobic bacteria isolated from a hot spring (Hammam Righa) in Algeria by using phenotypic and phylogenetic approaches (*16S* rDNA gene sequence analysis). In addition, extracellular hydrolytic activities of these strains were determined.

II. Materials and methods

II.1. Sample collection

Water samples were collected in march 2012, from a borehole of Hammam Righa hot spring(Fig.1), which is situated at 100 Km South-west of Algiers (Algeria) (2°24' East, 36°22' 60'' North), with an altitude of 550 meters, using 1 L sterile thermal glass bottles. Samples were stored in the laboratory at room temperature.

II.2. Measurements and analysis

Physico-chemical parameters (pH, temperature and conductivity) were measured in-situ, as soon as the samples were collected. Chemical analyses of geothermal spring waters, including calcium (Ca^{2+}), magnesium (Mg^{2+}), chloride (Cl^{-}), sodium (Na^{+}), potassium (K^{+}), sulfate (SO_4^{2-}) bicarbonate (HCO_3^{-}), nitrate (NO_3^{-}), nitrite (NO_2^{-}), iron (Fe), and phosphate (PO_4^{3-}) were performed in the laboratory using standard methods. Manganese (Mn), zinc (Zn), copper (Cu), fluorine (F), arsenic (As), nickel (Ni), and lead (Pb) were

Fig. 1. *Identificationofthesiteofsamplingisolation.*

II.3. Isolation of microorganisms

Enrichment cultures and isolation were performed in MG medium containing (in g/L): glucose (3,6); NH_4Cl (1); K_2HPO_4 (0,3); KH_2PO_4 (0,3); NaCl (1); KCl (0,1); $CaCl_2{\cdot}2H_2O$ (0,1); $MgCl_2{\cdot}6H_2O$ (0,25); yeast extract (1); biotrypcase (2). pH was adjusted to 7.0 with 10 M NaOH before autoclaving. Enrichment cultures were subcultured several times under the same conditions. Submerged cultures were carried out in 250 mL shake flasks with 50 mL of medium. The flasks were inoculated and incubated in an orbital shaker at 60 ºC and 150 rpm for 48 hrs. From each sample, 100 µL aliquot was plated by spreading on MG medium plates (five replicates) and incubated for 24, 48 h at 60°C. Different colonies were selected and restreaked several times to obtain pure cultures which were stored in nutrient agar (in g/L): peptone 10, meat extract 5, NaCl 5, agar 20) at 4°C until used.

II.4. Characterization of the isolates

II.4.1. Morphological and biochemical studies

The colony morphologies were determined using cultures grown aerobically on nutrient agar (NA). Cell morphology and motility were examined microscopically in exponentially growing liquid cultures after 18-24h of incubation at 60°C.

The thermophilic isolates were identified by the use of conventional tests. These latter were; Gram reaction, catalase and oxidase production. Acids production from carbohydrates and hydrolyses of some polymers were determined using API 20E and 50 CHB (bioMérieux) as recommended by the manufacturer.

II.4.2. Physiological tests

The temperatures tested were 30, 40, 50, 60, 70, and 80 °C. Salinity tolerance was investigated for 1.0 to 7.0% (w/v) NaCl. The pH growth range was examined between 4.0 and 12.0. All the physiological tests were determined in nutrient agaronly exception of the pH dependence of growth at pH 4.0 and the temperature growth at 80 °C which were performed in nutriment broth.

II.4.3. *16S* rDNA sequence analysis

The *16S* rDNA gene was amplifiedby PCR using universal primers Fd1(5'-AGAGTTTGATCCTGGCTCAG-3') and R6 (5'-TACGGTTACCTTGTTACGAC-3'). Methods for purification of the DNA and sequencing of the*16S* rDNA gene were described previously[20]. The partial sequences generated were assembled using BioEdit v. 5.0.9 [21] and the consensus sequence was corrected manually for errors. The sequence was compared with available sequences in GenBank (version 178) using a BLAST search[22]). The consensus sequence was then manually adjusted to conformto the*16S* rDNA secondary structure model [23]. Nucleotide ambiguities were omitted and evolutionary distances were calculated using the Jukes and Cantor option [24]. Phylogenetic trees were constructed with the Tree Con program using the neighbour joining [25]. Tree topology was evaluated by a bootstrap analysis using 2,000 resamplings of the sequences [26]. Ist topology was also supported using the maximum-parsimony and maximum-likelihood algorithms. The *16S* rDNA sequence of each strain has been deposited in the GenBank.

II.5. Screening ofhydrolyticactivities

II.5.1. Amylases

Each colony was streaked on a nutrient agar plate that contained 1% starch and incubated at 60°C for 48 hours. After the incubation period, plates were flooded with Lugol's iodine to detect the presence of clear halos around those bacterial colonies capable of secreting amylase [27]

II.5.2. Proteases

For protease activity, skimmed milk agar (SMA) medium was prepared and the nutrient broth culture of bacterium after 24 h of incubation was spot inoculated following agar well method. After inoculation the SMA plate was incubated at 60°C for 48 h. The colonies with a clear zone formed by the hydrolysis of milk casein were evaluated as protease producers [28].

II.5.3. Cellulases

Eachcolony was streaked on a nutrientagarplatethatcontained 1% (w/v) carboxymethylcellulose (CMC) andincubatedat 60°C for 48 hours. After incubation, plateswere floodedwith 0.1% (w/v) Congo red solutionfor 1 to 2 min followed by washing the plate with 1 M NaC1 to detect the presence of clear halos around bacterial colonies that secrete cellulases[29].

II.5.4. Xylanases

To observe xylanase production, isolates were cultured on nutrient agar plates containing 1% (w/v) oat spelt xylan. After incubation at 60°C for 48 h, the zone of hydrolysis was visualized by staining the plates with aqueous solution of 0.2% (w/v) Congo red for 15 min, and then destained with 1 M NaCl[30].

III. Results and discussion

III.1. Measurementsandanalysis

The results of physico-chemical characteristics of the water are presented in Table 1. Temperature and pH of the sample were 68 °C and 6.92, respectively. The measured values of conductivity of these waters are investigative of their richness in mineral salts.

They include numerous ions (mg/L): Ca^{2+} (320), Mg^{2+} (30.60), Cl^{-} (333), Na^{+} (320), K^{+} (14), HCO_3^{-} (260.104), and SO_4^{2-} (670). The major ions (Ca^{2+}, Mg^{2+}, Cl^{-}, Na^{+}, K^{+}, $SO4^{2-}$, and HCO_3^{-}) are naturally very variable due to local geological, climatic, and geographical conditions [31]. Nitrite concentration analysis revealed that the waters had very stumpy nitrite content by World Health Organization WHO standards (0.1 mg/L). Nitrate content was also lower than the WHO recommended limit (50 mg/L). The phosphate concentration is in the admissible limit of 0.5 mg/L. Trace metal concentrations mg/L (F, 0.01, As, 0.01 Ni, 0.01, Pb, 0.002) are generally within the permissible limits [32]. From the results of Table 1, we notice that the thermal waters of Hammam Righa have minimal concentrations of heavy metals indicating the absence of urban and industrial pollution.

Table 1. *Physical and chemical properties of Hammam Righa Hot spring. The concentrations are represented in mg/L except temperature, pH and Conductivity*

.

Variable	Water Righa hot spring
Temperature (°C)	68
pH	6.92
Conductivity (µS/cm)	2500
Ca^{2+}	320
Mg^{2+}	30.60
Cl^{-}	333
Na^{+}	200
K^{+}	14
HCO^{3-}	260.104
SO_4^{2-}	670
NO_2^{-}	0.01
NO_3^{-}	1.52
PO_4^{3-}	0.03
Fe	0,13
Mn	0.071
Zn	0.21
Cu	0.013
F, As, Ni	0.01
Pb	0.002

III.2. Isolation of microorganisms

The bacterial strains isolated in this study grew aerobically on nutrient agar. They are not exigent. A total of 40 thermophilic bacterial isolates were isolated with optimum temperature of growth occurring at 60 °C.

III.3. Characterizationoftheisolates

Among 40 isolates, only 14 have been the subject of physiological and biochemical study. Morphologically, the strains showed great variation in the color, shape and texture of the colonies (Fig. 2 and Table 2). The pigmentation of the colonies varied from cream, beige, yellow and orange. All the isolates were rods. Gram staining revealed a majority of Gram-positive bacteria with only three isolates (B5GN, HB14, and M2R) being Gram negative.

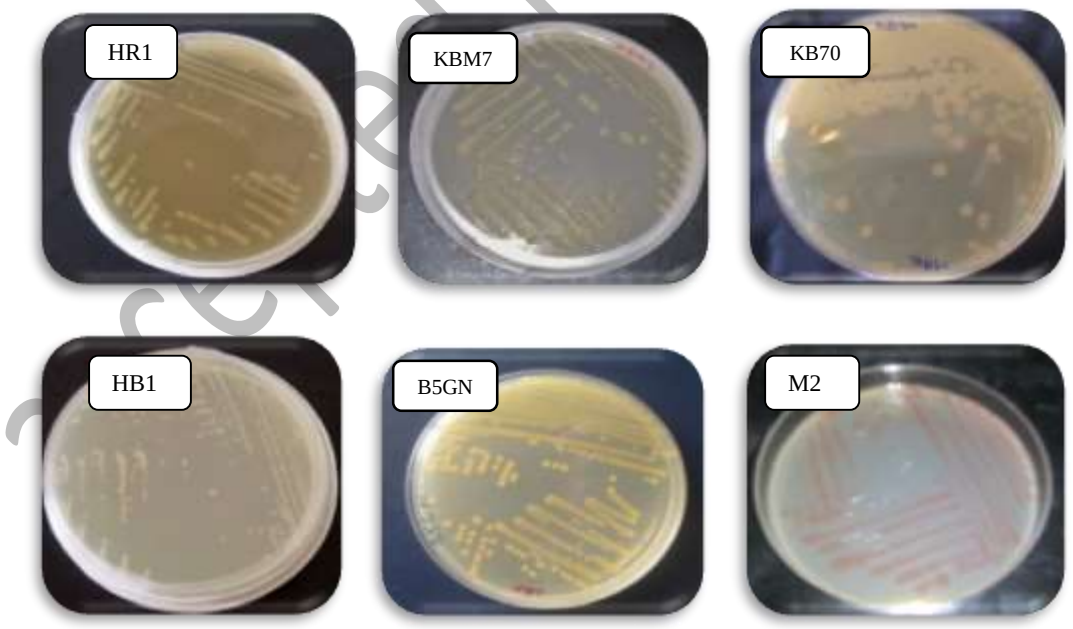

Fig. 2. *Macroscopic appearance of some strains.*

Table 2. *Phenotypic features of isolates.*

Strains	Phenotypic features						
	Colony morphology	Colony density	Pigmentation	Cell shape	Cell arrangement	Motile	Gram
ATAM	Circular	Translucent	Beige	Rod	Single	+	+
M1V	Circular	Translucent	Beige	Rod	Short chains	+	+
P2S	Circular	Translucent	Beige	Rod	Single	+	+
BHIA	Circular	Translucent	Beige	Rod	Short chains	+	+
KBM1	Circular	Opaque	Beige	Rod	Single	+	+
KBM7	Circular	Opaque	Beige	Rod	Single/Paired	+	+
B5GN	Circular	Translucent	Yellow	Rod	Single/Paired	-	-
KB70	Circular	Translucent	Cream	Rod	Single	+	+
HR1	Circular	Opaque	Cream	Rod	Single	+	+
HR2	Rhizoid	Opaque	Beige	Rod	Short chains	+	+
HB14	Circular	Opaque	Cream	Rod	Single	-	-
HR6	rhizoid	Opaque	Beige	Rod	Single	+	+
HR10	Circular	Opaque	Cream	Rod	Short chains	+	+
M2R	Circular	Opaque	Orange	Rod	Single	-	-

As shown in Table 3, strains KB 70, ATAM, M1V, P2S, HR6, HR1, HR2, HR10, BHIA and KBM7 were catalase, oxidase and nitrate reduction positive, and endospore-forming. KBM1, B5GN, and HB14 were catalase, oxidase, and nitrate reduction positive, and asporulated. Strains P2S, HB14, and BHIA1 were the only ones to show positive results for ortho nitrophenyl-β-D galactopyranosidase (ONPG). Strains M1V, KB 70, HR1, ATAM, M2R, HR2, BHIA, and B5GN showed negative results regarding arginine dihydrolases (ADH), lysine decarboxylase (LDC), ornithine decarboxylases (ODC), H_2S production and urease.

In addition, all isolates presented an immense variability in their aptitude to use sugars and polymers that reflect inter- and intra-specific polymorphism (Tables 3-4).

Table 3. *Biochemical characteristics of isolates (Analytical profiling index 20E).* ***1:*** *ATAM,* ***2:*** *M1V,* ***3:*** *P2S,* ***4:*** *BHIA,* ***5:*** *KBM1,* ***6:*** *KBM7,* ***7:*** *B5GN,* ***8:*** *KB70,* ***9:*** *HR1,* ***10:*** *HR2,* ***11:*** *HB14,* ***12:*** *HR6,* ***13:*** *HR10,* ***14:*** *M2R.*

Isolates test	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sporulation	+	+	+	+	-	+	-	+	+	+	-	+	+	-
ß-galactosidase	-	-	+	+	-	-	-	-	-	-	+	-	-	-
Arginine Dihydrolase	-	-	+	-	+	+	-	-	-	-	+	+	+	-
Lysine Decarboxylase	-	-	+	-	-	-	-	-	-	-	-	+	+	-
Ornithine Decarboxylase	-	-	+	-	-	-	-	-	-	-	-	+	+	-
Citrate	-	-	+	-	-	-	-	-	-	-	-	+	+	-
H_2S	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Urease	-	-	+	-	-	-	-	-	-	-	+	-	-	-
Tryptophane Desaminase	-	-	-	-	-	-	-	+	-	+	-	-	-	-
Indol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges proskauer	-	-	+	+	+	+	+	-	-	-	+	+	+	+
Gelatine	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	-	+	+	-	+	+	-	-	+	+
Mannitol	-	-	+	-	+	+	-	-	-	-	-	-	+	+

Inositol	-	+	+	-	+	+	-	-	-	-	-	-	-	+
Sorbitol	-	-	-	-	+	+	-	-	-	-	-	-	-	+
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Saccharose	+	+	+	-	-	+	-	-	-	-	-	+	-	-
Melibiose	+	-	-	-	-	-	-	-	-	-	-	+	-	+
Amygdaline	+	-	+	+	+	+	-	-	-	-	-	+	-	+
Arabinose	-	+	+	+	-	-	-	-	-	-	-	-	-	+

Table 4. *Biochemical characteristics of isolates: API 50 CHB.* ***1:*** *ATAM,* ***2:*** *M1V,* ***3:*** *P2S,* ***4:*** *BHIA,* ***5:*** *KBM1,* ***6:*** *KBM7,* ***7:*** *B5GN,* ***8:*** *KB70,* ***9:*** *HR1,* ***10:*** *HR2,* ***11:*** *HB14,* ***12:*** *HR6,* ***13:*** *HR10,* ***14:*** *M2R.*

Carbohydrate	**Symbol**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	GLY	-	+	+	-	-	-	+	-	-	-	-	+	+	-
Erythritol	ERY	-	+	+	-	-	-	-	-	-	-	-	-	-	-
D-arabinose	DARA	-	+	+	+	-	-	-	-	-	-	-	-	-	+
L-arabinose	LARA	+	+	+	-	-	+	-	-	+	-	-	+	+	-
D-ribose	RIB	+	+	+	-	-	+	-	-	+	-	-	+	+	-
D-xylose	DXYL	-	+	+	+	-	+	-	-	-	-	+	+	+	-
L-xylose	LXYL	-	+	+	+	+	-	-	-	-	-	-	-	-	-
D-adonitol	ADO	-	+	+	+	-	-	-	-	-	-	-	-	-	-
Methyl-ßDXylopyrano	MDX	-	+	+	-	-	-	-	-	-	-	-	-	-	-
D-galactose	GAL	+	+	+	-	-	+	-	-	+	-	-	+	+	-
glucose	GLU	+	+	+	+	-	+	+	-	+	-	-	+	+	+
D-fructose	FRU	+	+	+	-	+	+	+	-	+	-	+	+	+	-
D-mannose	MNE	+	+	+	-	-	+	-	-	+	-	-	+	+	-
L-Sorbose	SBE	-	+	+	-	-	-	-	-	-	-	-	-	-	-
L-rhamnose	RHA	+	+	+	-	-	-	-	-	+	-	-	-	-	-
Dulitol	DUL	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Inositol	INO	-	+	+	-	+	+	-	-	-	-	-	-	-	+
D-mannitol	MAN	-	-	+	-	+	+	-	-	-	-	-	+	-	+
D-sorbitol	SOR	-	-	-	-	+	+	-	-	-	-	-	-	-	+
α-Methyl-D-Mannoside	MDM	-	+	+	-	-	-	-	-	-	-	-	-	-	-
α-Methyl-D-Glucoside	MDG	+	+	+	-	-	+	-	-	+	-	-	+	+	-
N-Acetyl Glucosamine	NAG	+	+	+	-	-	-	-	-	+	-	-	-	-	-
Amygdalin	AMY	+	-	+	+	+	+	-	-	-	-	-	+	-	+
Arbutin	ARB	+	+	+	-	-	+	-	-	-	-	-	-	+	-
esculin	ESC	+	+	-	-	-	+	-	+	+	+	-	+	+	-
Salicin	SAL	+	+	+	-	+	+	-	-	+	-	-	+	-	-
D-cellobiose	CEL	+	+	+	+	+	+	-	-	+	-	-	+	+	-
D-maltose	MAL	+	+	+	+	+	+	+	-	+	-	-	+	+	-
D-lactose	LAC	+	+	+	+	+	-	-	-	+	-	-	-	-	-
D-melibiose	MEL	+	-	-	-	-	-	-	-	-	-	-	+	-	+
D-saccharose	SAC	+	+	+	-	-	+	-	-	+	-	-	+	-	-
D-trehalose	TRE	-	+	+	-	-	+	+	-	-	-	-	+	+	-
Inulin	INU	-	+	+	-	-	-	-	-	-	-	+	-	-	-
D-melezitose	MLZ	-	+	+	-	-	-	-	-	+	-	+	+	+	-
D-raffinose	RAF	+	+	+	-	-	+	+	-	+	-	+	+	+	-
Starch	AMD	+	+	+	-	-	+	+	-	+	-		+	+	-
glycogen	GLYG	+	+	+	-	-	+	+	-	+	-	+	-	-	-
Xylitol	XLT	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Gentiobiose	GEN	+	+	+	-	-	+	-	-	+	-	-	-	-	-
D-turanose	TUR	-	+	+	-	-	+	+	-	-	-	-	+	+	-
D-lyxose	LYX	-	+	+	-	-	-	-	-	-	-	-	-	-	-
D-tagatose	TAG	-	+	+	-	-	-	-	-	+	-	-	-	-	+
D-fucose	DFUC	-	+	+	-	-	-	-	-	-	-	-	-	-	+

L-fucose	LFUC	-	+	+	-	-	-	-	-	-	-	-	-	-	+
D-arabitol	DARL	-	+	+	-	-	-	-	-	-	-	-	-	-	-
L-arabitol	LARL	-	+	+	-	-	-	-	-	-	-	-	-	-	-
Gluconate	GNT	+	-	-	-	-	-	-	-	+	-	-	-	-	-
2- keto-gluconate	2KG	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5- keto -gluconate	5KG	-	-	-	-	-	-	-	-	-	-	-	-	-	-

As shown in Table 5, All strains grew in a wide range of temperature (from 40 to 60 °C) while strains M1V, P2S, BHIA, KBM7, B5GN, KB 70, HR1, HR2, HR6, and HR10) grew up to 80 ° C. The pH range for growth of all isolates is between 6 and 9. However, four strains (ATAM, HR1, HR2, and HR10) are able to grow under slightly acidic conditions at pH 5 and are considered as acido-tolerant. Strain B5GN grows at a pH range from 6 to 12 thus suggesting its alkali-tolerance. The isolates were all able to grow in the presence of 1 and 2% NaCl but not at 7%.

Table 5. *Physiological characteristics of isolates.*

Strains	**Physiological characteristics**																		
	Temperature (°C)						**pH**							**Salinity (%)**					
	30	40	50	60	70	80	4	5	6	7	9	11	12	1	2	3	4	5	6	7
ATAM	+	+	+	+	-	-	-	+	+	+	+	-	-	+	+	+	+	+	+	-
M1V	+	+	+	+	+	+	-	-	+	+	+	-	-	+	+	+	+	-	-	-
P2S	+	+	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	-	-
BHIA1	+	+	+	+	+	+	-	-	+	+	+	-	-	+	+	+	-	-	-	-
KBM1	+	+	+	+	-	-	-	-	+	+	+	-	-	+	+	+	-	-	-	-
KBM7	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	-	-	-
B5GN	-	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	-	-	-
KB70	+	+	+	+	+	+	-	-	+	+	+	+	-	+	+	+	+	+	-	-
HR1	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	+	-
HR2	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	-
HB14	+	+	+	+	-	-	-	-	+	+	+	-	-	+	+	+	+	+	+	-
HR6	+	+	+	+	+	+	-		+	+	+	-	-	+	+	+	-	-	-	-
HR10	+	+	+	+	+	+	-	+	+	+	+	-	-	+	+	+	-	-	-	-
M2R	-	+	+	+	+	-	-	-	+	+	+	-	-	+	+	-	-	-	-	-

The 14 strains shared more than 97% identity with their closest phylogenetic relative. They fall into three phyla (Fig. 3). 10 strains (KB 70, ATAM, M1V, P2S, HR6, HR1, HR2, HR10, BHIA, and KBM7) belonged to the family *Bacillaceae*, Strains HB14 (*Albidovulum* sp.), B5GN (*Hydrogenophilus* sp.), and KBM1 (*Tepidimonas* sp.) pertains to the class β-proteobacteria while strain M2R is closely related to the family *Thermaceae* with *Meiothermus ruber* as its closest phylogenetic relative.

Most of the thermophilic aerobic bacteria which inhabit this Algerian spring belong to *Geobacillus*, *Anoxybacillus* and *Bacillus*. The observed dominance of *Bacillaceae* in the samples was in agreement with preceding reports with regard to the microbial communities inhabiting hot springs [33, 34]. The colonization of such extreme environments by endospore forming bacilli has been well documented [35-38].

Anoxybacillus species are widely distributed and readily isolated from geothermally heated environments [39]. They have been isolated from hot environments in Russia, Italy, Saudi Arabia, and Turkey [40-43].

Among the genus *Bacillus*, *Bacillus licheniformis* is widely distributed in all places environment. It is Gram-positive, spore forming, rod-shaped cells, and aerobic or facultative anaerobic bacteria which can survive at high temperatures [44, 45]. *Bacillus licheniformis* strains were isolated from hot springs in Tunisia [5], Moroccan hot springs[33] the Sonoran Desert (Arizona) in USA [46], hot springs in Turkey [47], Saudi Arabia [38], and Indonesia [48].

Strain B5GN has 100% similarity with *Hydrogenophilus thermoluteolus* which has been isolated, for the first time, from soil around a hot spring in Izu peninsula, Shizuoka Prefecture, Japan [49].

Strain M2R share 100% sequence identity with *Meiothermus ruber*. In natural environments, *Meiothermus* strains have been found exclusively in thermal limnetic systems, predominantly in terrestrial hot springs [50, 51]. Strains of *Meiothermus ruber* have been isolated from geothermal areas worldwide, even from man-made thermal environments, while the distribution of the other *Meiothermus* species seems to be regional [52, 53].

Strains HB14 and KBM1 shares 100% sequence identities with *Albidovulum inexpectatum*, *Tepidimonas taiwanensis*, respectively. *Albidovulum inexpectatum* is an aerobic moderate thermophile isolated from a marine hot spring in the Azores [54], whereas *Tepidimonas taiwanensis* was isolated from Sih-Chong-Si hot spring in Taiwan [55].

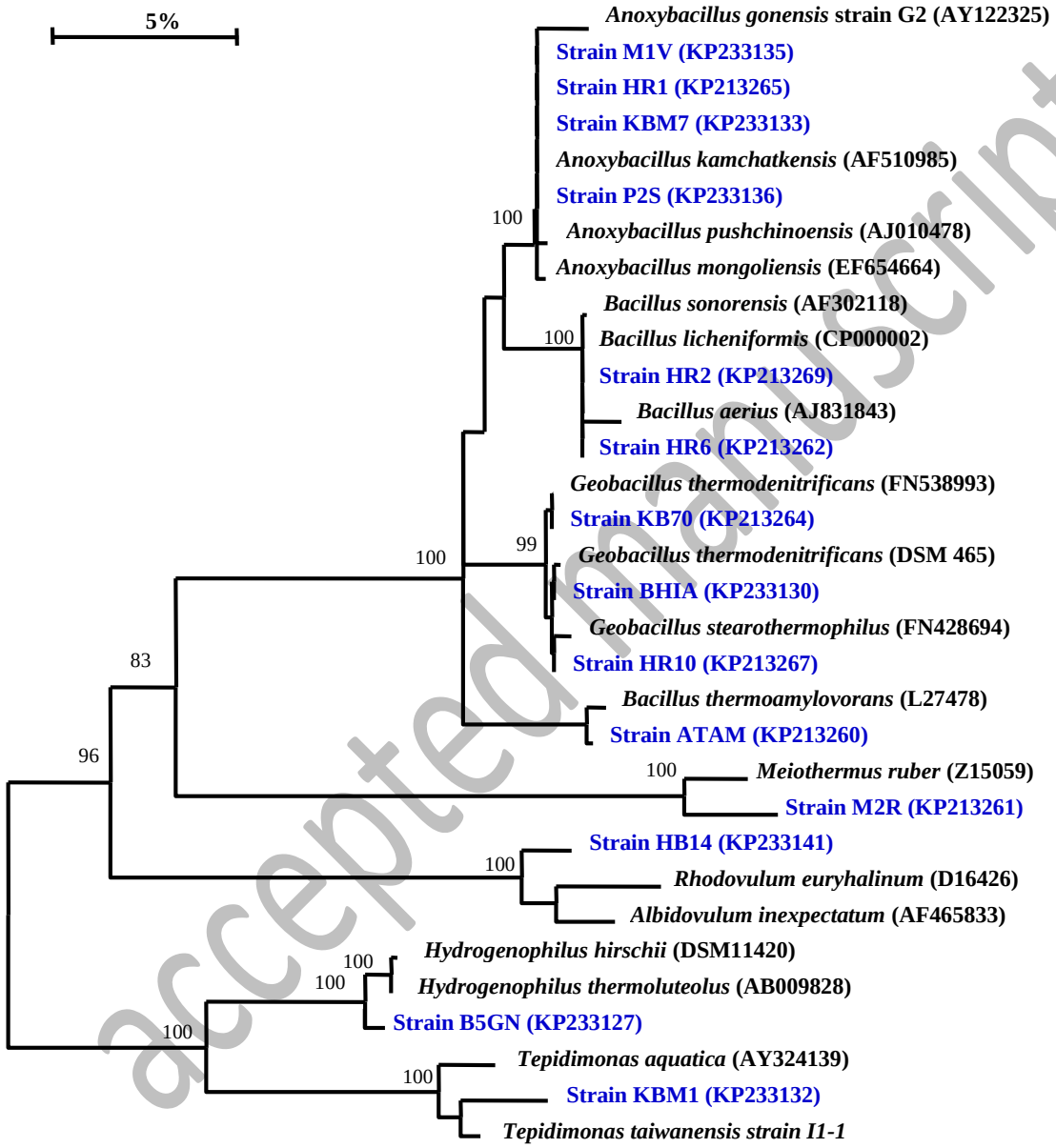

Fig. 3. *Molecular phylogeny of fourteen selected bacteria and the most related type strains species using partial 16S* rDNA *sequences.*

3.3. Hydrolase activities

Every one of the isolate was screened for amylase, protease, cellulase and xylanase activity at 60 °C (Fig. 4).

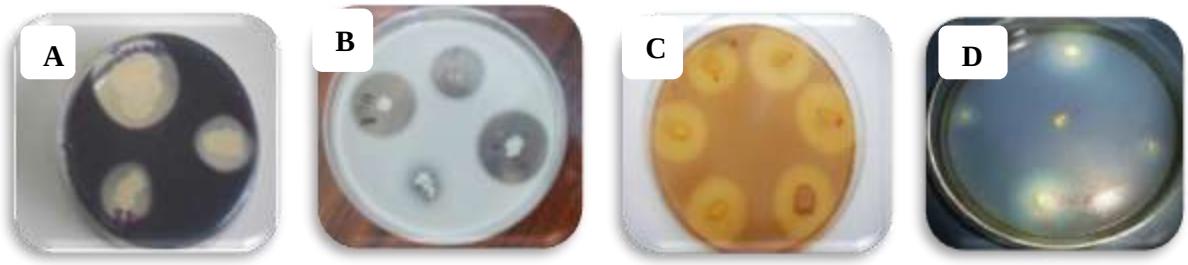

Fig. 4. *Detection of some extracellular enzymatic activities.* ***A****. Amylase,* ***B****. Protease,* ***C.*** *cellulase,* ***D****. Xylanase.* The enzyme screening studies were experienced three times for each isolate.

As shown in Table 6, Among the 14 strains selected in this study, 11 strains displayed amylase and protease activities. 12 strains (ATAM, M1V, P2S, BHIA, KBM7, B5GN, KB70, HR1, HR2, HR6, HR10, and M2R) use CMC and 8 strains (ATAM, M1V, P2S, BHIA, KBM1, KBM7, B5GN, and M2R) consume xylan. In a recent study, a novel thermostable protease (named SAPA) produced from *Anoxybacillus kamchatkensis* strain M1V was purified and biochemically and structurally identified. The promising potential of this enzyme for biotechnological applications was also well explored. SAPA is as bio-additive in detergent formulation and a candidate for shrimp waste valorization for the chitin recovery as well as for bioactive protein and peptide recuperation [56-58].

Table 6. *Enzymatic activities of isolates at 60 °C.*

Strains	Hydrolytic activities			
	Amylase	Protease	Cellulase	Xylanase
AT AM	+	+	+	+
M1V	+	-	+	+
P2S	-	+	+	+
BHIA	+	+	+	+
KBM1	+	+	-	+
KBM7	+	-	+	+
B5GN	+	-	+	+
KB 70	+	+	+	-
HR1	-	+	+	-
HR2	+	+	+	-
HB14	+	+	-	-
HR6	+	+	+	-
HR10	+	+	+	+
M2R	+	+	+	+

The presence of a multiplicity of biopolymer-degrading enzymes detected in isolates from Hammam Righa hot spring suggest major contribution of these bacteria to the hydrolysis of the major organic constituents (proteins, polysaccharides). In this way, they may contribute notably to carbon and nitrogen cycling in the Algerian springs. The activity of the enzymes at elevated temperatures is one of the vital mechanisms for adaptation of microorganisms to hot environments [59].

Species belonging to the genus *Bacillus* are known for their huge production of enzymes like proteases, amylases, cellulases, xylanases, and bioactive molecules representing scientific, therapeutic, and biotechnological interest [60, 61]. Among the collection of 161 strains of thermophilic *Bacillus* isolated from different samples of thermal water in Tunisia, 35 strains produced amylases, 37-proteases, 43-cellulases, 31-xylanases, and 37-mannanases [5, 6].

To our knowledge, few records are presently available on the enzymes from *Meiothermus* [53], *Tepidimonas*, *Albidovulum*, and *Hydrogenophilus* [62-65]. Recently, the purification, biochemical, and molecular characterization of a novel extracellular thermostable and alkaline α-amylase from *Tepidimonas fonticaldi* strain HB23 have been reported [66]. The identification of a novel chitinase from *Hydrogenophilus hirschii* strain KB-DZ44 have been also reported [67].

In the current research, we detected several promising thermophilic bacteria regarding the enzymes that they possess that could be applied in the industry. More attention should be paid to them and to other uncultivated microorganisms originating from hot ecosystems which may be of industrial significance.

IV. Conclusion

This study is the first investigation of thermophilic aerobic bacteria originating from Hammam Righa hot spring. The results showed an obvious dominance of thermophilic *Bacillaceae*. Our results extend our knowledge of microbial diversity existing in hot springs. They suggest that there are biological markers of hot springs. Moreover, they showed that new thermophilic populations can be found in these hot environments. They provide evidence and indication that the Hammam Righa possesses a rich microbial diversity to be explored by further surveys based on microbiological and metagenomics approaches with the aim to explore the uncultivated microorganisms. Additional studies are currently in progress in order to determine the diversity of thermophilic bacteria (aerobic and anaerobic) and their ability to produce extracellular hydrolytic enzymes in other Algerian hot springs and to select for the best hydrolytic enzymes producers to be used in biotechnology.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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