MOL2NET'21, Conference on Molecular, Biomedical & Computational Sciences and Engineering, 7th ed.
MODECO-06: Molec. Diversity, Environ. Chem., and Economy Congress, Paris, France-Ohio, USA, 2021

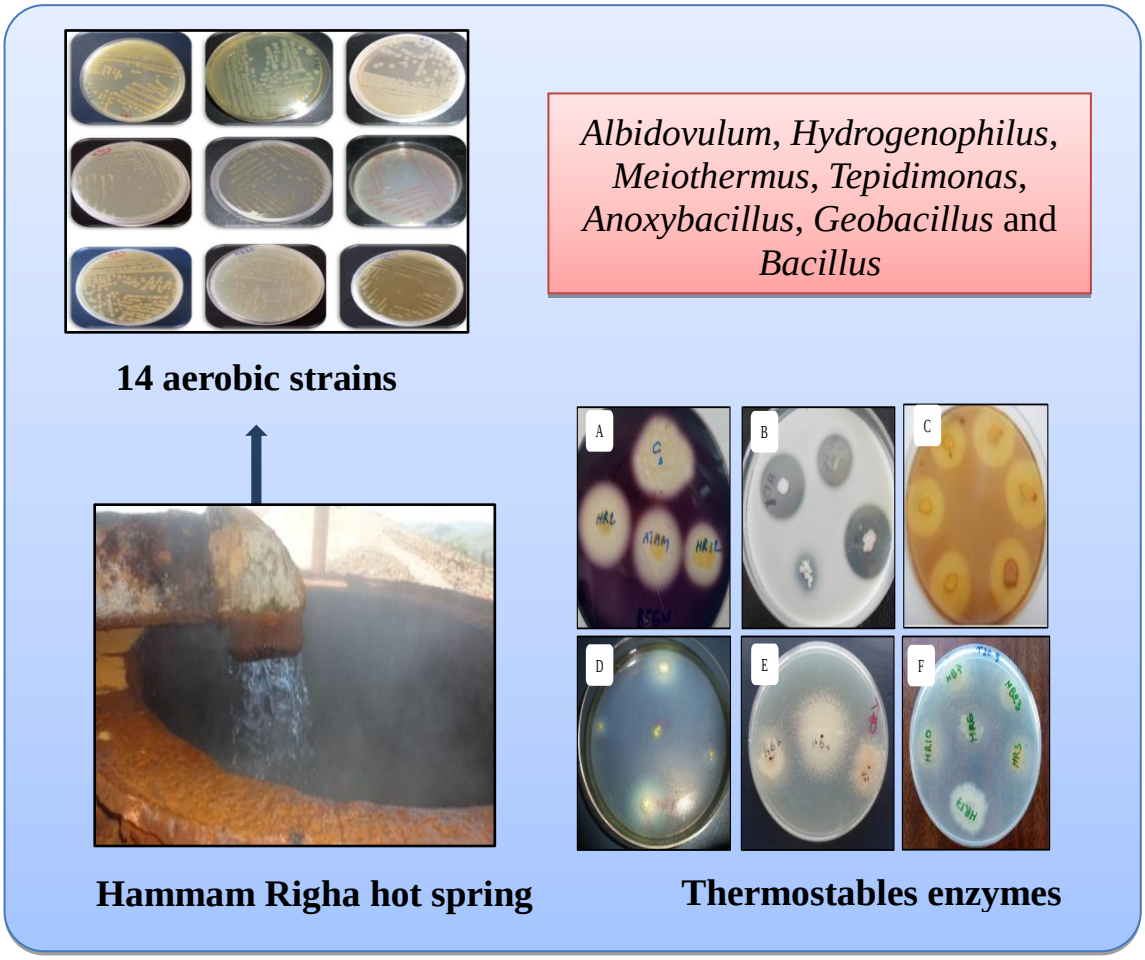
Isolation and characterization of thermophilic bacteria as producers of enzymes from Hammam Righa hot spring

Khelifa Bouacem [a,*], Amel Bouanane-Darenfed [a]

**E-mail addresses*: khelifa.bouacem@yahoo.fr; kbouacem@usthb.dz

[a] *Laboratory of Cellular and Molecular Biology (LCMB), Microbiology Team, Faculty of Biological Sciences, University of Sciences and Technology of Houari Boumediene (USTHB), PO Box 32, El Alia, Bab Ezzouar, 16111 Algiers, Algeria*

Graphical Abstract



Extreme environment have been an interesting object for microbiological research to study the diversity of extremophilic microorganisms. The ability to grow in extreme conditions makes the microorganisms unique. Thermophilic bacteria are bacteria that can grow at temperatures of 45-60°C. These organisms, each containing a catalog of enzymes and other bioproducts, could provide a highly valuable resource for biotechnological developments and applications. Biodiversity in geothermal springs in Algeria appears scanty and has not been thoroughly investigated. Geothermal springs are scattered in several areas in Algeria. The present study was conducted to isolate, identify, characterize and to determine the enzymatic activities of the thermophilic aerobic bacteria isolated from Hammam Righa Hot Spring. A total of 40 thermophilic bacterial strains were isolated from this Hammam. The optimum temperature of isolates was 60°C. Totally 14 bacterial strains were selected for this study. The phenotypic characterization (morphological, physiological, and biochemical tests) of those isolates was confirmed by genotypic method using 16S rRNA sequence analysis. *16S rDNA* gene analysis found them related to *Bacillus*, *Anoxybacillus*, *Geobacillus*, *Tepidimonas* and *Hydrogenophilus* genera. Positive results on several enzymes such as amylase, caseinase, cellulase, chitinases and xylanase of most isolates are indication of potential applications of these bacterial products in biotechnology.

Keywords: *Hot spring*; *Isolation*; *Thermophilic bacteria*; *16S rDNA; Enzymes.*

1. Introduction

Thermophilic prokaryotes are known to grow optimally at temperatures higher than 60 °C with hyperthermophiles possibly growing above 80 °C (Pandey et al., 2015). 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 (Baker et al., 2001; Bertoldo & Antranikian, 2002). Many terrestrial hot springs exist on Earth. Thermophilic microorganisms associated with these ecosystems have received considerable interest in recent years (Bouacem, 2016; Bouacem et al., 2018; Thebti et al., 2016) as they are of peculiar interest for regarding the production of thermostable enzymes like protease, cellulase, xylanase and amylase to be possibly used in the detergent, leather, pulp and paper industries. These enzymes are still active at temperatures which are even higher than the optimum temperatures for the growth of the microorganisms themselves (Saboto et al., 1999).

Algeria possesses more than 240 thermal sources with number increasing when approaching the Algerian North-eastern with temperatures ranging from 19 °C to 98 °C. These sources (Hammams) are most often used for therapeutic purposes and curative effects. For somme of them, they are known to harbor large communities of thermophilic anaerobic and aerobic bacteria (Bouacem, 2016).

While experiments have been undertaken in these springs to isolate novel anaerobic thermophiles possessing thermostable enzymes of industrial interest (Bouacem et al., 2014; Bouacem et al., 2016; Bouacem et al., 2015) there is no information with regard to indigenous thermophilic aerobic microorganisms inhabiting these extreme environments so far. The main objective of this study was to isolate and characterize thermophilic aerobic bacteria from a hot

spring (Hammam Righa) in Algeria by using phenotypic (morphological, biochemical and physiological features) and phylogenetic approaches (16S rRNA gene sequence analysis). Moreover, hydrolytic activities of these isolates that we obtained were determined.

2. Materials and Methods

2.1. Substrates and chemicals

Unless specified, all substrates, chemicals, and reagents were of the analytical grade or highest commercially available purity, and were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Sample collection

Water samples were collected from Hammam Righa hot spring, 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.

2.3. Isolation of microorganisms

Enrichment cultures and isolation were performed in MG medium containing as described by authors(Bouacem, 2016). Different colonies were selected and restreaked several times to obtain pure cultures which were stored in nutrient agar at 4 °C until used.

2.4. Characterization of the isolates

2.4.1. Morphological 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 18-24 h of incubation at 60 °C.

2.4.2. Biochemical tests

The thermophilic isolates were identified by the use of conventional tests (Gaudin et al., 2008; Hernndez et al., 2000).

2.5. 16S rRNA sequence analysis

Methods for purification of the DNA, PCR amplification, and sequencing of the 16S ribosomal RNA (rRNA) gene were described previously (Ben Dhia Thabet et al., 2004). The partial sequences generated were assembled using BioEdit v. 5.0.9 (Hall, 1999) and the consensus sequence was corrected manually for errors. The sequence was compared with available sequences in GenBank (version 178) using a BLAST search (Altschul et al., 1997)). The consensus sequence was then manually adjusted to conform to the 16S rRNA secondary structure model (Winker & Woese, 1991). Nucleotide ambiguities were omitted and evolutionary distances were calculated using the Jukes and Cantor option (Jukes, 1969). Phylogenetic trees were constructed with the Tree Con program using the neighbour joining (Saitou & Nei, 1987). Tree topology was evaluated by a bootstrap analysis using 2,000 resamplings of the sequences (Felsenstein, 1985). Its topology was also supported using the

maximum-parsimony and maximum-likelihood algorithms. The 16S rRNA sequence of each strain has been deposited in the GenBank.

2.6. Hydrolytic enzymes

2.6.1. Amylases

Each colony was streaked on a nutrient agar plate that contained 1% starch and incubated at 60 °C for 48 h. 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 (Burhan et al., 2003).

2.6.2. 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 (Priest et al., 1988).

2.6.3. Cellulases

Each colony was streaked on a nutrient agar plate that contained 1% (w/v) carboxymethyl cellulose (CMC) and incubated at 60 °C for 48 h. After incubation, plates were flooded with 0.1% (w/v) Congo red solution for 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 (Teather & Wood, 1982).

2.6.4. 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 (Silveira et al., 1997).

3. Results and discussion

3.1. Characterization of the isolates

Among 40 isolates, only 14 were subjected to various biochemical and physiological tests. Morphologically, the isolates showed great variation in the color, shape and texture of the colonies. The 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.

The 14 strains shared more than 97% identity with their closest phylogenetic relative. They fall into three phyla (Fig. 1). 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.

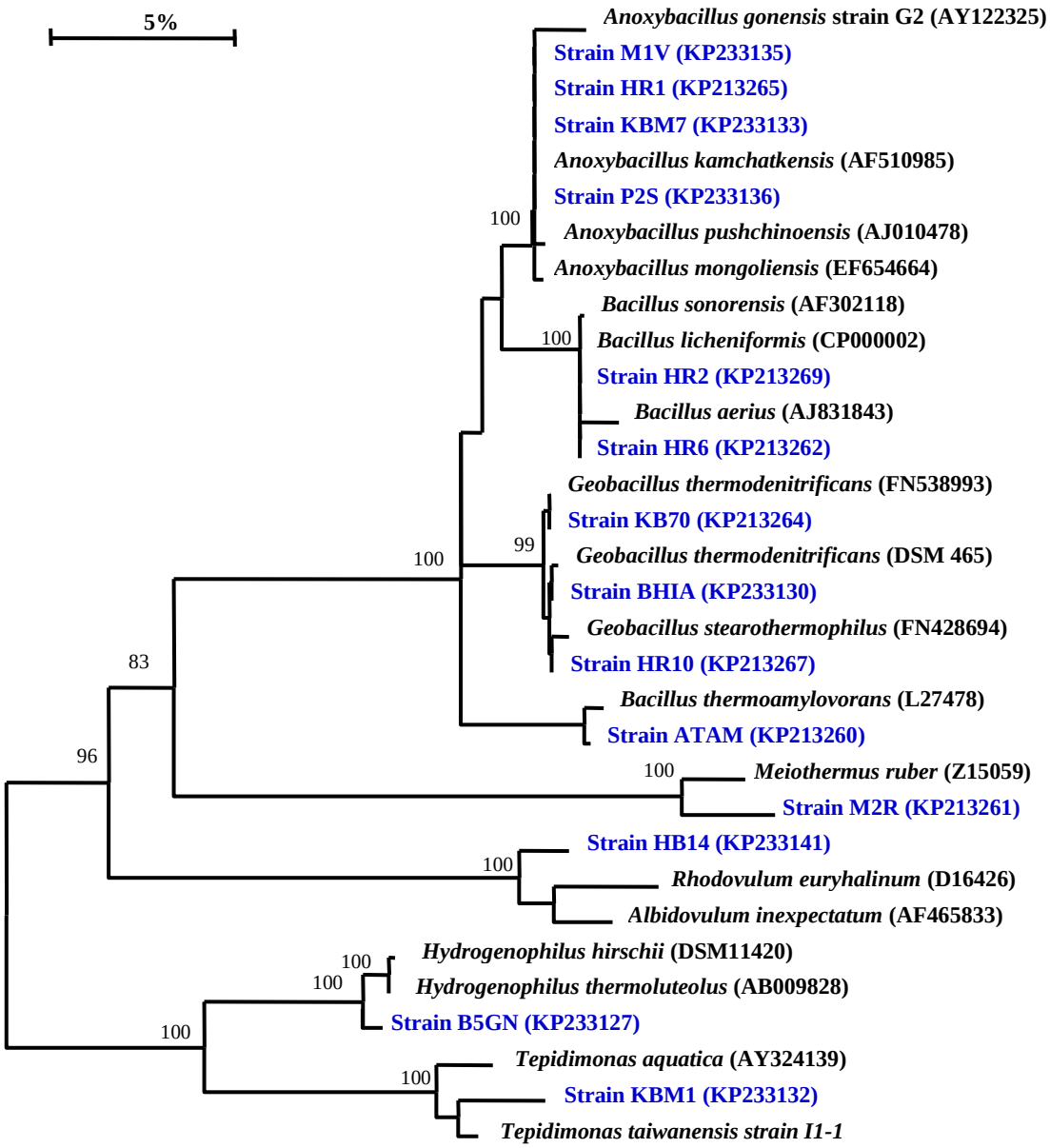


Fig. 1. Molecular phylogeny of fourteen selected bacteria and the most related type strains species using partial 16S rDNA sequences.

3.2. Hydrolase activities

All the isolates were screened for amylase, protease, cellulase and xylanase activity at 60 °C (Fig. 4). The enzyme screening studies were performed three times for each isolate. As shown in Table 2, Among the 14 strains selected in this study, 11 strains exhibit 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.

Table 2 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	+	+	+	+

4. Conclusion

In conclusion, this is the first investigation of thermophilic aerobic bacteria originating from Hammam Righa hot spring. The results showed a clear dominance of thermophilic *Bacillaceae*.

5. References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids research*, **25**(17), 3389-3402.

Baker, G.C., Gaffar, S., Cowan, D.A., Suharto, A.R. 2001. Bacterial community analysis of Indonesian hot springs. *FEMS Microbiol Lett*, **200**(1), 103-9.

Ben Dhia Thabet, O., Fardeau, M.-L., Joulian, C., Thomas, P., Hamdi, M., Garcia, J.-L., Ollivier, B. 2004. Clostridium tunisiense sp. nov., a new proteolytic, sulfur-reducing bacterium isolated from an olive mill wastewater contaminated by phosphogypse. *Anaerobe*, **10**(3), 185-190.

Bertoldo, C., Antranikian, G. 2002. Starch-hydrolyzing enzymes from thermophilic archaea and bacteria. *Curr Opin Chem Biol*, **6**(2), 151-60.

Bouacem, K. 2016. Caractérisation de souches bactériennes isolées à partir de sources thermales du Nord-Algérien: Etude des propriétés enzymatiques *Thèse de Doctorat 3ème cycle LMD en Microbiologie Appliquée* **USTHB**(189p).

Bouacem, K., Bouanane-Darenfed, A., Boucherba, N., Joseph, M., Gagaoua, M., Hania, W.B., Kecha, M., Benallaoua, S., Hacène, H., Ollivier, B. 2014. Partial characterization of xylanase produced by Caldicoprobacter algeriensis, a new thermophilic anaerobic bacterium isolated from an Algerian hot spring. *Applied biochemistry and biotechnology*, **174**(5), 1969-1981.

Bouacem, K., Bouanane-Darenfed, A., Jaouadi, N.Z., Joseph, M., Hacene, H., Ollivier, B., Fardeau, M.-L., Bejar, S., Jaouadi, B. 2016. Novel serine keratinase from Caldicoprobacter algeriensis exhibiting outstanding hide dehairing abilities. *International journal of biological macromolecules*, **86**, 321-328.

Bouacem, K., Bouanane-Darenfed, A., Laribi-Habchi, H., Ben Elhoul, M., Hmida-Sayari, A., Hacene, H., Ollivier, B., Fardeau, M.-L., Jaouadi, B., Bejar, S. 2015. Biochemical characterization of a

detergent-stable serine alkaline protease from Caldicoprobacter guelmensis. *International journal of biological macromolecules*, **81**, 299-307.

Bouacem, K., Laribi-Habchi, H., Mechri, S., Hacene, H., Jaouadi, B., Bouanane-Darenfed, A. 2018. Biochemical characterization of a novel thermostable chitinase from Hydrogenophilus hirschii strain KB-DZ44. *International journal of biological macromolecules*, **106**, 338-350.

Burhan, A., Nisa, U., Gökhan, C., Ömer, C., Ashabil, A., Osman, G. 2003. Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic Bacillus sp. isolate ANT-6. *Process Biochemistry*, **38**(10), 1397-1403.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 783-791.

Gaudin, V., Juhel-Gaugain, M., Moretain, J.P., Sanders, P. 2008. AFNOR validation of Premi Test, a microbiological-based screening tube-test for the detection of antimicrobial residues in animal muscle tissue. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*, **25**(12), 1451-64.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic acids symposium series*. pp. 95-98.

Hernndez, A., Martro, E., Matas, L., Martin, M., Ausina, V. 2000. Assessment of in-vitro efficacy of 1% Virkon against bacteria, fungi, viruses and spores by means of AFNOR guidelines. *J Hosp Infect*, **46**(3), 203-9.

Jukes, T.H. 1969. Evolutionary pattern of specificity regions in light chains of immunoglobulins. *Biochem Genet*, **3**(2), 109-17.

Pandey, A., Dhakar, K., Sharma, A., Priti, P., Sati, P., Kumar, B. 2015. Thermophilic bacteria that tolerate a wide temperature and pH range colonize the Soldhar (95 °C) and Ringigad (80 °C) hot springs of Uttarakhand, India. *Annals of Microbiology*, **65**(2), 809-816.

Priest, F.G., Goodfellow, M., Todd, C. 1988. A numerical classification of the genus Bacillus. *Journal of general microbiology*, **134**(7), 1847-1882.

Saboto, D., Nucci, R., Rossi, M., Gryczynski, I., Gryczyniski, Z., Lakowicz, J. 1999. The b-glycosidase from the hyperthermophilic archaeon Sulfolobus solfataricus: enzyme activity and conformational dynamics at temperatures above 100 C. *Biophys. Chem*, **81**, 23-31.

Saitou, N., Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*, **4**(4), 406-25.

Silveira, F.Q.d.P., Melo, I.S., Ferreira Filho, E.X. 1997. Carbohydrate-hydrolysing enzyme activity production by solid-state cultures of Trichoderma harzianum strains. *Rev. microbiol*, **28**(3), 152-6.

Teather, R.M., Wood, P.J. 1982. Use of Congo red-polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from the bovine rumen. *Appl Environ Microbiol*, **43**(4), 777-80.

Thebti, W., Riahi, Y., Belhadj, O. 2016. Purification and Characterization of a New Thermostable, Haloalkaline, Solvent Stable, and Detergent Compatible Serine Protease from Geobacillus toebii Strain LBT 77. *Biomed Res Int*, **2016**, 9178962.

Winker, S., Woese, C.R. 1991. A Definition of the Domains Archaea, Bacteria and Eucarya in Terms of Small Subunit Ribosomal RNA Characteristics. *Systematic and Applied Microbiology*, **14**(4), 305-310.