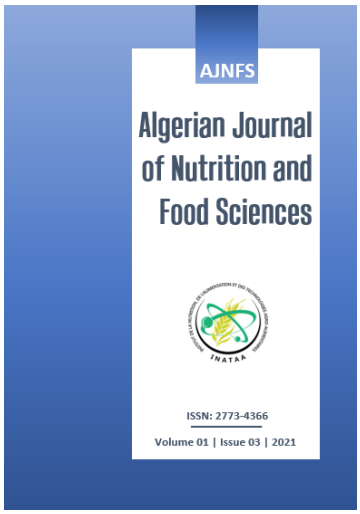

Algerian Journal of
Nutrition and Food Sciences
(AJNFS)

ISSN: 2773-4366

Journal homepage: fac.umc.edu.dz/inataa/revue/

Screening of extracellular glycoside hydrolases production thermophilic bacteria isolated from the hot spring of Hammam Salhine in Azzaba region (Skikda, Algeria)

Bouaita S., Bouguerba R., Gomri M.A.

Published online: November 9, 2021.

To cite this article: Bouaita, S., Bouguerba, R., Gomri, M.A. 2021. Effects of extraction speed, sample/solvent ratio and extraction time on the extraction of anthocyanins from strawberries. *Algerian Journal of Nutrition and Food Sciences*, 1(3), 1–10

To link to this article: https://fac.umc.edu.dz/inataa/revue/files/ajnfs0103001.pdf

Algerian Journal of Nutrition and Food Sciences (AJNFS) – Open Access – ISSN: 2773-4366
Volume 01, Issue 03, 2021: 1-10

Original Article

Screening of extracellular glycoside hydrolases production by thermophilic bacteria isolated from the hot spring of Hammam Salhine in Azzaba region (Skikda, Algeria)

Bouaita Sarra[1], Bouguerba Rayane[1], Gomri Mohamed Amine[2*]

[1] INATAA, Université Frères Mentouri Constantine 1 (UFMC1) (Algérie)
[2] Équipe Métabolites des Extrêmophiles (METEX), Laboratoire de Recherche Biotechnologie et Qualité des Aliments (BIOQUAL), INATAA, UFMC1 (Algérie)

Received September 24, 2021 Accepted October 11, 2021 Available online November 9, 2021

Abstract *Thermophilic microorganisms isolated from natural hot environments can represent an important source of microbial biodiversity with high value-added biomolecules. Among these biomolecules, glycoside hydrolases are of great interest for the food industry because of their unique reaction properties at high temperatures, their resistance to high salinity and their stability under different environmental conditions. In order to study some of their hydrolytic potential, 11 aerobic and facultative aerobic thermophilic bacterial strains were isolated from water and sediment samples taken from Hammam Salhine hot spring in Azzaba (wilaya of Skikda). The study of the physicochemical characteristics of this spring showed that it had a neutral pH, water is mesothermal and characterized by a strong mineralization. A phenetic characterization was carried out, and allowed to have access to some morphological, biochemical and physiological properties of these microorganisms and which were used for a numerical taxonomy and thereby construction of a phenotypic tree. The bacterial strains form Gram-positive rods with the presence and/or absence of endospores. They are moderate thermophilic, aerobic, neutrophilic and non-halotolerant bacteria. Four types of hydrolases were identified using three different substrates. The cellulolytic and/or xynalolytic activities were more encountered followed by the pectinolytic and amylase activities. The quantification assay of the amylolytic activity revealed that all positive strains show a significant activity, particularly AS7. Numerical analysis allowed to classify the 11 strains of the study in 3 main phenons with low phenetic variability, but it is necessary to perform phylogenetic analysis for confirmation.*

Keywords *Hot spring, Screening, Hydrolytic Activities, Thermophiles, Glycoside hydrolase*

Résumé *Les microorganismes thermophiles isolés à partir des environnements chauds naturels peuvent être une source importante de biodiversité microbienne ayant des actifs biotechnologiques à grande valeur ajoutée. Parmi ces actifs, les glycoside hydrolases sont d'un intérêt majeur en industrie agroalimentaire en raison de leurs propriétés réactionnelles uniques à températures élevées, leur résistance aux fortes salinités et leur stabilité à différentes conditions environnementales. Dans la perspective d'étudier une partie de leur potentiel hydrolytique, 11 souches bactériennes thermophiles aérobies et aérobies facultatives ont été isolées à partir d'échantillons d'eau et de sédiments prélevés au niveau de la source chaude de Hammam Salhine de Azzaba (wilaya de Skikda). Les résultats de l'étude des caractéristiques physico-chimiques de cette source montre qu'elle possédait un pH neutre, les eux sont mésothermales et caractérisées par une forte minéralisation. Une caractérisation phénétique a été réalisée, et a permis d'avoir accès à certaines propriétés morphologiques, biochimiques et physiologiques de ces microorganismes et qui ont servi à une taxonomie numérique, qui a mené à la construction d'un arbre phénotypique. Les souches bactériennes forment des bâtonnets à Gram positif avec la présence et/ou absence d'endospores. Elles sont thermophiles modérées, aérobies, neutrophiles et non halotolérants. Quatre types d'hydrolases ont été mises en évidence par l'utilisation de trois différents substrats. Les activités cellulolytique et/ou xynalolytique ont été les plus rencontrées suivie par les activités pectinolytique et amylasique. Les résultats du dosage de l'activité amylolytique ont révélé que toutes les souches productrices présentent une activité importante notamment la souche AS7. L'analyse numérique a permis de clustériser les 11 souches de l'étude en 3 principaux phénons présentant une faible variabilité phénétique, mais il est nécessaire d'effectuer une analyse phylogénétique pour confirmation.*

Mots clés *Source géothermale, Screening, Activités hydrolytiques, Thermophiles, Glycoside hydrolase*

* Corresponding author:
Gomri Mohamed Amine
Email address: gomrima@umc.edu.dz
INATAA, UFMC1
7e Km Route de Sétif, RN 5, 25000 Constantine (Algeria)

Introduction

Extremophiles and their enzymes have been identified as suitable means for greener, more efficient and cost-effective biotechnology (Krüger *et al.*, 2018). Thermophiles are a type of extremophiles that can grow at high temperatures between 45 °C and 122 °C (Kambourova, 2018). Enzymes from thermophilic microorganisms are referred to as thermozymes. The latter are considered ideal alternatives for industrial bioprocesses taking place at high temperatures. Indeed, thermoenzymes have several advantages. In particular, allowing faster reactions, increased solubility of substrates, decreased viscosity and elimination of microbial contamination (Kumar *et al.*, 2019; Ghosh *et al.*, 2020).

Glycoside hydrolases are among the most widely used biocatalysts in the world. Among them cellulases, xylanases, pectinases, amylases, arabinase, chitinases, etc. These enzymes are used in many industrial applications including hydrolysis of plant materials (cellulases, xylanases) for production of value-added components, applications in agriculture, in food industries, usage in paper and pulp industry, in detergent manufacturing, bioremediation, biorefining and healthcare sectors (Urbieta *et al.*, 2015; Shrivastava, 2020). Hence for, glycoside hydrolases produced by thermophilic microorganisms are of particular interest as promising thermoenzymes with wide biotechnological potential (Abdel-Azeem *et al.*, 2020).

Thermophilic microorganisms inhabit various natural and man-made environments and terrestrial hot springs can be an attractive source for these microorganisms (Mehta and Satyanarayana, 2013) and their glycolytic thermoenzymes (Benammar *et al.*, 2020; Msarah *et al.*, 2020).

Algeria is one of the richest countries of terrestrial hot springs in the world. There are more than 280 geothermal springs with temperatures rising up to 45 °C and higher.

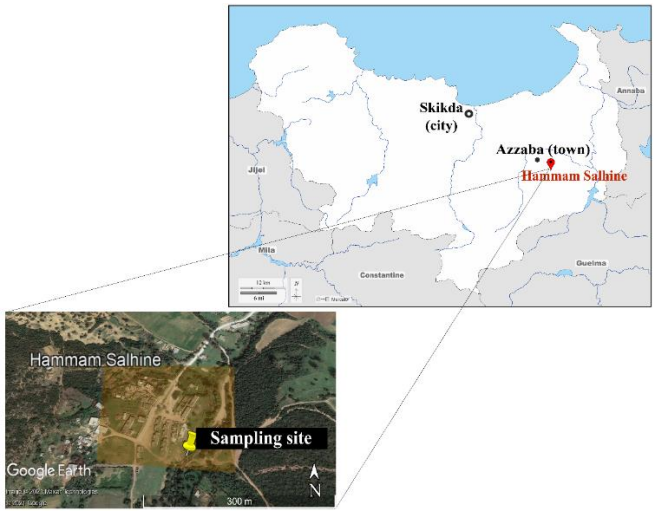

Figure 1. Localization of Hammam Salhine (source: d-map.com and Google Earth)

These springs are mainly used as thermal spas for balneology because of their therapeutic properties and, on a smaller scale, for space heating and greenhouses (Fekraoui and Kedaid, 2005; Adjeroud *et al.*, 2020; Lebbihiat *et al.*, 2021). However, the majority of these sites is so far still unexplored (Adjeroud *et al.*, 2020; Benammar *et al.*, 2020).

In this study, we isolate thermophilic bacteria from environmental samples collected from the hot spring of Hammam Salhine located in the Wilaya of Skikda in order to test the purified isolates for the production of some extracellular glycoside hydrolases (cellulases/ xylanases, pectinases and amylases) and to quantify the production of the most interesting enzymes by the selected isolates.

Materials and methods

Studied site description

The choice was made on the thermal spring of Hammam Salhine, located in the wilaya of Skikda. In fact, this ecosystem has not been the subject of any study on this aspect before. On the other hand, it is considered as an extreme environment, since it is a spring with a temperature ranging from 41 °C to 47 °C (Bekkouch and Benhamza, 2016).

Hammam Azzaba (also called Hammam Salhine) is sited near the municipality of Ain Cherchar, in the South- Est of Skikda state (North-East of Algeria) and at 7Km from Azzaba. It is located 20 m far from the left bank of Oued Hmimine at 36°72' N; 7.17' E and 104 m of altitude (Fig. 1). Water of the spring is used for therapeutic purposes. Its chemical composition is very rich in minerals with a calcic sulfate chemical facies (Bekkouche, 2013; Bekkouch and Benhamza, 2016).

Sampling

Sampling was carried out in February 2021. Water and sediment samples were collected using a disinfected dipper. The recovered samples were transferred into sterile bottles of 50 mL. Two liters of spring water were also collected from the same point and filtered in the laboratory. This water was used for physicochemical measurements and for the preparation of isolation media. The samples were transported in an isothermal bag and stored at 4°C.

Measurement of physicochemical characteristics of samples

In situ measurements of water and ambient air temperature were performed using a total immersion rod thermometer with mercury (-10 °C to 150 °C). At laboratory, both water and sediment samples were tested for pH values using a CRISON model GLP 21 pH meter. Total Dissolved Solids (TDS) and conductivity were measured by Jenway 430 pH/conductivity system. All measurements were done in duplicates.

Isolation

To induce the growth of heterotrophic aerobic thermophilic bacteria, two methods were adopted in order to vary the initial concentrations of microbial cells. For water samples, we performed a surface spreading of 1 mL of decimal dilutions (10^{-1}; 10^{-2}; 10^{-3}) on two different culture media (C1: 17.5 g/L dehydrated PCA medium; 10 g/L starch; 20 g/L agar; and C2: 30 g/L agar; 10 g/L CMC; 4 g/L peptone; 4 g/L yeast extract) (Atlas, 2005). In the case of sediments, we inoculated 9 mL of enrichment broth (C3: 5 g/L glucose; 4 g/L peptone; 4 g/L yeast extract) with 1 g of sediments followed by incubation for 18h at 60°C. Then, 0.5 mL of the enrichment broth was spread on the surface of C1 and C2 media. All Petri dishes were incubated at 60 °C for 18 to 38 hours.

Enumeration

After incubation, strain enumeration was performed by using a colony counter for quantitative determination of bacterial populations. The standard plate count method for determining the number of bacteria described by Harley (2002) was adopted.

Purification

The first isolates were selected and purified. Thus, 2 to 3 colonies were selected from each Petri dish based on their macroscopic aspects and then subcultured at least 2 times on the same isolation medium either on the C4 preservation medium (17.5 g/L dehydrated PCA; 20 g/L agar) using depletion streaks technique. Incubation was done at the isolation temperature and the dishes were covered by plastic bags to avoid desiccation of the culture medium (Abdollahi *et al.*, 2021).

Morphological characterization of isolates

Macroscopic characterization

The macroscopic characters of the colonies (size, shape, opacity, consistency, pigmentation) were visually appreciated. Strains were cultured on C4 medium and observed for different morphological aspects after 18 to 38 h incubation at 60 °C.

Microscopic characterization

Microscopic characters of the isolates were determined by Gram and endospore staining following the protocols of Gram (1884) and Schaeffer-Fulton (1930), respectively (Prescott *et al.*, 2003). Observations were made using a Motic type light microscope using an X100 objective under immersion.

Screening of extracellular glycoside hydrolases production

The isolates were screened for extracellular glycoside hydrolases production on a basic medium containing 30 g/L agar; 3 g/L peptone; 2 g/L yeast extract; 2 g/L NaCl; 1 g/L $CaCl_2$; 1 g/L $MgSO_4$ modified by three different polysaccharides (10 g/L starch, cellulose or pectin). After inoculation on compartmentalized plates, the isolates were incubated at 60 °C for 72 h. All experiments were done in duplicate to confirm results and conducted according to the standard protocols described below.

Cellulose degradation

Basal medium supplemented with 0.5% (w/v) carboxy-methyl cellulose (CMC) (C5) was used to select bacterial strains with cellulolytic or xylanasic activities. After incubation at 60 °C for 72 h, plates were filled with 0.1% (w/v) Congo red solution and set for 30 min at the incubation temperature. Then, they were washed with 1M NaCl solution and kept for 15 min at room temperature to detect the enzymatic activity. Clear zones around growing bacterial colonies indicated cellulose/ xylane hydrolysis and growth without clearing around the colonies was evaluated as negative (Bragger *et al.*, 1989).

Starch degradation

The starch hydrolysis method was used to evaluate the amylase activity of the isolates. Bacteria were cultured in basal medium added with 1% (p/v) soluble starch (C6) and incubated at 60 °C for 72h. after incubation, the medium was covered with a solution of lugol for 30 seconds and the results of discoloration were examined. The appearance of a clear zone around the colonies indicated the presence of amylase activity (Bragger *et al.*, 1989; Logan *et al.*, 2009).

Pectin degradation

C7 medium supplemented with 1% (w/v) pectin was used for selection of pectinase-producing bacterial strains. After incubation at 60 °C for 72h, the plates were flooded with a solution of diode and potassium iodide to detect clear zones. Pectinase production was examined by the appearance of a clear zone around the colonies (positive result) (Anisa *et al.*, 2013).

Phenotypic characterization of isolates

Physiological characterization

The influence of temperature, pH and NaCl concentration on growth were determined on solid medium by varying one of the parameters while the other two ones were held constant. Indeed, all the isolates were incubated at different temperatures (0-80 °C), different pH levels (4.0-9.0) and variable NaCl concentrations (up to 5% w/v). Growth was periodically supervised between 24 and 72 hours of incubation. For all tests, the medium was adjusted with a phosphate buffer, shaken and autoclaved for 15 minutes at 105°C before use.

The use of sugars as a unique source of carbon and energy was further tested on a solid culture medium containing NH_4Cl (0.5 g/L), Na_2HPO_4 (0.005 g/L), agar(30 g/L) and the test sugar (10 g/L): D (+)-glucose, D (+)-galactose, D (+)-fructose, D (+)-lactose, D (+)-dextrin, D (+)-sucrose, D (+)- maltose, D (+)-mannose. Determination of respiratory type and use of citrate were also performed according to Harley (2002) protocols.

Biochemical characterization

The isolated strains were subjected to different biochemical tests. Respiratory enzymes tests (Cytochrome oxidase and Catalase) and search for tryptophanase were performed according to Joffin and Leyral (2006). Methyl red reaction (MR), Voges Proskauer reaction (VP), the use of sugars on Triple Sugar Iron (TSI) medium and fermentation of sugars on liquid medium were performed according to Harley (2002). Finally, nitrate reduction was implemented following the protocol mentioned in Bergey's Manuel of Systematics (Logan *et al.*, 2009).

Determination of amylolytic activity

The strains that showed a positive result in the examination of amylase production were selected. The assay is performed using the protocol cited by Bernfeld (1955), and adapted by Mamo and Gessesse (1997) and Ait Kaki- El Hadef El Okki (2017).

The preculture was prepared by inoculating 3 mL of enrichment broth (5 g/L glucose; 4 g/L peptone; 4 g/L yeast extract; 2 g/L NaCl; pH 7.0 ± 0.2) with the selected strains and then incubated at 60 °C for 24 hours. After that, enzyme production was run in 20 mL of production medium containing 10 g/L soluble starch; 4 g/L peptone; 2 g/L yeast extract; 2 g/L glucose; 2 g/L NaCl; 2g/L Na_2HPO_4; 0.1 g/L $CaCl_2$ and 0.1 g/L $MgSO_4$ (pH 7.0 ± 0.2) and incubated for 72 hours at 60 °C with continuous shaking. The production medium was after that centrifuged at 4000 g for 15 minutes. The supernatant was collected and considered as the enzyme extract. Later, 0.5 mL of this extract was added with 0.5 mL of substrate solution (1% soluble starch solution buffered at pH 7.0) and incubated at 60 °C for 15 min. The reaction was stopped by adding 1 mL of 3,5-dinitrosalicylic acid (DNS) reagent. The reaction mixture was then subjected to heat for 5 min at 100°C, then allowed to cool in ice before dilution with distilled water (10 mL). Hence, the absorbance was measured by using UV-visible spectrophotometer at 540 nm against the blank.

The enzymatic activity was determined based on a calibration curve established with glucose concentrations ranging from 0 to 2 mg/mL. One enzyme unit is considered as the amount of enzyme that catalyzes the production of 1 mg of glucose per minute at 60°C.

All the tests were performed in triplicates.

Numerical taxonomy

The relationship based on the phenotypic traits of the strains were studied by constructing a phenogram using NTSYS-pc software version 2.02i. Numerical analysis of the data was performed after coding the results of 73 phenotypic traits: 1 for "positive or present", 0 for "negative or absent". The degree of similarity between the strains in the study was assessed by calculating the Simple Matching (SM) index (Sokal, 1958). The SM similarity matrix was used for the grouping of isolates by the UPGMA (Unweighted Pair Group Method with Arithmetic mean) classification method. In this method, relationships were identified in the order of their similarity and the construction of the tree representing these links was done step by step thanks to this order (Perrière and Brochier-Armanet, 2010).

Figure 2. Sampling point of Hammam Salhine (Azzaba)

Results and discussion

Sampling

Four water samples and two sediment samples were taken at the geothermal spring of Hammam Salhine (Fig. 2). At the time of sampling, the spring water was clear and transparent, with no particular odor and no apparent signs of pollution. In addition, the presence of some vegetation and algae around the site was observed.

Measurement of the physico-chemical characteristics of the samples

The physicochemical characteristics and/or climatic parameters of the environment of thermal waters can influence their microbial diversity especially ion concentrations, temperature, pH, and electrical conductivity (Chiriac *et al.*, 2017). The results of physicochemical analyses of water and sediment are shown in table 1. The temperature values of the water and the surrounding air measured at the time of sampling are 45°C and 10°C, respectively. The values recorded for the spring water does not contradict those described by Bekkouche (2009, 2013). According to his studies, the water temperature varied between 41°C and 47°C at 6 sampling points in Hammam Salhine. These waters can therefore be described as mesothermal. Moreover, Bahri *et al.* (2011) measured the water temperature at 36°C. These variations may be due to seasonal fluctuations and measurements at different points.

The pH values of the samples in this study are neutral. It averaged between 7.28 ± 0.00 for the water and 7.49 ± 0.021 for the sediments. The electrical conductivity and TDS of the water have average values of 2.05 dS/m

Table 1. Average values of physico-chemical parameters of water, sediments and surrounding air at the study site

	T (°C)	pH	TDS (mg/L)	Cond (dS/m)
Water	45 ± 0	7.28 ± 0.00	1327 ± 12.72	2.05 ± 0.02
Sediments	-	7.49 ± 0.02	-	-
Ambient air	10 ± 0	-	-	-

T: temperature of the water at emergence or temperature of the surrounding air at the time of sampling; TDS: Total Dissolved Solids; Cond: conductivity; -: not determined.

and 1327 mg/L, respectively. In general, thermal waters in the Azzaba region are all acidic and have medium to high conductivity (Bekkouche, 2013). According to our results, the pH of Hammam Salhine water tends towards neutrality rather than acidity, but has a significant conductivity value. Likewise, to the majority of thermal springs in Algeria, this spring is characterized by a high mineralization with a TDS falling in the range of 0.4 to more than 10 g/L of organic and inorganic substances dissolved in the water (Ouali and Moundji, 2018). Compared to the data provided by Bahri *et al.* (2011), the TDS of our study is significantly lower than the latter (2700 mg/L). Differences in these parameters may be due to seasonal variations (Rodier, 1996).

Isolation, enumeration and purification of isolates

44 colonies were counted from the water and sediment samples on two different culture media (C1 and C2) at 60 °C. After that, 11 isolates were selected based on their morphological variability. Nevertheless, a greater number of isolates originate from sediment samples and C2 allowed a better growth for all isolates but they were less elected because their cultures were all uncountable and sometimes invasive.

Morphological characters of the isolates

The macroscopic and microscopic characters of the selection strains were determined and are presented in table 2.

Macroscopic characters

Macroscopic observation of colonies on C2 or C4 agar revealed two different colony patterns: a) Smooth type colonies: this cultural type is the most common (9 strains), characterized by umbilical or flat colonies; with cream consistency; opaque, translucent or transparent and cream pigmentation. Their size varies between 0.5 mm and 12 mm; b) Mucous type colonies: only two strains have this type of culture (AS1 and AS3). They have flat or umbilicate shape, mucous; transparent and vary in size from 1 to 11 mm.

Microscopic characters

Strains were generally Gram positive, rod-shaped, single or grouped in chains. Endospores were often observed, in central or in terminal position with either deforming or non-deforming cells. Gram negative strains occasionally encountered have probably modified their Gram while forming endospores as it is known that endospore-forming bacilli species can lose their Gram-positive or have variable Gram (Logan *et al.*, 2009).

Production of extracellular glycoside hydrolases on solid media

All 11 isolates were screened for amylolytic, cellulolytic/xylanatic and pectinolytic activity. Data from table 3 revealed that 9 strains were capable of degrading one substrate or more. Indeed, carboxymethylcellulose (CMC) was hydrolyzed by 7 strains which means that they possess a cellulase

Table 2. Macroscopic and microscopic characters of the strains

Strain	Morphological characters						Microscopic characters					
	Size (mm)	Aspect	Shape	Opacity	Consistency	Pigmentation	Gram	Cell shape	Cell arrangement	Endospore	Cell deformation	Endospore position
AS1	1-10	Mucus	Plate	Transparent	Mucus	Transparent	+	Rod	In chain	Non-observed	Non-observed	Non-observed
AS2	1-12	Smooth	Plate	Translucent	Creamy	Cream	+	Rod	In chain	+	Non-deforming	Terminal
AS3	1-11	Mucus	Umbilical	Transparent	Mucus	Transparent	-	Rod	Single	+	Non-deforming	Central
AS4	0,5-5	Smooth	Plate	Opaque	Creamy	Cream	+	Rod	In chain	+	Non-deforming	Terminal
AS5	1-4	Smooth	Plate	Opaque	Creamy	Cream	+	Rod	In chain	+	deforming	Terminal
AS6	1	Smooth	Plate	Opaque	Creamy	Cream	+	Rod	In chain	Non-observed	Non-observed	Non-observed
AS7	1-5	Smooth	Umbilical	Translucent	Creamy	Cream	-	Rod	Single	+	Non-deforming	Terminal
AS8	0,5-7	Smooth	Umbilical	Translucent	Creamy	Cream	+	Rod	Single	Non-observed	Non-observed	Non-observed
AS9	1-9	Smooth	mbilical	Translucent	Creamy	Cream	+	Rod	Single	+	Non-deforming	Terminal
AS10	0.5-8	Smooth	Umbilical	Translucent	Creamy	Cream	+	Rod	Single	+	Non-deforming	Terminal
AS11	1-4	Smooth	Umbilical	Translucent	Creamy	Cream	+	Rod	In chain	+	Non-deforming	Terminal

Table 3. Results of the screening for extracellular glycoside hydrolase activities

Strain	CMC	Starch	Pectin
AS1	+	+	+
AS2	+	+	-
AS3	+	-	+
AS4	+	+	+
AS5	+	+	+
AS6	-	+	+
AS7	-	+	+
AS8	-	-	-
AS9	-	-	-
AS10	+	-	-
AS11	+	-	-

CMC : carboxymethylcellulose

and probably a xylanase since these two substrates are usually found intermixed in nature (Bajaj and Mahajan, 2019). Starch and pectin were hydrolyzed by 6 strains each and thus, they produce amylase and pectinase enzymes. Moreover, three isolates co-degrade the three substrates. While four strains co-degrade two different substrates and two strains degrade only one substrate (CMC). The presence of combined hydrolytic activities in thermophiles has been reported in numerous studies conducted in geographically separated warm ecosystems (Gomri, 2012; Bouacem, 2016; Benkahoul *et al.*, 2017; Benammar *et al.*, 2020; Saeed *et al.*, 2021; Uluçay, 2021). Results of glycoside hydrolases activity screening are shown in figure 3.

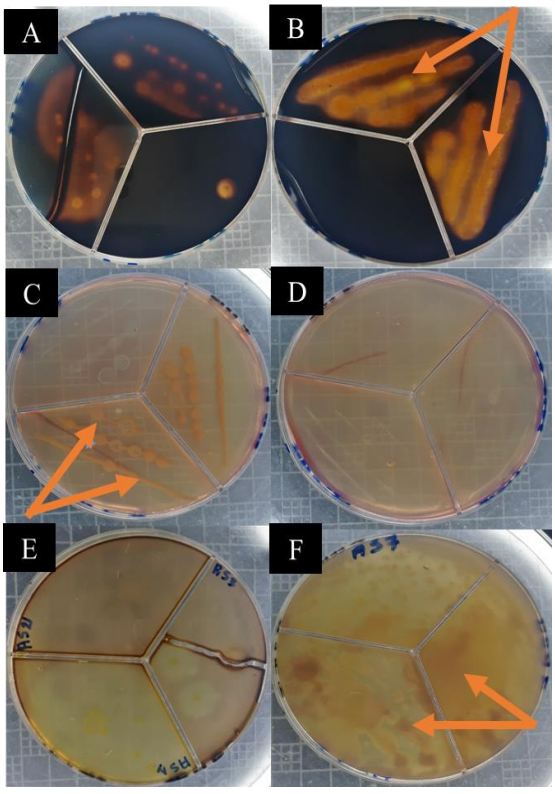

Figure 3. Examples of detected hydrolytic activities
A. Starch hydrolysis by strains AS1, AS2, AS3; B. Hydrolysis of starch by strains AS9, AS10, AS11; C. Hydrolysis of pectin by strains AS7, AS9, AS11; D Hydrolysis of pectin by strains AS8, AS10; E. Hydrolysis of CMC by strains AS1, AS2, AS3; F. Hydrolysis of CMC by strains AS4, AS5,AS7. The arrows indicate the lysis zones.

Physiological characters

Tolerance to temperature, pH and salinity

Growth was observed in the majority of strains at 70°C after 24 hours' incubation and at 50°C after 48 hours' incubation. In general, strains grow in the temperature range 50 °C to 70 °C. Therefore, it is likely that they are moderate thermophiles (Ito and Ino, 2013).
Almost all the strains can grow in a pH range between 5 and 9, but all grow optimally at pH 7. The strains in the study can also grow in the absence of NaCl and are unable to grow at 5% (w/v) NaCl. Hence, the majority of them grow at 1% (w/v) NaCl and tolerate concentration up to 3% (w/v) NaCl. therefore, strains of the present study fall within the range of non-tolerant or mild halophiles.
The strains also show a remarkable range of growth temperatures, between 30 °C and 70 °C and pH values from 5 to 9. This means that these strains are possibly polyextremophiles, *i.e.* thermoacidophiles or thermo-alkaliphiles, and that they exhibit molecular adaptations that allow them to exist in these harsh environments (Egamberdieva *et al.*, 2018; Ghosh *et al.*, 2020; Salwan and Sharma, 2020). Results of growth limiting conditions are presented in table 4.

Use of sugars as unique source of carbon

From table 5, it is evident that the majority of the strains can use one or several sugars as a unique source of carbon. Thus, some similarities and differences in substrate specificity among strains were observed. In fact, this variable capability to use different carbon substrates depends on the specific enzymes baggage, central metabolic pathways and specific transporters that allow the assimilation of substrates found in their environment (Rampelotto, 2016). Likewise, citrate was not used by any strain and manifestly do not possess the enzyme responsible for its assimilation (citrate permease) (Joffin and Leyal, 2006).

Respiratory type

Hot springs harbor both aerobic and anaerobic thermophilic bacteria (Counts *et al.*, 2017). In this study, the majority of isolates are strict aerobes. Only one strain is facultatively anaerobic (AS7).
A multitude of studies conducted in different countries around the world (Turkey, Egypt, Indonesia, Algeria, etc.) have been carried out on natural hot springs and the researchers were able to isolate bacteria with different respiratory types, strict aerobic, facultative aerobes, and anaerobics (Bouanane-Darenfed *et al.*, 2011; Bouacem *et al.*, 2014; Gomri *et al.*, 2018; Oztas and Arzu, 2020; Saeed *et al.*, 2021; Uluçay, 2021). In some cases, the geochemistry of the thermal environments studied may modulate the dominant metabolism (Egamberdieva *et al.*, 2018).

Table 4. Growth of strains at different temperatures, pH and NaCl concentrations

Strain	T (°C)							pH					NaCl % (p/v)				
	10	30	37	50	60	70	80	5	6	7	8	9	0	1	2	3	5
AS1	-	+	+	+	+	+	-	+	+	+	+	+	+	-	-	-	-
AS2	-	+	+	+	+	+	-	-	-	+	-	-	+	-	-	-	-
AS3	-	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-	-
AS4	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
AS5	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-
AS6	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
AS7	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-
AS8	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-
AS9	-	+	+	+	+	-	-	+	+	+	+	+	+	+	-	-	-
AS10	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-
AS11	-	-	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-

Table 5. Physiological characters of isolates (sugar utilization, citrate and respiratory type)

Strain	Glu	Gal	Fruc	Lac	Dex	Suc	Mal	Man	Cit	Respiratory type
AS1	-	-	-	-	+	-	+	-	-	Strictly aerobic
AS2	-	-	-	-	+	-	-	-	-	Strictly aerobic
AS3	-	-	-	-	+	-	-	-	-	Strictly aerobic
AS4	-	+	-	+	+	+	+	+	-	Strictly aerobic
AS5	-	-	-	+	+	+	+	+	-	Strictly aerobic
AS6	+	+	-	-	+	+	+	+	-	Strictly aerobic
AS7	-	-	-	-	+	+	-	+	-	Facultative anaerobic
AS8	-	+	-	+	+	+	-	+	-	Strictly aerobic
AS9	-	+	-	-	+	-	-	+	-	Strictly aerobic
AS10	-	+	-	+	+	+	+	+	-	Strictly aerobic
AS11	-	+	-	-	+	-	-	+	-	Strictly aerobic

Biochemical characters

All results of the biochemical characters of the isolates are listed in table 6. All the bacterial strains were able to produce catalase and/or oxidase. Most aerobic microorganisms possess these enzymes including thermophilic bacteria (Counts *et al.*, 2017). Accordingly, almost all bacteria in this study are strict aerobes. Moreover, they reacted negatively to RM, VP, TSI and sugar fermentation tests in most cases which means that these bacteria harbor aerobic metabolism (Gomri *et al.*, 2018). All strains were indole negative and therefore, do not possess the enzyme tryptophanase. Some strains produce nitrate reductase at the NO_2- stage, others produce nitrate reductase at the nitrogen stage.

Quantification of amylase production

Five isolates with a higher level of extracellular amylase activity were selected for enzyme production quantification assay. The results of the assay are shown in figure 4. Overall, all strains showed significant amylolytic activity ranging from 6.099±1.759 to 10.204U±2.285. However, AS7 presented the highest activity. Compared to similar studies (Taha *et al.*, 2020; Msarah *et al.*, 2020), the strains in our study might be considered highly amylase producing and are potentially exploitable for industrial applications especially strain AS7.

Numerical taxonomy

The relationship between the 11 strains was analyzed by the construction of a phenogram by UPGMA

Table 6. Results of biochemical characters of isolates

Stra	Sac/lac*	Glu*	*H_2S*	Gaz*	Nit	Oxi	Cat	Try	RM	VP	Glu	Fru	Gal	Suc	Lac	Mal	Man	Dex
AS1	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	+	-
AS2	-	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-
AS3	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+
AS4	-	-	-	-	-	+	+	-	-	+	+	+	-	-	+	+	-	-
AS5	-	-	-	-	+	+	+	-	-	-	+	+	-	-	-	+	-	+
AS6	-	-	-	-	+	+	+	-	-	-	+	-	-	-	-	+	-	-
AS7	-	-	-	-	-	-	+	-	-	-	+	-	-	+	-	+	-	+
AS8	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-
AS9	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-
AS10	-	-	-	-	+	+	+	-	-	+	+	-	-	+	-	-	+	-
AS11	-	-	-	-	+	+	-	-	-	-	+	-	+	-	-	-	-	+

Nit: nitrate, Oxi: oxidase, Cat: catalase, Try : tryptophane, RM : rouge de methyl, VP : Vogues Prausker, Glu: glucose, Gal:galactose, Fru: fructose, Lac: lactose, Dex : dextrin, Suc : sucrose, Mal : maltose, Man : Mannose.

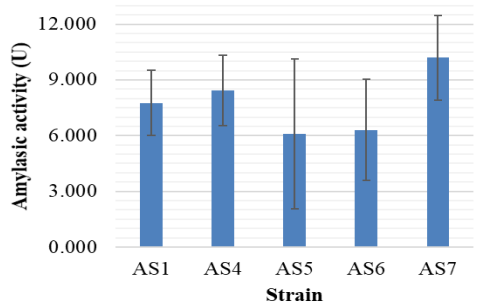

Figure 4. Results of the amylolytic activity determination

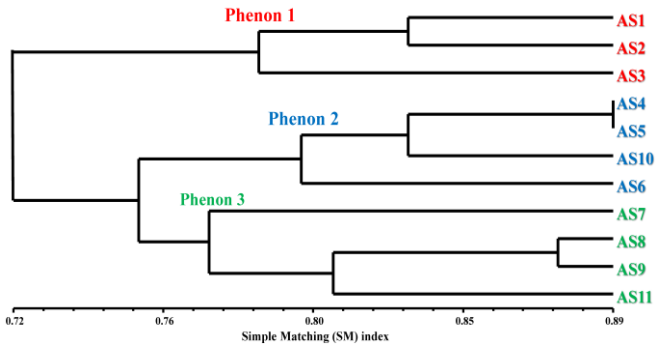

Figure 5. Phenotypic tree based on morphological, physiological and biochemical characters of strains isolated from the thermal spring of Hammam Salhine

method (fig. 5). This tree is based on the calculation of the Simple Matching Index including 73 phenotypic characters (morphological, physiological and biochemical) of the isolates.

In general, the clustering of the strains indicates a relatively low phenotypic variability. Indeed, three main phenons are clustered. They are formed at similarity percentages between 72% and 89%. The most taxonomically related strains are grouped under the same phenon. The strains of the first cluster are grouped at 79% similarity, this same cluster is linked to the second cluster (80% similarity) which is linked to the last phenon at 77% similarity. These taxonomic groups share similar phenotypic characters such as endospore formation, Gram, and a low physiological and biochemical variability: thermophilic, aerobic, neutrophilic with a wide pH range and non-halotolerant besides their glycoside hydrolases profile.

Conclusion

The present study's intended objectives described at the early parts of the article are perceived to be met. Actually, we were able to isolate thermophilic bacteria from water and sediment samples collected from the hot spring of Hammam Salhine, located in the wilaya of Skikda, and to characterize them phenotypically (morphologically, microscopically, physiologically and biochemically). The study of the physico-chemical characteristics of the thermal water the Hammam showed that the spring had a pH tending towards neutrality rather than acidity with a high mineralization.

A total of 11 bacterial strains were purified from the collected samples. These strains were then phenotyped and found to be rod-shaped, Gram-positive with the presence and/or absence of endospores. Usually they were moderate thermophiles, they possess a cytochrome oxidase and a catalase, and are strict aerobic or facultative anaerobic. They are physiologically and biochemically different, but share some characters, such as the ability to grow in the absence of NaCl, to grow at temperatures between 30 and 70°C and have a wide pH range (between 5 and 9) in most cases. They do not demand growth factors and their use of energy, carbon and/or nitrogen sources is variable. Some of them are able to ferment several sugars. However, all the isolated strains are unable to produce indole or gases by fermentation. Some strains are positive for methyl red and nitrate to nitrite reactions, but all are negative for Vosges-Proskauer reactions. Moreover, strains were screened for amylolytic, cellulolytic/xylanasic and pectinolytic activities on the same isolation medium supplemented with soluble starch, carboxymethylcellulose and pectin, respectively. The results from the test revealed that the majority of strains showed a remarkable hydrolytic activity with a predominance of cellulotic activity followed by amylolytic and pectinolytic activity. Some of the strains presented multiple activities at the same time. Additionally, quantification of the amylolytic activity showed high values in all tested strains notably the train AS7.

This work is a first step of a work that could be continued by performing several complementary tests in the short and medium term, including a more advanced polyphasic identification of the isolated strains, by combining the phenotypic approach (morphological, physiological, biochemical and chemotaxonomic, etc.) with the molecular approach (sequencing of molecular markers, phylogenetic and genomic study, etc.); the study of the properties of the enzymes of the selected strains, in particular the amylases of the AS7 strain, which must be completed in order to determine the profitability of their exploitation; further optimization of growth and enzyme production parameters on economically viable culture media; and the constitution of an enzyme library and a gallery of extremophilic strains, isolated, identified and indexed from the different extreme environments of the Algerian territory with the aim of exploiting them in industrial processes of transformation in agro-food as well as in other industrial and analytical fields.

Acknowledgments

We would like to thank Dr. Amel Ait-Kaki from the laboratory of research on Microbiological Engineering and Applications (University of Frères Mentouri Constantine 1, UFMC1) and Dr. Djamel-Eddine Mekhancha from the laboratory of research ALNUTS (UFMC1 and University of Salah Boubnider Constantine 3) for their technical assistance.

References

Abdel-Azeem, A.M., El-Mansy, S.A., Nahas, H.A.H.A., Mousa, M.K., Gandal, H.E., Hamdy, S.E., ..., Abdel-Azeem, M.A. 2020. Thermophilic Chaetomium in Biotechnology. *In* Recent Developments on Genus Chaetomium (pp. 421-439). Springer, Cham.

Abdollahi, P., Ghane, M., Babaeekhou, L. 2021. Isolation and Characterization of Thermophilic Bacteria from Gavmesh Goli Hot Spring in Sabalan Geothermal Field, Iran: *Thermomonas hydrothermalis* and *Bacillus altitudinis* isolates as a potential source of thermostable protease. *Geomicrobiology Journal*, 38(1), 87–95. https://doi.org/10.1080/01490451.2020.1812774

Adjeroud, M., Escuder-Rodríguez, J.-J., González-Siso, M.-I., Kecha, M. 2020. Metagenomic investigation of bacterial and archaeal diversity of Hammam Essalihine hot metagenomic investigation of bacterial and archaeal diversity of Hammam. *Geomicrobiology Journal*, 37(9), 804–817.https://doi.org/10.1080/01490451.2020.1783035

Ait Kaki - El Hadef El Okki, A. 2017. L'α-Amylase d'une nouvelle souche de *Rhizopus oryzae* FSIS4 isolée de blé des zones arides. Thèse de doctorat. Université Frères Mentouri Constantine 1. 174p.

Anisa, S.K., Ashwini, S., Girish, K. 2013. Isolation and screening of *Aspergillus spp.* for pectinolytic activity. *Electronic Journal of Biology*, 9(2), 37–41.

Atlas, R.M. 2005. Handbook of media for environmental microbiology; sec. Ed. Taylor.

Bahri, F., Saibi, H., Cherchali, M.E.H. 2011. Characterization, classification, and determination of drinkability of some Algerian thermal waters. *Arabian Journal of Geosciences*, 4(1–2), 207–219. https://doi.org/10.1007/s12517-010-0127-0

Bajaj, P., Mahajan, R. 2019. Cellulase and xylanase synergism in industrial biotechnology. *Applied Microbiology and Biotechnology*, 103(21–22), 8711–8724. https://doi.org/10.1007/s00253-019-10146-0

Bekkouch, M.F., Benhamza, M. 2016. Hydrochemical characteristics of the thermal sources in the extreme northeastern part of Algeria. *AIP Conference Proceedings*, 1758. https://doi.org/10.1063/1.4959431

Bekkouche, M.F. 2009. Caractéristiques des sources thermales de la région d'Azzaba. Thèse de Doctorat. Université de Annaba-Badji Mokhtar.

Bekkouche, M.F. 2013. Caractéristiques des sources thermales de la région d'Azzaba. Séminaire International sur l'Hydrogéologie et l'Environnement. 5–8.

Benammar, L., İnan Bektaş, K., Menasria, T., Beldüz, A.O., Güler, H.I., Bedaida, I.K., Gonzalez, J.M., Ayachi, A. 2020. Diversity and enzymatic potential of thermophilic bacteria associated with terrestrial hot springs in Algeria. *Brazilian Journal of Microbiology*, 51(4), 1987–2007. https://doi.org/10.1007/s42770-020-00376-0

Benkahoul, M., Talhi, A., Boulefkhad, N. 2017. Bactéries des environnements chauds Algériens : Isolement et mise en évidence de la production d'hydrolases. Université Frères Mentouri Constantine, 1, 25–35.

Bernfeld, P. 1955. Amylases, alpha and beta. *Methods in enzymology*, 1, 149-158.

Bouacem, K. 2016. Caractérisation de souches bactériennes isolées à partir de sources thermales du Nord-Algérien "étude des propriétés enzymatiques". Thèse de Doctorat. Université des sciences de la Technologie, Houari Boumedien, 248 p.

Bouacem, K., Bouanane-Darenfed, A., Boucherba, N., Joseph, M., Gagaoua, M., Ben Hania, W., Kecha, M., Benallaoua, S., Hacène, H., Ollivier, B., Fardeau, M.L. 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), 1969–81. https://doi.org/10.1007/s12010-014-1153-2

Bouanane-Darenfed, A., Fardeau, M.L., Grégoire, P., Joseph, M., Kebbouche-Gana, S., Benayad, T., Hacene, H., Cayol, J.L., Ollivier, B. 2011. *Caldicoprobacter algeriensis* sp. nov. a new thermophilic anaerobic, xylanolytic bacterium isolated from an Algerian hot spring. *Current Microbiology*, 62(3), 826–832. https://doi.org/10.1007/s00284-010-9789-9

Bragger J.M., Daniel R.M., Coolbear T., Morgan H.W. 1989. Very stable enzymes from extremely thermophilic archae bacteria and eubacteria. *Appl. Microbiol Appl. Microbiol. and Biotech.*, 31, 556–561.

Chiriac, C.M., Szekeres, E., Rudi, K., Baricz, A., Hegedus, A., Coman, C. 2017. Differences in temperature and water chemistry shape distinct diversity patterns in thermophilic microbial communities. August. https://doi.org/10.1128/AEM.01363-17

Counts, J.A., Zeldes, B.M., Lee, L.L., Straub, C.T., Adams, M.W.W., Kelly, R.M. 2017. Physiological, metabolic and biotechnological features of extremely thermophilic microorganisms. 2. https://doi.org/10.1002/wsbm.1377

Egamberdieva, D., Birkeland, N.K., Panosyan, H., Li, W.J. 2018. Extremophiles in Eurasian Ecosystems: Ecology, Diversity. Springer.

Fekraoui, A., Kedaid, F.-Z. 2005. Geothermal resources and uses in Algeria: A country update report. Proceedings world geothermal congress-Antalya, Turkey, 24-29 April 2005.

Ghosh, S., Lepcha, K., Basak, A., Mahanty, A.K. 2020. Thermophiles and thermophilic hydrolases. In Physiological and Biotechnological Aspects of Extremophiles. INC. https://doi.org/10.1016/b978-0-12-818322-9.00016-2

Gomri, M.A. 2012. Screening d'activités hydrolytiques extracellulaires chez des souches bactériennes aérobies thermophiles isolées à partir de sources thermales terrestres de l'Est algérien. Mémoire de Magistère Université Frères Mentouri Constantine 1, 137 p.

Gomri, M.A., Khaldi, T.M., Kharroub, K. 2018. Analysis of the diversity of aerobic, thermophilic endospore-forming bacteria in two Algerian hot springs using cultural and non-cultural methods. *Annals of Microbiology*, 68, 915–929. https://doi.org/10.1007/s13213-018-1401-8

Harley, J., Harley, J. 2002. Laboratory exercises in microbiology. The McGraw− Hill.

Itoh, T., Iino, T. 2013. Phylogeny and biological features of thermophiles. In Thermophilic Microbes in Environmental and Industrial Biotechnology: Springer, p. 249-270. https://doi.org/10.1007/978-94-007-5899-5_9

Joffin J.N., Leyral G. 2006. Microbiologie Technique : Dictionnaire de techniques. 4e Ed. Espagne : Centre régional de documentation pédagogique d'Aquitaine. 363 p.

Kambourova, M. 2018. Thermostable enzymes and polysaccharides produced by thermophilic bacteria isolated from Bulgarian hot springs. *Engineering in Life Sciences*, 18(11), 758–767. https://doi.org/10.1002/elsc.201800022

Krüger, A., Schäfers, C., Schröder, C., Antranikian, G. 2018. Towards a sustainable biobased industry – Highlighting

the impact of extremophiles. *New Biotechnology*, 40, 144–153. https://doi.org/10.1016/j.nbt.2017.05.002

Kumar, S., Dangi, A. K., Shukla, P., Baishya, D., Khare, S. K. 2019. Bioresource Technology Thermozymes: Adaptive strategies and tools for their biotechnological applications. *Bioresource Technology*, 278, 372–382. https://doi.org/10.1016/j.biortech.2019.01.088

Lebbihiat, N., Atia, A., Arıcı, M., Meneceur, N. 2021. Geothermal energy use in Algeria: A review on the current status compared to the worldwide, utilization opportunities and countermeasures. *Journal of Cleaner Production*, 302, 126950. https://doi.org/10.1016/j.jclepro.2021.126950

Logan, N. A., Berge, O., Bishop, A. H., Busse, H. J., De Vos, P., Fritze, D., Heyndrickx, M., Kämpfer, P., Rabinovitch, L., Salkinoja-Salonen, M. S., Seldin, L., Ventosa, A. 2009. Proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria. *International Journal of Systematic and Evolutionary Microbiology*, 59(8),2114–2121. https://doi.org/10.1099/ijs.0.013649-0

Mamo, G., Gessesse, A. 1997. Thermostable amylase production by immobilized thermophilic *Bacillus sp*. *Biotechnology Techniques*, 11(6), 447-450.

Mehta, D., Satyanarayana, T. 2013. Diversity of hot environments and thermophilic microbes. In *Thermophilic microbes in environmental and industrial biotechnology*, pp. 3-60. Springer, Dordrecht. https://doi.org/10.1007/978-94-007-5899-5_1

Msarah, M. J., Firdose, A., Ibrahim, I., Aqma, W.S. 2020. Isolation and characterization of thermophilic *Bacillus licheniformis* sungc2 as producer of α-amylase from Malaysian hot spring. *Pertanika Journal of Science and Technology*, 28(special issue 2), 121–134. https://doi.org/10.47836/pjst.28.S2.10

Ouali, S., Moundji, H.M. 2018. Cartographie et caractérisation des ressources géothermiques de l ' Algérie Cartographie et caractérisation des ressources géothermiques de l'Algérie. *Revue Des Energies Renouvelables*, 21(January), 54–61.

Oztas, E., Arzu, G. 2020. Identification and Characterization of Novel Thermophilic Bacteria from Hot Springs, Erzurum , Turkey. *Current Microbiology*, 77(6), 979–987. https://doi.org/10.1007/s00284-020-01880-0

Perrière, G., Brochier-Armanet, C. 2010. *Concepts et méthodes en phylogénie moléculaire*. Springer.

Prescott, L. M., Harley, J. P., Klein, D. A., Bacq-Calberg, C., Dusart, J. 2003. Microbiologie. 2e Ed. *Bruxelles : De Boeck*, 1139.

Rampelotto, P.H. 2016. Grand Challenges in Biology and Biotechnology (Titles in this series). http://www.springer.com/series/13485?detailsPage=titles

Rodier, J, Bazin, C, Broutin, JP, Chambon, P, Champseur, H, Rodi, L. 1996. L'analyse de l'eau : Eaux naturelles, eau résiduaire, eau de mer. Eighth ed. Dunod, Paris.

Saeed, A.M., El-shatoury, E.H., Sayed, H.A.E. 2021. Statistical factorial designs for optimum production of thermostable α-amylase by the degradative bacterium *Parageobacillus thermoglucosidasius* Pharon1 isolated from Sinai, Egypt. 4.

Salwan, R., Sharma, V. 2020. Overview of extremophiles. In Physiological and Biotechnological Aspects of Extremophiles. Academic Press. pp. 3-11.

Shrivastava, S. 2020. Industrial Applications of Glycoside Hydrolases. In Industrial Applications of Glycoside Hydrolases. https://doi.org/10.1007/978-981-15-4767-6

Sokal, R.R. 1958. A statistical method for evaluating systematic relationships. *Univ. Kansas, Sci. Bull., 38*, 1409-1438.

Taha, G.H., El-Shatoury, E.H., Tolba, S.T., Ibrahim, M.K. 2020. Potential enzyme activity of thermophilic bacteria from hot spring in Egypt. *African Journal of Biological Sciences*, 16(1), 197-206.

Uluçay, O. 2021. Identification, Characterization and Hydrolase Producing Performance of Thermophilic Bacteria: Geothermal Hot Springs in Eastern and Southeastern Anatolia Region of Turkey.

Urbieta, M.S., Donati, E.R., Chan, K., Shahar, S., Sin, L.L., Goh, K.M. 2015. Thermophiles in the genomic era: Biodiversity, science, and applications. Biotechnology Advances, 2014. https://doi.org/10.1016/j.biotechadv.2015.04.007