Vol.64: e21190020, 2021
https://doi.org/10.1590/1678-4324-2021190020
ISSN 1678-4324 Online Edition

Review - Agriculture, Agribusiness and Biotechnology

Screening and Characterization of *Bacillus* Strains Producing Highly Thermostable Amylase from Various Hot Springs of Algeria

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Editor-in-Chief: Paulo Vitor Farago
Associate Editor: Luiz Gustavo Lacerda

Received: 2019.01.14; Accepted: 2020.11.15.

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HIGHLIGHTS

- Screening extremophile *Bacillus* strains from various Hot Springs
- Characterization Of *Bacillus* Strains Producing Highly Thermostable Amylase
- Genetic identification of the best strains

Abstract: Currently thermostable Amylase represents a broad biotechnological interest and desired by a various industries. In this study, forty-six bacterial strains belonging to the genus Bacillus were isolated from various hot springs in the North West of Algeria based on their ability to degrade starch and produce amylase in Starch Agar medium. The majority of isolates showed a positive amylolytic activity. In order to select the most thermostables amylase the effect of temperature on enzymes was estimated, therefore the study of amylase thermostability was culminated by the selection of Four Strains having an interesting optimum of activity and range of stability, reaching 75 °C for the strains HBH1-2, HBH1-3, HBH3-1and 85 °C for the strain HC-2, This indicates that the Enzyme produced by retained strains have optimum activity at high temperature. The identity of the selected strains was established on the basis of the morphological, biochemical characteristics and phylogenetic position as determined by 16S Ribosomal DNA gene sequencing. The whole strains belonged to the genus Bacillus and their phylogeny were also reported in this study.

Keywords: amylase; Bacillus; hot springs; thermostability;16S rDNA gene.

INTRODUCTION

Amylase, is an extremely important industrial enzyme that amounts to around a quarter of the enzyme market [1], and has almost completely replaced chemical hydrolysis of starch in various processing industries [2]. Amylases have an Enzyme Commission number of 3.2.1.1 and symbolize enzymes that catalyses the hydrolysis of internal α-1,4-glycosidic linkages in starch to low molecular weight products, such glucose, maltose and maltotriose units [3,4].It is currently being used in the sugar, animal nutrition, leather, paper and pulp, textile, detergents, baking, brewing, and distilling industries; production of cakes and starch syrups; preparation of digestive aids; and pharmaceutical industries [1,5].

Amylase can be obtained from several sources, such as plants, animals and microorganisms [6]. The major advantages of using microorganisms for production of Amylases are in economical bulk production capacity and microbes are also easy to manipulate to obtain enzyme with desired characteristics [7].

Using ordinary enzyme as a catalyst in chemical reactions has been very cost ineffective, due to its low stability at high temperatures and need of additional enzyme. However, with the use of thermostable enzymes, not only we can expect reduction of cost, but also other merits [8].Thermostable enzymes are more versatile than thermolabile [9], as they have higher operational stability and a longer shelf life at elevated temperatures [10].

Nowadays thermostable Amylases are gaining wide industrial and biotechnological interest due to the fact that their enzymes are better suited for harsh industrial processes [11,12,13]. With the availability of thermostable enzymes a number of new possibilities for industrial processes have emerged. Thermostable Amylase, which have been isolated mainly from thermophilic organisms, have found a number of commercial applications because of their overall inherent stability [14] .Therefore, there is a need to screen the microorganisms for the production of thermostable amylase, which can be used in a wide array of industrial processes.

Members of the genus *Bacillus* are heterogeneous and they are very versatile in their adaptability to the environment. They are the source of enzyme genes used in the production of the majority of the microbial industrial enzymes produced. Amylases have been purified earlier from various *Bacillus* species such as *Bacillus megaterium* [15], from *Bacillus subtilis* [16] and from *Bacillus licheniformis* SPT 27 [17]. *Bacillus stearothermophilus* and *Bacillus licheniformes* amylases are well characterized and heavily used in the starch processing industry [8]. Since thermostability is an important factor in the use of amylolytic enzymes in starch processing, amylases from thermophilic and hyperthermophilic bacteria are of special interest as a source of novel thermostable enzymes [18].

The Algerian north is a region characterized by the presence of a multitude of hotsprings and geothermal stations showing a rich biodiversity which remains unexplored microbiologically. That's why it's interesting to explore these special niches and screen new thermophilic bacteria and new thermostable amylase. The present study describes the screening of new thermophilic *Bacillus* strains then testing them for the amylolytic activity and the selection of the thermostable amylases produced by the isolated strains followed by a molecular identification by 16s rDNA gene sequencing.

MATERIAL AND METHODS

Sampling

View the ubiquity of bacteria belonging to the genus *Bacillus* and in order to isolate *Bacillus* amylolytic strains elaborating thermostable amylase, different samples were collected aseptically from various hot springs and geothermal stations in the North of Algeria as illustrated in the Table 1. From each sampling site temperature and pH were measured then 50 mL of water was collected in sterile Bags and transported at 4 °C to the laboratory.

Table 1. Description of sampling sites and characteristics of geothermal water

Origin of Samples	Codification	Temperature/pH	Number of isolated Strains
Geothermal station of Boughrara In Tlemcen, Algeria	*HBG*	55 °C/ 8.18	7
Chiguer hot spring in Maghnia Algeria	HC	57 °C/ 8.02	6
Hot Spring of Sidi aiede in Ain Temouchente, Algeria	HS	65 °C/ 7.9	9
Hot spring Of Bouhanifia in Mascara Algeria	HB	65 °C/ 8.41	7
Hot spring of Bouhjar in Ain Temouchente, Algeria	HBH	75 °C/ 8.12	6
Geothermal station of *Ouarsenis in Tissemsilt*	HO	55 °C/ 7.89	11

The physicochemical proprieties of water's hot springs vary from geothermal station to another which can increase the biodiversity of isolated strains and also theirs amylases.

Microorganism Isolation and Characterization

In order to isolate a Bacterial amylolytic strains belonging to the genus *Bacillus*, a serial dilutions up to 10^{-5} was made from each sample and the last dilutions have been subjected to heat shock 10 minutes at 80 °C in order to eliminate the vegetative cells [19]. A volume of 0.1 mL aliquot from each dilution thermally treated was inoculated on starch agar plate and incubated at 37 °C for 72 hours [20]. After incubation the zone of hydrolysis of starch was revealed by flooding the plates with iodine solution. The development of blue color indicated the presence of starch, while the areas around the amylolytic bacteria appeared clear [21].

Before purification and conservation, the appurtenance to the genus *Bacillus* of the colonies showing a positive hydrolysis zone has been rapidly verified by preliminary tests, such as positive Gram straining, presence of spores and a positive Catalase [22].

Selection of the thermostable Amylase

In order to select the most efficient amylolytic strains from all the *Bacillus* bacteria isolated previously the effect of temperature on amylase activity was investigated using the Dinitrosalysilyc Acid method [23],which the principle depend on the estimation of the enzymatic reaction in liquid medium by the measurement of the reducing sugar rate. The experiment was conducted above 75 °C to select enzymes with optimal activity at high temperature.

Inoculum Preparation

Bacterial suspension was prepared for each isolate in nutrient broth and incubated for 24 hours, then theirs optic density were measured at 600nm and adjusted to 0.5 Mc Farland to bring them under the same conditions of Initial biomass and standardize to conditions.

Culture medium inoculation

Erlenmeyer flask containing 100 mL of sterile starch fermentation broth were inoculated with 1% volume/volume of adjusted inoculum of each strain then stirred in agitator at 46 °C with 75 rpm for 72 hours.

Effect of temperature on Amylase activity

After 72 hours of incubation, 2 mL were recuperated from each inoculated Erlenmeyer flask and centrifuged at 4000 rpm for 10 minutes. α-Amylase was assayed by adding 0.5 mL of supernatant to 0.5 ml soluble starch 1% weight/volume in 0.1 M phosphate buffer, pH 7.0 for 30 minutes at different Temperatures ranging from 45 to 95 °C. The reaction was stopped and the reducing sugars determined with dinitrosalicylic acid according to the method of [23]. An enzyme unit is defined as the amount of enzyme releasing 1 mg of glucose equivalents from the substrate per minute [24].

Identification of the efficient strains

Firstly the appurtenance of the isolates to the genus *Bacillus* was done by a phenotypic identification. After the selection of the most efficient strain, the chosen bacteria were identified at the species level with API 50CHB system according to the manufacturer's instructions, Biomerieux, France.

Molecular identification

PCR amplification and 16S rDNA sequencing analyses

Genomic DNA was extracted from the cultures growing on Nutrient Agar for 18 hours using boiling DNA preparation technique [25]. The gene encoding 16S rRNA was amplified by PCR with the 16S bacteria specific primers; 27F(5'AGAGTTTGATCC- TGGCTCAG3'),and the1492R reverse primer (5'-GGTTACCTTGTTACGACTT-3') as described previously [26]. Amplification of DNA was carried out with a Perkin Elmer thermal cycler (PE corporation, USA) under the following conditions: initial denaturation at 94 ºC for 7 minutes; 35 cycles of denaturation at 94 ºC for 1minute, annealing at 53 ºC for 45 secondes, extension at 72 ºC for 45 secondes, and a final extension at 72 ºC for 7 minutes. The amplified PCR product was analyzed on a 1.5% agarose gel colored with Midori green®. Amplified DNA was purified using QIA quick® gel extraction kit and sequenced with (BigDye terminator, Applied Biosystems, UK).

Nucleotide sequences accession numbers

After the assembly and the correction, the nucleotide sequence data of the strains, HBH1-2, HBH1-3, HBH3-1 and HC-2 were submitted to Genbank at NCBI, (http://www.ncbi.nlm.nih.gov/) under the accession numbers: KP779643, KP835199, KP835200 and KP835201 respectively.

Phylogenetic analysis of the strains

In order to determine the identity of the selected strains a homology search was carried out using the basic BLASTN search program at the NCBI Web-site. After that, multiple sequence alignments were performed with CLUSTAL W program [27]. Furthermore MEGA 6.0 software [28] was used to construct interactive phylogenetic trees and estimate evolutionary distances on the basis of the neighbor joining method [29]. With 1000 bootstrap values and Maximum Composite Likelihood (MCL) method to estimate the evolutionary distances between all pairs of sequences [30].

RESULTS

The study of the six samples collected from different hot springs and geothermal stations localized in the North of Algeria was culminated by the isolation of forty six bacterial strains belonging to the genus *Bacillus.* Strains were screened for their potential amylolytic activity and all of them are found to be positive for amylase production on starch agar medium. The isolated amylolytic strains were gram positive, rod shaped, aerobic, catalase positive and spore forming as described in Roger and coauthors [22] and Bergey's manual of determinative bacteriology.

Concerning the study the effect of temperature on enzyme activity and the selection of the most thermostable amylases, the isolated *Bacillus* strains exhibit a good activity at high temperature. We can notice that the optima of amylases are ranging from 60 °C for the amylase of the strain HBG-6 and 85 °C for the amylase of the strain HC-2. The comparative study of yields of amylases of isolates *Bacillus* strains, theirs optima and theirs range of thermostability are presented in Table 2.

Table 2. Some representatives of the isolated amylolytic strains and their thermostability characteristics

Isolated strains	Amylase yield (U*/ml)	Optimum Temperature (°C)	The range of thermostability (°C)
HBH-1-1	0.548	75	70-80
HBH1-2	0.548	75	70 -80
HBH-1-6	0.618	70	65 -75
HBH3-1	0.226	75	70 -80
HBH3-2	0.472	75	70 -80
HBH-3-7	0.591	75	70 -80
HBH-3-5	0.456	75	65 -80
HC-1	0.336	75	65 -75
HC-2	0.444	85	70 -85
HC-5	0.591	70	65 -75
HBG-5	0.473	70	60 -75
HBG-6	0.213	60	55 -70
HBH-2-1	0.635	75	70 -80
HBH-2-2	0.536	70	60 -75

*: One unit (U) of activity as determined by the DNS assay is defined as an average of 1 mg of glucose equivalents released per min in the assay reaction.

From all the isolated amylolytic *Bacillus* and based on high optima of temperature and an interesting range of thermostability we observe that the amylase of the strains HBH1-2 , HBH3-1 HBH3-2 have an optimum of temperature of 75 °C and are stable from 70 °C to 80 °C and the amylase of the strains HC-2 have an optimum temperature of 85 °C and range of thermostability from 70 °C to 85 °C, the effect of the temperature on the activity of amylases of the best four strains are illustrated in the (Figure 1).

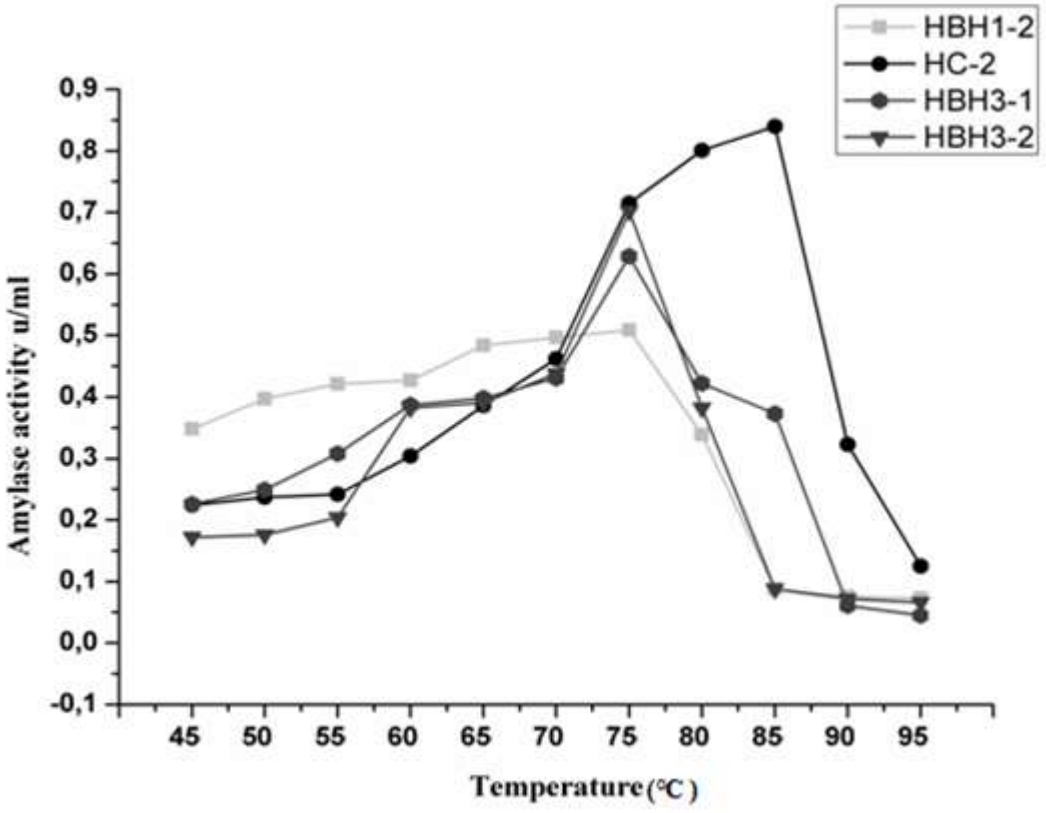

Figure 1. Effect of temperature on amylase activity and stability of the retained strains

Identification of the efficient strains

The identity of the selected strains was established on the basis of the morphological, biochemical characteristics and phylogenetic position as determined by 16S ribosomal DNA gene sequencing.

Molecular identification

The PCR amplification of 16S rDNA genes from the selected strains show that sequences have 1500bp approximately (Figure 2). According to the DNA sequencing of the amplified 16S rDNA genes the identity of the strains was determined and presented in Table 3. Multiple sequence alignments were carried out using ClustalW [27] by comparing with16S rDNA sequences of some related strains which were obtained from the NCBI. A phylogenetic tree was constructed by the neighbor joining method using MEGA 6.0 [28] as illustrated in (Figure 3).

Table 3. Selected strains identification based on the homology comparison analyses of the 16S rDNA sequences

Strain	Accession Number	Product size	Closely Related	Similarity
HBH1-2	KP779643	1412 bp	*B. subtilis*	98%
HBH3-1	KP835199	1418 bp	*B. licheniformis*	98%
HBH1-3	KP835200	1405 bp	*B. sp.H-46*	97%
HC-2	KP835201	1440 bp	*B. sp. hswX58*	97%

16S rDNA sequences amplified with 27f and 1492r primers and sequenced were used to identify the retained strains.

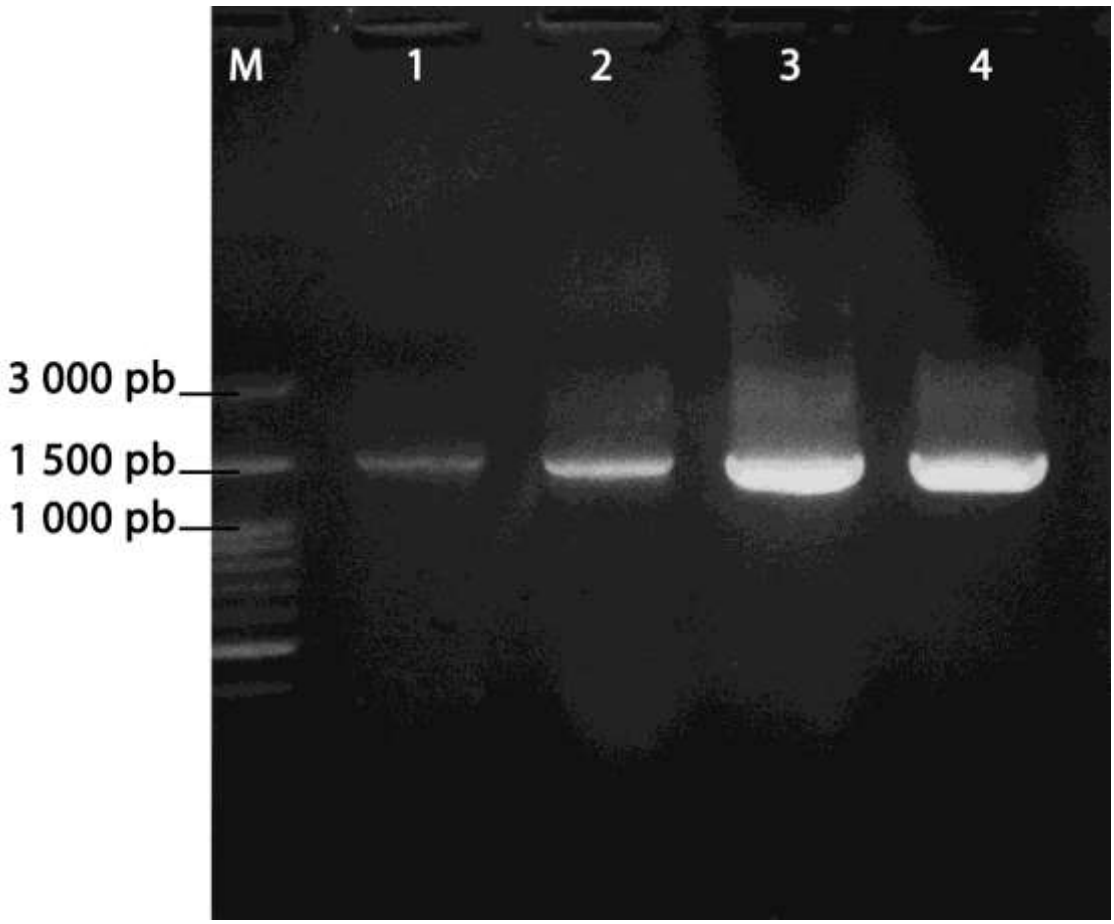

Figure 2. Gel picture of the amplified 16s rDNA genes of the selected strains; lane M: 3kb DNA ladder, lane1:HBH1-2, lane2: HBH1-3, lane 3:HBH3-1, lane 4: HC-2. (1.5% agarose concentration, revealed with Midori green®)

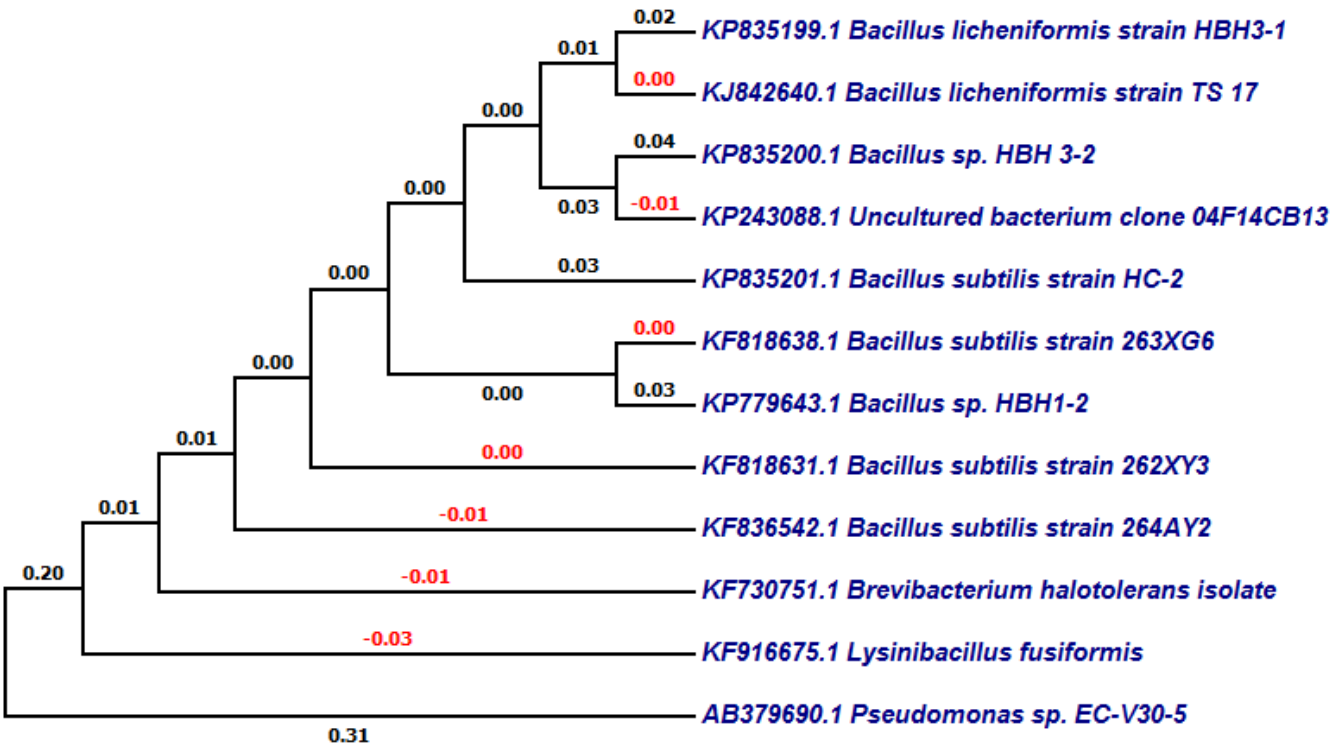

Figure 3. Neighbor-joining evolutionary distance phylogenetic tree based on the 16S rRNA gene sequences of the selected strains HBH1-2, HBH1-3, HBH3-1, HC-2 and some representative members of related genera of the family 'Bacillaceae'. Bootstrap values = 1000 replicates and Bar indicates 0.01 substitutions per nucleotide position.

DISCUSSION

Thermophilic bacterial communities inhabiting the hot springs of Algeria have not been thoroughly studied yet. In this study, the screening of amylolytic *Bacillus* strain from hot springs and geothermal stations were done on starch agar medium, similar method has been used earlier by Ahmed [24] and Wisdom [31] in order to screen the microorganisms for amylase production. Heat shock method was adapted as isolation strategy to eliminate the vegetatives cells and enhance the growth of spore-forming bacteria indeed this technique provide good efficiency for the isolation of Bacterial *Bacillus* strains, this is in concordance with [19].

The exploration of the wide geothermal stations and hot springs in the North of Algeria have been culminated by the isolation of forty six *Bacillus* strains all of them were positive for amylase production according to starch agar plate assay. However, the enzyme activity was different between the strains, as confirmed by DNS method. The production of amylase by *Bacillus* strains has been thoroughly investigated and is affected by a variety of physicochemical factors reported in similar studies such as salinity, pH effect and the influence of inhibitors and different metal ions on purified enzyme activity [32]. Most notable among these are the temperature which seems not to be related to the growth of the organism [4].

In our study a selective step was done to estimate the thermostability of the amylases by testing theirs activity at different temperatures from 45 °C to 95 °C. We Remarque that variations exist in the relationship between the effect of temperature and amylolytic activities of the isolated strains and we have obtained high optima of temperature which approximates 75 °C and a range of stability between 70 °C and 80 °C for the strains HBH1-2, HBH3-1 and HBH3-2. But the highest optimum of temperature is achieved by the amylase of the strain HC-2, 85 °C and a range of stability between 70 °C to 85 °C. Similar results were obtained with *Bacillus licheniformis* with an optimum of 65 °C to 95 °C and *Bacillus subtilis* from 50 °C to 75 °C [8]. This observation shows this isolates could be a potential sources for the production of thermostable amylase for various industries, especially in the food industry for the preparation of maltose syrup and clarification of various drinks. In addition, amylase is generally used in other industries like biorefinery, paper, detergents, textiles and pharmaceuticals [33,34].

The four strains producing the most thermostable amylase were retained for the molecular identification and phylogenetic analysis. The comparison of the 16s rDNA sequences with those in the GenBank database indicated that all of them were belonged to the genus *Bacillus*. The identity of the selected strains and a phylogenetic study were also investigated and reported in this work.

CONCLUSION

Amylases are among the most important enzymes used in industrial processes. With the advent of new frontiers in biotechnology, the spectrum of α-amylase applications has also expanded and to meet the high demands it's very interesting to search for novel sources of this valuable enzymes especially the thermostable. Hot springs of Algeria have not been thoroughly studied yet and we have shown in this work that the newly isolated *Bacillus* strains are potential producers of thermostable amylases and can operate up to 85 °C. This result is promising and confirms the existence of other bacterial strains with high amylolytic activity and desired properties. The newly isolated *Bacillus* strains merit further importance as potential producer of thermostable amylase by the characterization of the enzyme in order to know in which domain it can be applicated.

Acknowledgments: This work was financially supported by The Ministry of Higher Education and Scientific Research of Algeria and the laboratory of applicated microbiology and proteomics.

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