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Screening and characterization of thermo-active enzymes of biotechnological interest produced by thermophilic *Bacillus* isolated from hot springs in Tunisia

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As part of the contribution to the global efforts in research of thermostable enzymes being of industrial interest, we focus on the isolation of thermophilic bacteria from Tunisian hot springs. Among the collection of 161 strains of thermophilic *Bacillus* isolated from different samples of thermal water in Tunisia, 20% are capable of growing at 100°C and the rest grow at 70°C or above. Preliminary activity tests on media supplemented with enzyme-substrates confirmed that 35 strains produced amylases, 37 – proteases, 43 – cellulases, 31 – xylanases and 37 – mannanases. The study of the effect of temperature on enzyme activity led to determination of the optimal temperatures of activities that vary between 60 and 100°C. Several enzymes were active at high temperatures (80, 90 and 100°C) and kept their activity even at 110°C. Several isolated strains producing enzymes with high optimal temperatures of activity were described for the first time in this study. Both strains B62 and B120 are producers of amylase, protease, cellulase, xylanase, and mannanase. The sequencing of 16S DNA identified isolated strains as *Geobacillus kaustophillus*, *Aeribacillus pallidus*, *Geobacillus galactosidasus* and *Geobacillus toebii*.

Key words: temperature, amylase, protease, cellulase, xylanase, mannanase, *Geobacillus,* Tunisian hot springs

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INTRODUCTION

The growing interest in thermophilic microorganisms and biotechnological applications they harbor explains the increasing number of studies of extremophilic microorganisms. These microorganisms produce unique biocatalysts that function under extreme conditions comparable to those prevailing in various industrial processes (Niehaus *et al.*, 1999). The order *Bacillales* includes thermophilic members of the genera *Geobacillus*, *Alicyclobacillus*, *Aneurinibacillus*, *Anoxybacillus*, *Bacillus*, *Brevibacillus*, *Caldalkalibacillus*, *Sulfobacillus*, *Thermobacillus*, *Ureibacillus* and *Vulcanibacillus*. They are responsible for the degradation of organic matter in hot environments. Very often they are acidophilic or alkaliphilic bacteria (Logan & Allan, 2008; Cihan *et al.*, 2011).

Running biotechnological processes at elevated temperature has many advantages. The increase of temperature has a significant influence on the bioavailability and solubility of organic compounds. Therefore, higher reaction rates due to smaller boundary layers are expected (Becker *et al.*, 1997; Niehaus *et al.*, 1999). Consequently, several thermo-active enzymes have been isolated and characterized, like amylases from *Bacillus subtilis* (Junge *et al.*, 1959) and *Bacillus licheniformis* ATCC 9945a (Božić *et al.*, 2011). Thermostable proteases isolated from *Geobacillus caldoproteolyticus* (Chen *et al.*, 2004) or *Bacillus alveayuensis* CAS 5 (Annamalai *et al.*, 2014) are of a great interest in the field of detergents and biodegradation of wastes. Cellulases, xylanases and mannanases are used in the paper industry, juice clarification, bakery industry and even the production of biofuel (Mawadza *et al.*, 2000; He *et al.*, 2008; Bajaj and Manhas, 2012).

Tunisia is a country of a great biological and geological diversity. There are also extreme ecosystems there such as sebkhas, desert soils and especially hot springs exploited for their therapeutic benefits. However, these springs have been little-studied from biodiversity-oriented point of view and it was only recently that we began to focus on the microorganisms that inhabit these local environments. In this study we focused on the screening of extracellular-polymer-degrading enzymes, such as amylases, proteases, cellulases, xylanases and mannanases.

MATERIALS AND METHODS

Sampling and isolation of thermophilic bacteria. The water samples were collected from many localizations distributed throughout the thermal springs of the north and south part of Tunisia. Samples were chosen to vary the nature and location of the sampling. *In situ* measurements of temperature and pH were performed. Isolation of bacteria was performed according Adiguzel and coworkers (Adiguzel *et al.*, 2009) through serial dilution method. 1 ml of spring water was added to 10 ml of sterile distilled water and then mixed by gentle vortexing. Subsequently, the serial dilutions were performed. 100 µl of the mixture was plated on Petri dishes containing TSA and then incubated at 70°C for 24 to 48 hours. Strains which were able to grow were purified by five successive sub-cultures on TSA. Thereafter the second selection of thermophilic bacteria was performed in liquid media at 100°C.

Morphological and biochemical characterizations of isolates. Cell and colony morphology, Gram reactions, the presence of catalase and oxidase were in-

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Abbreviations: TSA, tryptic soy agar; TSB, tryptic soy broth; CMC, carboxy methyl cellulose; LB, Lauria Bertanie; LBG, locust bean gum; TCA, Tri chloro acetic acid; DNS, 3,5-Di nitro salicylic acid

vestigated according to the methods described by Carl Lamanna (Lamanna, 1940).

Screening of the activity of some hydrolases. The purified thermophilic *Bacillus* strains were grown in TSB and incubated at 70°C for 24h. The cultures were used for the various preliminary tests of activity. The screening of the amylase activity was performed according to the method of dissemination through wells on agar starch media containing 1% soluble starch, 0.2% yeast extract, 0.5% peptone, 0.05% $MgSO_4$, 0.05% NaCl, 0.015% $CaCl_2$ and 2% agar at pH 7.0. The presence of amylase activity was confirmed by the appearance of a clear halo around the well after the staining with Lugol (Teodoro & Martins, 2000). Screening of protease activity was performed according to the method of dissemination through wells on milk agar medium at pH 9 containing; 0.5% tryptone, 0.3% yeast extract, 1.5% agar and 25% skimmed milk. Protease activity was confirmed by the appearance of a clear zone around the well indicating degradation of milk casein (Benkiar *et al.*, 2013). Determination of the cellulase activity was performed on a medium containing 0.2% carboxymethylcellulose (CMC) 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.02% peptone, and 2% agar. Cellulase activity was identified as the appearance of a clear halo around the tested strain after treatment with Gram's iodine (Kasana *et al.*, 2008). Testing strains for xylanolytic activity was done on LB agar medium supplemented with 0.5% xylane. The presence of xylanase activity was confirmed by the appearance of a clear zone around the strain tested following the staining with Congo Red (Yang *et al.*, 1995). Identification of strains displaying mannanase activity was performed on the LB solid medium supplemented with 0.5% LBG. After 24 h of incubation at 55°C plates were treated with Congo Red and mannanase activity was confirmed by the appearance of a clear halo around the tested strain (Yin *et al.*, 2012).

Production of the enzymes and assessment of their activity. Strains which had a halo of starch degradation were sub-cultured on production medium containing 1% peptone, 1% NaCl, 0.5% yeast extract and 1% starch at pH 7 and then incubated in a water bath at 70°C, 150 rpm for 96 h. The supernatant obtained after centrifugation at 10000 rpm, 4°C for 20 min was used as crude extract for the amylase activity assay. 500 µl of 1% starch solution prepared in 0.1 M phosphate buffer pH 7 was mixed with 500 µl of enzyme extract. The reaction mixture was incubated for 10 min at 70°C. The reaction was stopped by adding 1 ml of 0.1N HCl, then 100 µl of Lugol was added. One unit of amylase activity was defined as the amount of enzyme that reduces the absorbance of the iodine-starch complex at 620 nm by 1% per minute (Konsoula & Liakopoulou-Kyriakides, 2007).

Production of proteases was carried out at pH 8 in a medium containing 0.5% peptone, 0.5% yeast extract, 0.5% NaCl, 1% gelatin, 0.02% $CaCl_2$ (Sinha and Khare, 2013). Production was performed on a shaker (150 rpm) for 96 h at 70°C then the culture was centrifuged at 12000 rpm for 20 min at 4°C and the cell-free supernatant was used as a crude extract for estimation of protease activity.

Proteolytic activity was determined by using casein as substrate. One ml of 1% casein in 50 mM glycine-NaOH buffer pH 8 was mixed with 900 µl of glycine-NaOH. Reaction was initiated by the addition of 100 µl of enzyme and tubes were placed in a water bath at 70°C. After 20 min of incubation 2 ml of 10% TCA was added to stop the reaction. The reaction mixture was centrifuged at 12000 rpm for 10 min and absorbance was measured at 280 nm (Shrinivas & Naik, 2011). One unit of protease activity was defined as the amount of enzyme required to release 1 µg of tyrosine per min under experimental conditions.

For determination of the cellulase activity, strains were grown on production medium composed of 0.2% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.05% KCl, 0.2% CMC and 0.02% Peptone. pH was adjusted to 7. After 4 days of incubation at 55°C, 150 rpm, the supernatant was recovered by centrifugation at 12000 rpm for 20 min at 4°C. Enzyme extract, 500 µl, was added to 500 µl of 1% CMC solution prepared in 50 mM citrate-phosphate buffer pH 7. The mixture was incubated for 1h at 70°C. Then 3 ml of DNS was added to stop the reaction and the mixture was placed in boiling water for 10 min and after that cooled in water to stabilize the coloration. The absorbance was measured at 550 nm. One unit of activity was defined as the amount of enzyme which liberated 1µmole of glucose per minute. (Assareh *et al.*, 2012).

For xylanase activity testing strains were grown in a production medium containing 0.12% $NaNO_3$, 0.3% KH_2PO_4, 0.6% K_2HPO_4, 0.005% $CaCl_2$, 0.001% $MgSO_4$, 0.0001% ZnSO4 and 1% Xylane (Nascimento *et al.*, 2002) . Production was performed at 55°C for 96 h next the culture was centrifuged at 12000 rpm for 20 min at 4°C. The cell-free supernatant was used as a crude extract for estimation of xylanase activity. Crude extract, 50 µl, the was incubated with 450 µl of 1% xylane solution prepared in 50 mM sodium phosphate buffer pH 7 for 10 min at 70°C. 750 µl of DNS was added to stop the reaction. Tubes were subsequently placed in boiling water for 10 min. Absorbance was measured at 590 nm. One unit of activity was defined as the amount of enzyme required to liberate one micromole of xylose per minute (Breccia *et al.*, 1998).

The production of mannanases was performed on a medium composed of 1% LBG, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.2% $MgSO_4$, 0.2% $(NH_4)_2SO_4$ and 0.03% yeast extract for 96 h at 55°C . Afterward, the supernatant was recovered by centrifugation at 14000 rpm for 30 min at 4°C to be used as crude extract for the assay. The mannanase activity was measured according to the DNS method by determining the amount of reducing sugars released from LBG. 1.8 ml of 0.5% LBG prepared in 50 mM phosphate buffer and 200 µl of enzyme extract were incubated for 30 min at 70°C. 3 ml of DNS was added. The tubes containing the mixtures were incubated in boiling water for 5min. After cooling at room temperature the absorbance was measured at 540 nm. One unit of activity was defined as the amount of enzyme required to liberate one micromole of mannose per minute (Yin *et al.*, 2012).

Determination of protein concentration. The determination of the amount of protein present in each enzymatic extract was performed according to Bradford (Bradford, 1976) using Bovine Serum Albumin as standard.

Characterization of the effect of temperature on the amylase, protease, cellulase, xylanase and mannanase activities. The effect of temperature was determined by incubating the reaction mixtures at different temperatures ranging from 55 to 110°C and determining the relative activity. For each strain the highest activity was considered as 100%. Temperatures above 100°C were reached using an oil bath.

Table 1. Summary table of sample properties, strains and revealed activity.

Hot Springs	Temperature (°C)	PH	Number of strains	Number of active strains				
				Amylase	Protease	Cellulase	Xylanase	Mannanase
Elmahassen	65	7.2	14	5	8	3	4	2
Sidi Bouhlel	72	7.2	9	1	1	3	2	4
Fatnassa	63	7	5	0	1	3	0	0
Souk Lahad	58	7	8	2	2	1	2	2
Hammet Tozeur	60	8	6	0	0	0	1	0
Nafta	60	7.3	7	0	1	3	1	2
Ras el Ain	54	8.1	10	4	4	5	3	3
Legtar	58	7	8	3	2	0	0	0
H.Ennegrez	54	6.8	9	2	1	3	3	3
H.el Atrous	58	6.7	7	1	2	2	3	2
H.Abdelkader	49	6.7	6	1	1	0	0	1
H.Bent Ejdidi	62	6.9	10	1	3	3	3	2
H.Ezzriba	49	6.9	8	1	0	0	0	0
Djbel Ouest	62	7.9	12	3	4	6	2	5
Ain Kelassira	50	6.6	19	5	3	4	3	5
Ain ElAtrous	62	6.7	7	2	1	1	1	1
Ain Sbia	56	7.2	6	1	1	2	1	2
Ain Echfa	49	7.5	10	3	2	4	2	3
Ain Elfakroun	43	6.6	0	0	0	0	0	0
Total			161	35	37	43	31	37

All experiments mentioned above were repeated at least three times, and each value represents the average of three repetitions.

Identification of thermophilic strains. Strains were identified based on the phenotypic characteristics of *Bacillus* genus and phylogenetic analysis of the 16S rDNA sequence. Genomic DNA was extracted as described by Marmur (Marmur, 1961). 16S rRNA gene sequences were amplified using the bacterial universal primers 16SF (5'AGAGTTTGATCCTGGCTCAG3') and 16SR (5'CTACGGCTACCTTGTTACGA3') (Piterina *et al.*, 2010). The following PCR program was used: 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 1.5 min, and a cycle at 72°C for 5 min. The amplified products were purified and the sequence of the 16S rRNA gene was determined by DNA sequencing with Sanger method (ABI 3730xl DNA analyzer). Sequence comparison with the databases was performed using BLAST program through NCBI website. A phylogenetic tree was constructed with MEGA version 6.06 using the neighbor-joining method.

RESULTS AND DISCUSSION

Sampling and isolation of thermophilic bacteria

The samples of thermal waters were collected from several Tunisian regions: Ichkeul, Jebel oust, Zriba, Korbos, Gafsa, Touzeur and Kebelli during January and February 2013. The physicochemical parameters of the samples are summarized in Table 1. The northern Tunisian waters were characterized by high temperature (49–64°C) and acidic pH with a trend towards neutrality (6.6 to 7.5) while the southern Tunisian waters were hotter (42–72°C) and had neutral pH with a trend towards alkalinity (7 to 8.1). In total we managed to collect 40 samples from different regions. From the samples we isolated 161 bacteria that grew at 70°C. 20% were able to grow at 100°C. Among the 32 strains able to grow at 100°C, 21.87% were isolated from thermal Hammam Ichkeul, 18.75% from Korbos and 18.75% from Elmahassen Touzeur. Therefore, the samples from springs located in the vicinity of the sea (Korbos & Ichkeul) presented the highest percentages of thermophilic *Bacillus*. This is consistent with the findings of Seung Seob Baeand coworkers (Bae *et al.*, 2005).

Morphological and biochemical characterizations of isolates

Morphologic investigation showed that all the strains were Gram positive, endospore forming and mobile. The strains showed different types of colonies; large, matt or granular with variable shapes (circular, regular edges or jagged) or colonies with wavy contours and creamy consistency. Biochemically, isolated strains were catalase positive giving variable responses to the test of oxidase (Slepecky & Hemphill, 2006).

Screening of hydrolases activity

Preliminary tests on media enriched with the substrate for each enzyme showed that among the studied collection 35 strains were producers of amylases, 37 – of proteases, 43 – of cellulases (Fig. 1), 31 – of xylanases and 37 – of mannanases (Fig. 2) (Table 1). The diameters of activity halos were different suggesting that some strains displayed higher activity than others. Strains developing

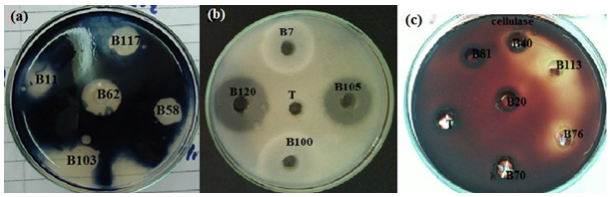

Figure 1. Determination of amylase (a), protease (b) and cellulase (c) activity.
The presence of amylase activity was confirmed by the appearance of a clear halo around the well after the staining with Lugol. The presence of protease activity was confirmed by the appearance of a clear halo around the well indicating degradation of casein milk. Cellulase activity resulted in the appearance of a clear halo around the tested strain after treatment with Gram's iodine.

the largest halos were chosen for the spectrophotometric determination of their activities.

Production of enzymes and assessment of their activities

The spectrophotometric assays at 70°C confirmed the results of preliminary tests and allowed selection of the most active strains. Indeed 47.45% of amylase producing strains had activity greater than 150 U/mg, 40.54% of protease producing strains had activity exceeding 190 U/mg, 30.43% of cellulase producing strains had activity above 80 U/mg, 36% of xylanase producing strains had activity greater than 60 U/mg and 22.22% of mannanolytic strains had activity higher than 40 U/mg.

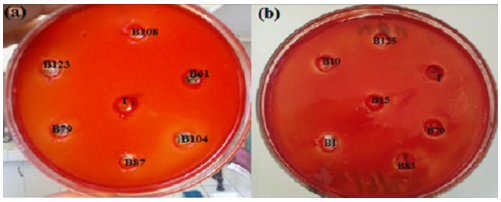

Figure 2. Determination of xylanase (a) and mannanase (b) activity.
The presence of activity was confirmed by the appearance of a clear zone around the tested strain following the staining with Congo Red.

Characterization of the effect of temperature on amylase, protease, cellulase, xylanase and mannanase activity

The study of the effect of temperature enabled us to identify the optimal temperature for each enzyme activity in the range from 55 to 110°C.

Effect of temperature on amylase activity

The strain B77, isolated from Hammam El Atrous Ichkeul had the optimum of activity at 70°C–170.07 U/mg. It kept its activity up to 100°C. These results are in perfect coincidence with those obtained with *Bacillus* sp (Fooladi & Sajjadian, 2010). B62 produced the most active amylase (390U/mg) with the optimal temperature of 90°C. This reminds the results observed in *Bacillus licheniformis* NH1 (Hmidet *et al.*, 2009). B62 and B22 had profiles distinguished by a large plateau which indicated the great resistance of their activity to the temperature. The most prominent output of the study was the successful isolation of a strain (B138) producing amylase with the highest thermoactivity (193.7 U/mg) at 100°C (Fig. 3).

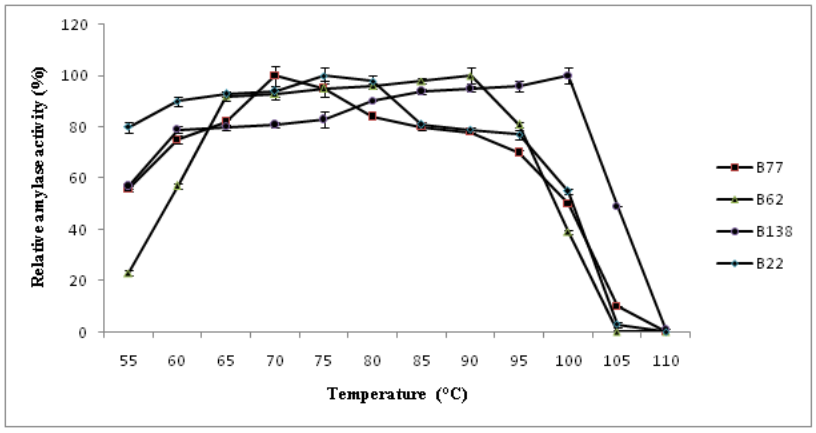

Figure 3. Effect of temperature on amylase activity.
The temperature profiles were determined by assaying amylase activity at temperature between 55 and 110°C. For each strain the highest activity was considered as 100%.

Effect of temperature on protease activity

Proteases from strains B77 and B95 had optimal activity at 100°C (440 U/mg and 127 U/mg respectively). For the first time we isolated strains with the optimum of protease activity at 100°C. The strain B11 produced high activity (590.56 U/mg) at 95°C. This result is consistent with that observed in *Bacillus* sp. MLA64 (Lagzian & Asoodeh, 2012). In fact both strains kept their activities beyond 110°C. The protease from the strain B46 showed optimal activity at 80°C and kept its activity up to 100°C (Fig. 4). This is similar to what was observed for *Bacillus* sp HUTBS62(H. AQEL, 2012).

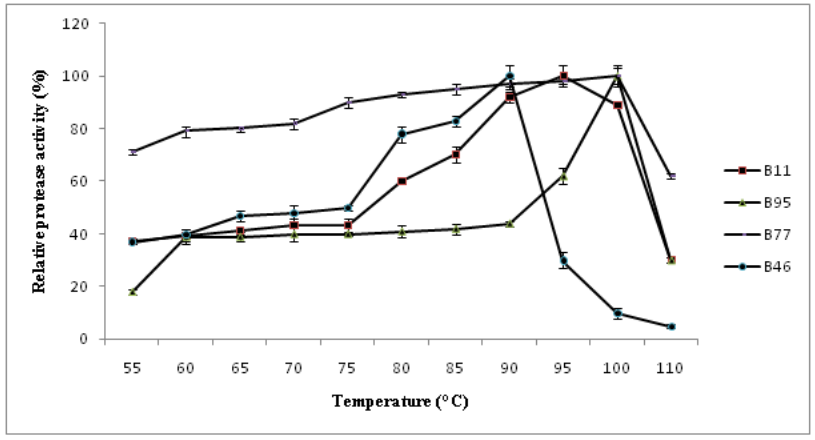

Figure 4. Effect of temperature on protease activity.
The temperature profiles were determined by assaying protease activity at temperature between 55 and 110°C. For each strain the highest activity was considered as100%.

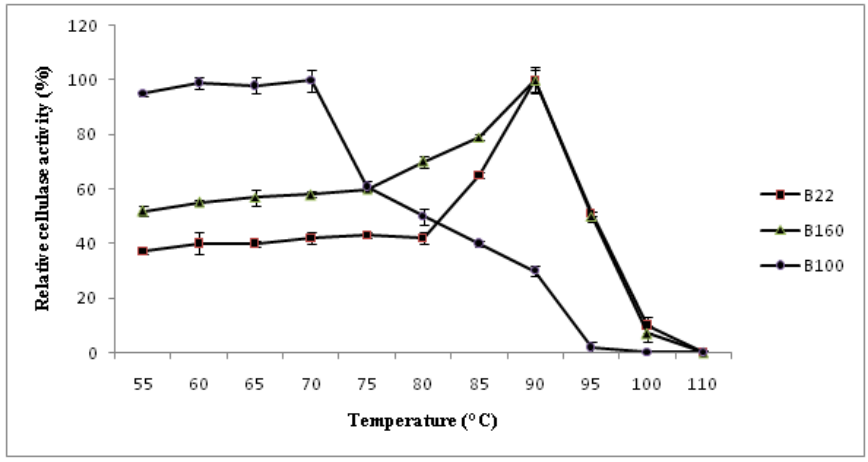

Figure 5. Effect of temperature on cellulase activity.
The temperature profiles were determined by assaying cellulase activity at temperature between 55 and 110°C. For each strain the highest activity was considered as 100%.

Effect of temperature on cellulase activity

Cellulase from B100 showed an almost identical behavior to that of *Geobacillus* sp.T1 (Assareh *et al.*, 2012). Indeed, both had an optimal activity at 70°C and kept their activities at 90°C. Cellulases from strains B22 and B160 had optimal activity at 90°C (161.15U/mg and 138.8U/mg, respectively) confirming their potential usefulness for biotechnological processes occurring in high temperatures (Fig. 5).

Effect of temperature on xylanase activity

Xylanase from the strain B138 had optimal activity of 138.08 U/ml, (specific activity of 211.35 U/mg) at 60°C. The strain *Bacillus licheniformis* P11 (Bajaj & Manhas, 2012) produces a xylanase with the same optimal temperature but B138 had greater activity. The B161 and B98 strains had the optimal activity at 80°C with 147.25 U/mg and 201.99 U/mg respectively. These results are similar to those observed in *Bacillus circulans* AB 16 (Dhillon *et al.*, 2000). The strain B104 is of particular importance with its high xylanase activity (110 U/mg) with the optimum of temperature at 90°C (Fig. 6).

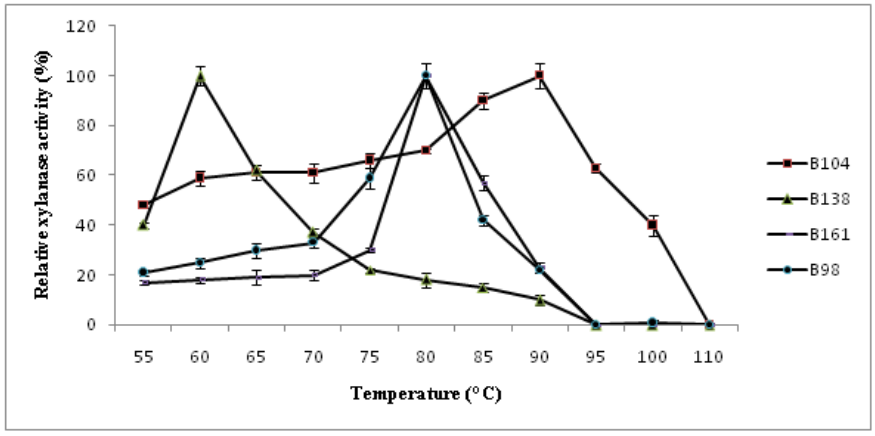

Figure 6. Effect of temperature on xylanase activity.
The temperature profiles were determined by assaying xylanase activity at temperature between 55 and 110°C. For each strain the highest activity was considered as 100%.

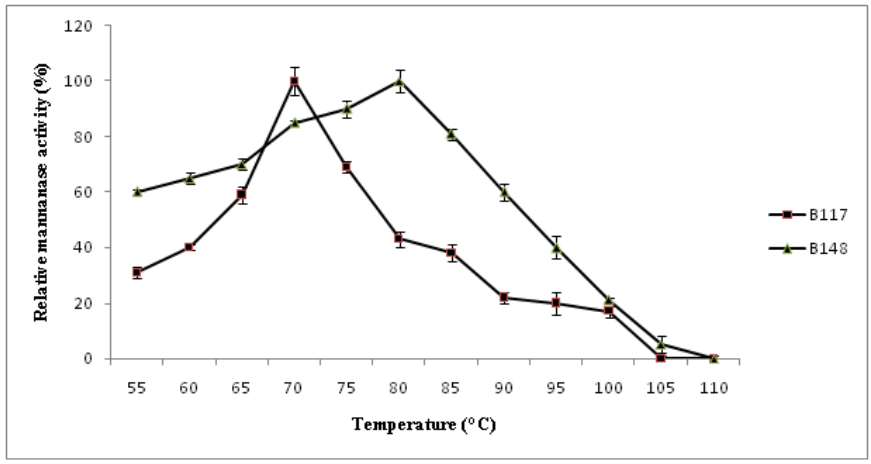

Figure 7. Effect of temperature on mannanase activity.
The temperature profiles were determined by assaying mannanase activity at temperature between 55 and 110°C. For each strain the highest activity was considered as 100%.

Effect of temperature on mannanase activity

The strain B117 produces mannanase with optimal activity of 123.22 U/mg at 70°C and its activity is kept at 100°C. This result was also found in *Bacillus* sp. N 165 (He *et al.*, 2008). B148 produced more thermoactive enzyme, which at 80°C displayed an activity of 135.44 U/mg and was active at 100°C (65.72 U/mg) (Fig. 7).

Some of the isolated thermophilic strains were active on several substrates. 47.87% of the strains were active on at least two substrates.

Eighteen strains were co-producing amylases and proteases activities. For example the strain B77 isolated from Hammam El Atrous Ichkeul, was a co-producer of amylases and proteases activities with optimal activity of amylase (170.07 U/mg) at 70°C and the activity was kept at 100°C. It also had the optimal protease activity (440 U/mg) at 100°C. This result is partly consistent with that reported in *Bacillus licheniformis* NH1 (Hmidet *et al.*, 2009).

Seven strains were co-producing xylanases and cellulases activities. The strain B94 is a

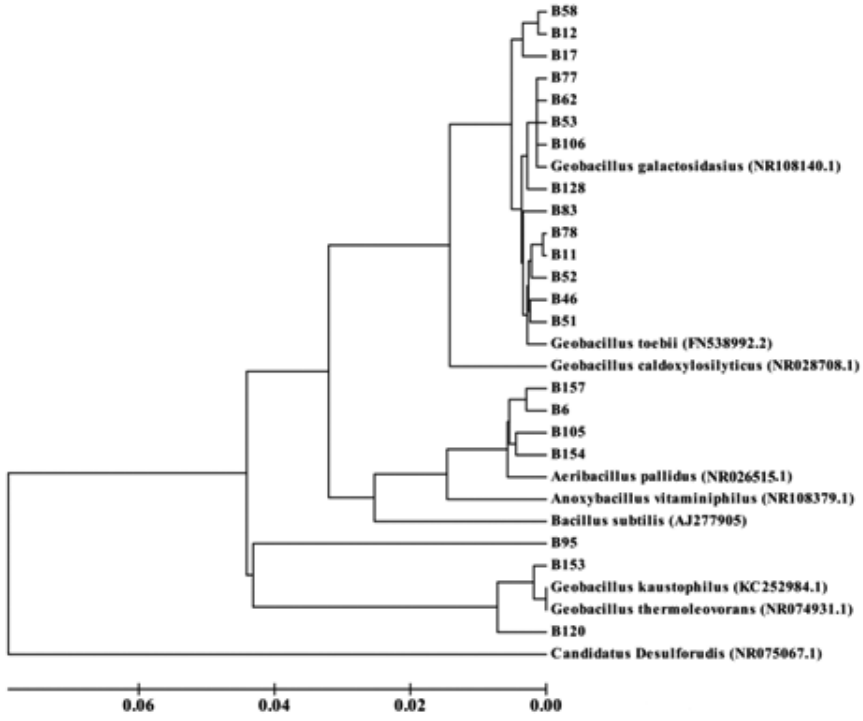

Figure 8. Dendrogram representing estimated phylogenetic relationship on the basis of 16S DNA sequences of thermophilic bacteria isolated from various hot springs in Tunisia.

fine representative of this group, with an optimum of xylanase activity (108 U/mg) at 70°C and its activity kept at 80°C. It also displayed an optimum of cellulase activity (193 U/mg) at 80°C. A similar result was reported in *Aspergillus ustus* (Shamala & Sreekantiah, 1986).

Five strains were co-producing cellulases and mannanases activities. They included the B148 strain postulated as the best example since it was the most active on LBG (135.44 U/mg) with the optimal activity at 80°C in addition to its high cellulase activity (128 U/mg) at 70°C. This reminds the results achieved with *Thermomyces lanuginosus* (Puchart *et al*., 1999).

Four strains were co-producing xylanases and mannanases activities. The strain B95 is a good example as it produced xylanase and mannanase, both active at 60°C with optimal activities of 95.21 and 105.11 U/mg respectively. In addition, it kept their activities at 90°C. Co-production of xylanase and mannanase has been reported for *Trichoderma atroviride* (Kovacs *et al*., 2009).

The two strains: B62 and B120 isolated from Hammam Ennegrez and Ain Sbia respectively were producing all five activities. This result shows the great importance of these two strains as enzyme reservoirs with great biotechnological potential. To our knowledge no similar result was described in the literature.

Identification of thermophilic strains

Phylogenetic analysis of 16S DNA sequences by the neighbor-joining method allowed construction of the phylogenetic tree. Phylogenetic tree enable to claim that the strains belong to five major groups (Fig. 8).

Group A contained two strains B153 and B120 related to *Geobacillus kaustophillus* (KC252984.1) with respectively 98% and 94% homology.

Group B contained two clusters. The first had 97% homology to *Aeribacillus pallidus* (NR026515.1) and composed of strains B157 and B6. The second displayed 96% homology with *Aeribacillus pallidus* (NR026515.1) (Rainey *et al*., 1994).

Group C contained a cluster composed of the strains B53, B62, B77, B106 with 98% homology to *Geobacillus galactosidasus* (NR_108140.1) (Poli *et al*., 2011).

Group D was the widest as it included 4 strains and 3 clusters linking with *Geobacillus toebii* (FN538992.2). Cluster 1 composed of the strains B51 and B46 exhibiting 97.4% homology. Cluster 2 consisted of the strains B58 and B12 with 98% homology. Cluster 3 consisted of the strains B78 and B11 showing 99.5% homology. the strains B17, B52, B83 and B128 had 96%, 97.5%, 96.5% and 97% homology with *Geobacillus toebii* (FN538992.2)(Coorevits *et al*., 2012).

The strain B95 showed no significant homology with any sequence from the NCBI database. It showed 57.5 % homology with *Candidatus desulforudis* (NR_075067.1)(Chivian *et al*., 2008). This suggests the possibility that the strain was described for the first time.

Conflict of interest

No conflict of interest declared.

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