

Characterization of Thermophilic Bacteria Isolated from Two Hot Springs in Jazan, Saudi Arabia

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The capability of thermophilic microorganisms to flourish at high temperatures makes their enzyme systems ideal for various biotechnological applications. Based on the proteolytic and amylolytic activities, two thermophilic bacteria were isolated from hot springs in Jazan, Saudi Arabia. The antibiotic and heavy metals susceptibility patterns of the bacterial isolates were performed. According to the metabolic fingerprint, the bacterial isolates were identified as *Brevibacterium linens* and *Bacillus subtilis*. Moreover, the impact of temperature, substrate concentration, and some metal ions on the production of proteases and amylases from the bacterial strains was investigated. The maximum protease production was achieved at 50°C while the greatest amylase production was recorded at 30°C for both strains at a constant pH of 7.5. The highest enzyme production was recorded at 5% skimmed milk for protease of *B. linens* and 10% for *B. subtilis* while 0.6% starch was the optimum substrate concentration of amylase production of the two strains. Furthermore, Ca²⁺ showed a stimulative influence on protease production from the two strains whereas Mg²⁺ and Mn²⁺ demonstrated minor effect. On the other hand, Ca²⁺, Mg²⁺ and Mn²⁺ demonstrated a positive effect on the amylase production from both strains.

Keywords: Thermophiles, Hot springs, Hydrolytic enzymes, Antibiotics, Heavy metals.

Bacteria are ubiquitous and highly diverse prokaryotes that can survive in adverse habitats. Most of bacteria present in the environment are still unexplored and hence remain obscure for their ecological functions. Thermophilic bacteria are microbes that mostly inhabit hot springs, live and survive in temperatures between 45 and 122 °C. Thermophiles including bacterial and archaeal species are found in various geothermally heated regions of the earth such as hot springs and deep-sea hydrothermal vents. In Saudi Arabia, there are about ten geothermal springs with varying deep temperatures of and different flow rates. They are distributed in Gizan and Al-Lith areas. Of these,

Ain Khulab, Ain Khulab Quwa, Ain Mijara Quwa, Ain ad Damad, Ain al Wagrah and Ain al Wagrah Dam are located in Gizan meanwhile Ain al Harra, Ain Jumah, Ain Markub, and Ain ad Darakah are located in Al-Lith area^{1,2}. Hot springs in Gizan are of volcanic origin and used as places for bathing and recreation by citizens and tourists.

Microbial communities inhabiting aquatic environments vary according to the physiochemical parameters including temperature, salinity, pH and nutrient loads. Extremophiles are microbes that can live and reproduce in harsh environments. It is well known that the enzymes of an organism are adapted to function optimally at or near its growth conditions, and so the range of extremes at which life is found defines the range of conditions at which enzyme activity might be detected^{3,4}. The phenotypic and genotypic characterization

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of thermophilic bacteria has been done for many geothermal areas in different parts of the world including Turkey⁵, Italy⁶, Bulgaria⁷, Greece⁸, China⁹, India¹⁰, and Morocco¹¹.

Several reports on the isolation of thermophilic bacteria from hot springs in Saudi Arabia have been published. In 2012, Khiyami and coauthors¹² have identified about 15 thermo-aerobic bacteria from Jazan and Al-Lith geothermal springs where *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus thermoamylovorans*, *Pseudomonas aeruginosa* and *Enterobacter sp.* were the dominant strains. Another study was conducted with Khalil (2011) who isolated thirteen thermophilic bacteria from hot springs in Saudi Arabia. Based on the biochemical characterization, all the isolates were lipase positive, 11 isolates showed amylase activity, while only 3 of them showed cellulase activity³.

It is possible to consider thermophiles as sources of industrially relevant thermostable enzymes. Many industrial processes require enzymes such as amylase, cellulase, xylanase, pectinase, protease, and lipase that are operationally stable at high temperatures^{13,14}. Such these enzymes are usually produced by Gram-positive bacteria and fungi. These organisms secrete enzymes directly into the fermentation broth which offers major advantages in their downstream processing. Gram-positive *Bacillus* species are among the bacterial champions in enzyme production. Proteases are a group of enzymes that perform protein catabolism by hydrolysis of peptide bond. They are among the most important class of industrial enzymes that have various applications including detergent, leather preparation, peptide synthesis, textile industry, food industry, pharmaceutical industry as well as in bioremediation process^{15,16,17}. Thermostable proteases are of greater advantage in applications because they remain active at high temperatures. *Bacillus spp.* of hot-springs has gained commercial significance as source of thermostable enzymes¹⁸. Amylases are one of the main enzymes used in industry. Their wide range of application in nutritional, detergent, alcohol, textile, food, paper, cosmetic and pharmaceutical industries increases their significance. Amylases are characterized by their ability to hydrolyze starch to generate glucose, maltose, a mixture of malto-oligosaccharides, and various α -limit dextrin-containing α (1-6) bonds¹⁹. Microorganisms are the predominant source

of amylase production due to optimum growth requirements, accessibility, efficient, ecofriendly, and cost-effective as compared to other resources such as animal and plant²⁰.

Due to the ability to produce thermostable enzymes, thermophilic microorganisms have gained great magnitude in pharmaceutical and industrial applications. The aim of the present study is the characterization of some thermophilic bacteria isolated from hot springs in Jazan, KSA in addition to the investigation of the optimum conditions for amylases and proteases production.

MATERIALS AND METHODS

Samples collection and isolation of bacteria

The hot springs chosen for the present study were Bani Malik and Al Khawbah. The first is located in Bani Malik mountains at a distance of about 150 km northeast of Jazan city (43° 042 E, 17° 332 N) while the second is located in Al Harth governorate at a distance of about 50 km southeast of Jazan city (43° 152 E, 16° 562 N). Water samples were collected from Bani Malik hot spring and Al-Khobah hot spring, Jazan, KSA. Samples were assembled in 500 mL glass screw cap bottles and brought to the laboratory. Serial dilution was made and 100 μ L of each dilution was inoculated into Nutrient Agar plates in triplicates. After the incubation of NA plates at 37 °C for 24 hrs the total bacterial count was recorded as CFU mL⁻¹ as described earlier²¹. The developed colonies were repeatedly streaked on NA plates for the isolation of pure cultures. The purified colonies were transferred into NA slants and kept in refrigerator for further study.

Identification of the bacterial isolates

The bacterial isolates were Gram stained and observed under a high power magnifying lens in light microscope. Then the bacterial isolates were identified using GEN III MicroPlate™ test panel of the Biolog System (Biolog, Inc., Hayward, USA). The test panel provides a “Phenotypic Fingerprint” of the microorganism which can then be used to identify them to a species level. The GEN III MicroPlates™ enable testing of Gram-negative and Gram-positive bacteria in the same test panel. The test panel contains 71 carbon sources and 23 chemical sensitivity assays. This test analyzes the ability of the cell to metabolize all major classes

of compounds, in addition to determining other important physiological properties such as pH, salt and lactic acid tolerance, reducing power, and chemical sensitivity. Fresh overnight cultures of the isolates were tested as recommended by the manufacturer. The bacterial suspensions were prepared by removing bacterial colonies from the plate surface with a sterile cotton swab and agitating it in 5 mL of 0.85% saline. 150 μ L of the suspension was dispensed into each well of a Biolog GEN III microplate. The plates were read in the MicroStation semi-automated reader after 20 h and results interpreted by the identification system's software (GEN III database, version 5.2.1).

Characterization of the bacterial isolates

Antibiotics susceptibility test

A single bacterial colony was picked using a sterile loop and streaked on the nutrient agar plate. A filter paper disk saturated with the following antibiotics (Mast Diagnostics, Bootle, UK); amikacin (30 μ g), gentamicin (10 μ g), cefepime (30 μ g), ticarcillin (75 μ g), piperacillin (100 μ g), imipenem (10 μ g), colistin (10 μ g), rifampicin (5 μ g), penicillin G (10 units μ g), erythromycin (15 μ g), cephalothin (30 μ g), clindamycin (2 μ g), cotrimoxazole (25 μ g) and ampicillin (10 μ g) were dispensed onto the media plate separately. Using a sterilized forceps, each disc was placed on the nutrient agar medium to ensure that the disc are attached and fixed on the agar. Nutrient agar medium containing antibiotic discs were incubated overnight at 35 °C²².

Heavy metals susceptibility test

Analytical grade salts of CdCl₂, CuSO₄, HgCl₂, AgNO₃, Pb(NO₃)₂, ZnSO₄ and K₂Cr₂O₇ (Merck) were used to prepare 0.1M stock solutions. Metal solutions were sterilized by using 0.45 μ m pore-size polysulfone sterile filters (Whatman). Susceptibility tests were conducted using well diffusion method according to Essa *et al.* (2005)²³. The metal solutions of Cr, Ag, Cd, and Hg were used at the concentrations 25, 50 and 100 mg/l while metal solutions of Pb, Cu and Zn were used at the concentrations 50, 100 and 150 mg/L. To each NA plate, 0.5 mL of metal solution was added in a central well of 1 cm in diameter and 4 mm in depth. Plates were then incubated for 24 hrs at 37 °C to allow diffusion of the metal into the agar. Then the plates were inoculated in radial

streaks with the tested bacterial isolates. Plates were incubated at 37 °C for 48 h. After incubation, the area of inhibition zone (mm) was millimeter²⁴.

Screening of bacterial isolates for protease and amylase production

Skim milk nutrient agar media were used for protease qualitative screening for several colonies using streaking method¹⁵. Colony forming transparent zone due to the partial hydrolysis of milk casein was selected. Regarding the screening of amylase, starch agar plates were inoculated with the bacterial isolates and incubated at 37 °C for 48 hours. Then the Petri dishes were flooded with iodine solution. The clear zone surrounding the colony was measured in millimeter²⁵. The bacterial isolates K2 (isolated from Bani Malik hot spring) and K10 (isolated from Al-Khobah hot spring) were chosen for study based on their elevated proteolytic and amylolytic activities.

Factors affecting the production of protease and amylase from thermophilic bacteria

Effect of temperature on both of protease and amylase production was studied at different temperatures (30, 37 and 50 °C). Also, the effect of substrate concentration on enzymes production was studied. For protease production; the skim milk substrate concentrations 5, 10 and 20% were added to nutrient agar. For amylase production; the starch substrate concentrations were 0.2, 0.4 and 0.6%. The effect of trace elements on both enzymes production was studied. Calcium, magnesium and manganese trace elements were used at concentrations 20, 40, 80 mg/L.

RESULTS

Bacteriological analysis of water samples

The total bacterial count of water samples from Bani Malek and Al-khoba hot springs was carried out. The bacterial count was 2000 CFU mL⁻¹ in Bani Malek sample and 300 CFU mL⁻¹ in Alkhoba sample. Based on the proteolytic and amylolytic activities, two bacterial isolates with maximum potential of starch and protein degradation were chosen; K2 from Bani Malek hot spring and K10 from Al-khoba hot spring.

Antibiotics and heavy metals resistance profile

The obtained results (Table 1 and Figure 1) showed the antibiotic resistance pattern of the two bacterial isolates. The first isolate (K10) recorded

a clear sensitivity against all the tested antibiotics with different degrees. Meanwhile the other isolate (K2) demonstrated resistance against ticarcillin, colistin, penicillin, cephalothin, clindamycin and ampicillin. Regarding heavy metals resistance profile, data shown in Table (2) clarified that both

Table 1. Physiological profile of the bacterial strains (K2 & K10) isolated from Bani Malek and Al-khoba hot springs, Jazan, KSA using Biolog System (Biolog, Inc., Hayward, USA)

Properties	K2	K10	Properties	K2	K10
Dextrin	-	+	Glycyl-L-Proline	-	+
D-Maltose	+	+	L-Alanine	-	+
D-Trehalose	-	+	L-Arginine	-	+
D-Cellobiose	-	+	L-Aspartic Acid	-	+/-
Gentiobiose	-	+	L-Glutamic Acid	-	+
Sucrose	-	+	L-Histidine	-	+
D-Turanose	-	+	L-Pyroglutamic Acid	-	-
Stachyose	-	-	L-Serine	-	+/-
pH 6	+	+	Lincomycin	-	-
pH 5	-	+	Guanidine HCl	+	-
D-Raffinose	-	-	Niaproof 4	-	-
D-Lactose	-	-	Pectin	+/-	+
D-Melibiose	-	+	D-Galacturonic Acid	-	-
β -Methyl-D-Glucoside	-	+	L-Galactonic Acid Lactone	-	+
D-Salicin	-	+	D-Gluconic Acid	-	+
N-Acetyl-d-Glucosamine	-	+	D-Glucuronic Acid	-	-
N-Acetyl- β -d-Mannosamine	-	-	Glucuronamide	+/-	-
N-Acetyl-d-Galactosamine	-	-	Mucic Acid	-	+
N-Acetyl Neuraminic Acid	-	-	Quinic Acid	+/-	-
1% NaCl	+	+	D-Saccharic Acid	-	+
4% NaCl	+	+	Vancomycin	-	-
8% NaCl	ND	+	Tetrazolium Violet	-	-
α -d-Glucose	-	+	Tetrazolium Blue	-	-
D-Mannose	-	+	p-HydroxyPhenylacetic Acid	+	-
D-Fructose	+	+	Methyl Pyruvate	-	+/-
D-Galactose	-	-	D-Lactic Acid Methyl Ester	-	-
3-Methyl Glucose	-	-	L-Lactic Acid	-	+
D-Fucose	-	-	Citric Acid	-	+
L-Fucose	-	-	α -Keto-Glutaric Acid	-	-
L-Rhamnose	-	-	d-Malic Acid	+	-
Inosine	-	-	L-Malic Acid	-	+
1% Sodium Lactate	+	+	Bromo-Succinic Acid	-	-
Fusidic Acid	-	-	Nalidixic Acid	-	-
D-Serine	-	-	Lithium Chloride	+	+
D-Sorbitol	-	+	Potassium Tellurite	-	+
D-Mannitol	-	+	Tween 40	-	-
D-Arabitol	-	-	γ -Amino-Butyric Acid	-	-
myo-Inositol	-	+	α -Hydroxy Butyric Acid	-	-
Glycerol	-	+	α -Hydroxy-d,l-Butyric Acid	-	-
D-Glucose- 6-PO ₄	-	-	α -Keto-Butyric Acid	+/-	-
D-Fructose- 6-PO ₄	+/-	+/-	Acetoacetic Acid	+/-	+/-
D-Aspartic Acid	-	-	Propionic Acid	-	-
D-Serine	-	-	Acetic Acid	-	-
Troleandomycine	-	-	Formic Acid	-	-
Rifamycine SV	-	+	Sodium bromate	-	+
Minocycline	-	-	Sodium butyrate	+	+

Table 2. The antibiotic sensitivity test of the bacterial strains (K2 & K10) isolated from Bani Malek and Al-khoba hot springs, Jazan, KSA

Antibiotic	Bacterial isolate K2	Inhibition zone (mm)	Bacterial isolate K10	Inhibition zone (mm)
Amikacin	S	24	S	26
Cefepime	S	25	S	25
Ticarcillin	R	0	S	20
Piperacillin	S	18	S	21
Gentamicin	S	25	S	32
Colistin	R	0	S	30
Rifampicin	S	10	S	26
Penicillin	R	0	S	26
Imipenem	S	35	S	50
Cephalothin	R	0	S	50
Clindamycin	R	0	S	20
Cotrimoxazole	S	10	S	30
Erythromycin	S	20	S	35
Ampicillin	R	0	S	22

isolates exhibited high resistance against chromium (100 µg/mL), zinc and copper (150 µg/mL). At the same time, the bacterial isolate K10 demonstrated a marked resistance against mercury (100 µg/mL), silver (100 µg/mL) and lead (150 µg/mL). On the contrary, isolate K2 exhibited sensitivity against the same heavy metals.

Identification of the bacterial isolates

The bacterial isolates (K2 & K10) were Gram-positive rod-shaped cells. According to the metabolic fingerprints (Table 3), the isolate K2 from Bani Malik hot spring was identified as *Brevibacterium linens* meanwhile the K10 isolate from Al-khoba hot spring was identified as *Bacillus subtilis*.

Optimization of protease and amylase production

In the present study, the production of proteases and amylases of *B. linens* and *B. subtilis* was assayed under various temperatures, substrate concentrations in addition to calcium, magnesium and manganese levels. The obtained data (Table 4) showed that protease production of the bacterial strains on skim milk agar was increased with the raise of temperature reaching the maximum rate at 50°C for the two strains. At the same time, 5% skim milk was the optimum concentration for the enzyme production. Regarding the impact of Ca, Mg & Mn on the protease production, the enzyme production was augmented via increasing calcium

concentration in the cultures for both strains up to 80 ppm. However the enzyme production of *B. subtilis* was decreased with increasing of magnesium and maximum protease production was recorded at 20 ppm. In case of Mn, the maximum protease production was achieved at 40 ppm for *B. linens* and 20 ppm for *B. subtilis*.

Data in Table (4) demonstrated that the amylase production was steadily decreased on starch agar via rising the temperature and the maximum enzyme production was recorded at 30°C for both strains. At the same time, 0.4 % & 0.6 % starch were the optimum concentrations for the amylase production from *B. linens* and *B. subtilis*, respectively. Regarding the impact of Ca on the amylase production, the maximum enzyme production was recorded at 80 ppm for *B. linens* and 40 ppm for *B. subtilis*. In case of Mg and Mn, the amylase production was increased for both strains via increasing Mg and Mn up to 80 ppm.

DISCUSSION

Geothermal areas which are favorable habitats for thermophilic organisms are limited to restricted sites all over the world. In Saudi Arabia there are about 10 geothermal springs that are distributed in Jazan and Al-Lith areas. The microorganisms in geothermal springs are fastidious and exist in limited number. The total count of

bacteria in hot spring water was low and ranged between 3×10^{-2} CFU mL⁻¹ in Alkhoba sample

Table 3. The heavy metals resistance profile of *Brevibacterium linens* and *Bacillus subtilis* where the sensitivity was calculated by measuring the diameter of clear zone (mm)

Isolate	Heavy metals	Concentration (µg/ml)		
		25	50	100
K2	HgCl ₂	5	8	13
K10		0	0	0
K2	CdCl ₂	13	17	19
K10		18	20	21
K2	AgNO ₃	9	17	21
K10		0	0	0
K2	K ₂ Cr ₂ O ₇	0	0	0
K10		0	0	0

Isolate	Heavy metals	Concentration (µg/ml)		
		50	100	150
K2	Pb(NO ₃) ₂	7	10	12
K10		0	0	0
K2	ZnSO ₄	0	0	0
K10		0	0	0
K2	CuSO ₄	0	0	0
K10		0	0	0

Table 4. Effect of temperature, substrate concentration in addition to calcium, magnesium and manganese concentrations on the production of protease and amylase enzymes from *Brevibacterium linens* and *Bacillus subtilis*. Experiments were carried out in triplicate and diameters of the halo zones were measured by millimeter

	Factors	<i>Brevibacterium linens</i> Protease production		<i>Bacillus subtilis</i> Amylase production	
		<i>Brevibacterium linens</i>	<i>Bacillus subtilis</i>	<i>Brevibacterium linens</i>	<i>Bacillus subtilis</i>
Temperature	30°C	36	37	17	25
	37°C	38	37	10	13
	50°C	40	41	8	11
Substrate	5%	37	39	0.2%	13
	10%	35	37	0.4%	28
	20%	35	37	0.6%	37
CaCl ₂	20 ppm	35	33	20 ppm	20
	40 ppm	38	35	40 ppm	28
	80 ppm	40	40	80 ppm	15
MgSO ₄	20 ppm	40	40	20 ppm	18
	40 ppm	39	35	40 ppm	20
	80 ppm	39	35	80 ppm	29
MnCl ₂	20 ppm	35	40	20 ppm	16
	40 ppm	38	35	40 ppm	23
	80 ppm	38	35	80 ppm	25

and 2×10^{-3} CFU mL⁻¹ in Bani Malek sample. A similar total count trend of thermophilic bacteria in water samples collected from geothermal springs in Jazan, Saudi Arabia were recorded¹². Also, a very low number of thermophilic bacteria were recorded in four hot springs in Morocco¹¹. The low density of the bacterial populations in hot springs could be attributed to the adverse conditions of these environments or due to the application of culture-dependent identification approaches that identify only a small portion of the total microbial communities²⁶.

Although the bacterial isolate (K10) exhibited sensitivity against all the tested antibiotics, the isolate (K2) demonstrated resistance against some antibiotics. Moreover, both thermophilic strains showed resistance against different heavy metals. The positive correlation between heavy metals resistance and antibiotic resistance could be ascribed to the co-existence of heavy metals and antibiotics in the environment. Some studies have clarified the relation between the abundance of antibiotic resistance genes and the elevated concentrations of antibiotic and heavy metals in environments^{27,28}. At the same time, low levels of heavy metals could play a role in the emergence

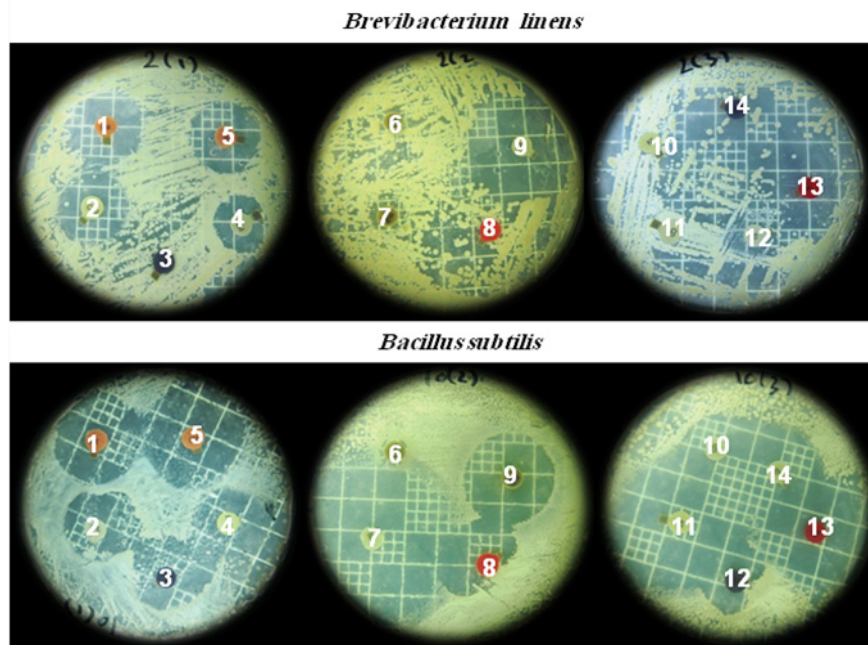


Fig. 1. Antibiotic resistance profile of *Brevibacterium linens* and *Bacillus subtilis* where 1, Amikacin (AK); 2, Cefepime (CPM); 3, Ticarcillin (TC); 4, Piperacillin (PRL); 5, Gentamicin (GM); 6, Colistin (CL); 7, Rifampicin (RIF); 8, Penicillin G (PG); 9, Imipenem (IMI); 10, Cephalothin (KF); 11, Clindamycin (CD); 12, Cotrimoxazole (TS); 13, Erythromycin (E); 14, Ampicillin (AP).

of antibiotic resistance in the resistant bacteria or induce antibiotic resistance in sensitive bacteria^{29,30}.

Based on the metabolic fingerprint, the bacterial isolates K2 and K10 were identified as *Brevibacterium linens* and *Bacillus subtilis*, respectively. In fact, *Brevibacterium* is a unique genus of the Brevibacteriaceae family located in the suborder Micrococccineae, order Actinomycetales, and class Actinobacteria. *Brevibacterium* is a Gram-positive bacterium that can tolerate high salt concentrations (8–20%), and is capable of growing in a broad pH range³¹. *B. subtilis* is a Gram-positive bacterium that is found in soil and the gastrointestinal tract of ruminants and humans. *B. subtilis* can form endospore allowing it to tolerate extreme environmental conditions. It can secrete proteins into the medium which is considered as a major advantage. This allows the accumulation of the native product with high yield and relatively in a pure form. Moreover, *B. subtilis* has no known pathogenic interaction with human or animals³².

Proteases production by microorganisms is affected by the medium compositions in

addition to the environmental conditions. Thus, it is important to explore the influencing factors to attain the maximum protease production. Cultivation temperature affects protein synthesis by influencing rate of biochemical reactions within the cell and consequently inducing or repressing enzyme production³³. The present study showed that the optimum temperature of protease production was 50°C and 5% skimmed milk at a constant pH of 7.5 for *B. linens* and *B. subtilis*. Meanwhile, the optimum amylase production was recorded at 30°C and 0.4–0.6% starch for the two strains. Previous studies have reported a similar finding on a *Bacillus* sp. with maximum protease production at 45 °C^{34,35}. At the same time, it was reported a lower optimum temperature of 30°C for protease production by *Bacillus* sp. The obtained data showed that 5% skim milk was the optimum concentration for protease production of the two strains and the increase above that level led to a decrease of protease yield³⁶. Protease is an inducible enzyme which needs a substrate to encourage the microbial cell to produce it. Consequently, the increase of skim

milk concentration in the fermentation medium led to increase of protease production until the skim milk concentration reduced the dissolved oxygen in fermentation medium resulting in a feedback inhibition of the protease production³⁷. Regarding the influence of metal ions on the protease production and activity, Ca²⁺ showed a positive effect on proteases of both strains meanwhile Mg²⁺ and Mn²⁺ demonstrated a minor effect. These results indicated that protease requires metal ions as cofactors. These findings correlate with the observations of Kunamneni *et al.*³⁸ and Sayem *et al.*³⁹ who found that the activity of protease from *Bacillus subtilis* and *Bacillus licheniformis* was accelerated by the addition of Mg²⁺, Ca²⁺ and Mn²⁺. It could be suggested that these metal ions could play a vital role in protecting the enzyme against thermal denaturation in addition to their task for maintaining the active conformation of the enzyme at higher temperatures⁴⁰.

This study clarified that maximum amylase production was recorded at 30 °C for both strains and the production rate was reduced by increasing the temperature. These findings are in accordance with those reported who recorded that 30°C was the optimum temperature for the production of amylase from *Bacillus subtilis*⁴¹. Similarly, It was showed that the maximum yield of amylase produced from *Brevibacterium linens* was achieved at 32.5 °C⁴². The decrease of the amylase production by increasing the temperature above 30 °C could be attributed to the loss of moisture in the substrate which adversely affects the metabolic activities of the microbes leading to reduced growth and decline in enzyme production⁴³. The obtained data showed that 0.4 – 0.6% starch was the optimum concentration for amylase production. Excess of starch in the medium could be responsible for feedback inhibition of the amylase production. The current study showed that Ca²⁺, Mg²⁺ and Mn²⁺ have a positive effect on the amylase production and activity of both strains. These results are in harmony with previous studies who recorded that the activities of amylases of *Bacillus subtilis* and *Bacillus amyloliquefaciens* were stimulated in the presence of some metal ions such as Ca²⁺, Ba²⁺, Mg²⁺, Li²⁺, Fe²⁺ and Mn²⁺^{44,45}. It is also well established that calcium has a vital task in the thermal stabilization of α -amylases^{46,47}.

CONCLUSION

Thermophilic bacteria and their hydrolytic enzymes have become a chief research subject due to their valuable biotechnological applications. The current study highlighted the capability of the thermophilic bacteria *Brevibacterium linens* and *Bacillus subtilis* for the production of amylolytic and proteolytic enzymes. The optimum conditions of the production of these enzymes were achieved at 50°C for both strains at 5% skimmed milk for protease of *B. linens* and 10% for *B. subtilis* while 0.6% starch recorded the highest amylase production for both strains. At the same time, Ca²⁺, Mg²⁺ and Mn²⁺ demonstrated a positive impact on the production rate of these enzymes. Further studies are in progress for the isolation and purification of protease and amylase from different thermophilic bacteria to evaluate their application in different commercial fields.

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REFERENCES

1. Al-Dayel, M. Geothermal resources in Saudi Arabia. *Geothermics*, 1988; **17**(2-3): 465 – 476.
2. Rehman, S., Shash, A. Geothermal resources of Saudi Arabia—country update report. *Proceedings World Geothermal Congress*, 2005.
3. Khalil, A. Isolation and characterization of three thermophilic bacterial strains (lipase, cellulose and amylase producers) from hot springs in Saudi Arabia. *African Journal of Biotechnology*, 2011; **10**(44): 8834-8839.
4. Panda, M.K., Sahu, M.K., Tayung, K. Isolation and characterization of a thermophilic *Bacillus* sp. with protease activity isolated from hot spring of Tarabalo, Odisha, India. *Iran J Microbiol*, 2013; **5**(2): 159–165.
5. Adiguzel, A., Ozkan, H., Baris, O., Inan, K., Gulluce, M., Sahin, F. Identification and characterization of thermophilic bacteria isolated from hot springs in Turkey. *J. Microbiology Method*, 2009; **79**: 321- 328.
6. Maugeri, T. L., Gugliandolo, C., Caccamo, D., Stackebrandt, E. A Polyphasic Taxonomic

- Study of Thermophilic Bacilli from Shallow, Marine Vents. *Systematic and Applied Microbiology*, 2001; **24**: 572-587.
7. Derekova, A., Mandeva, R., Kambourova, M. Phylogenetic diversity of thermophilic carbohydrate degrading Bacilli from Bulgarian hot springs. *World J. Microbiology and Biotechnology*, 2008; **24**: 1697-1702.
 8. Sievert, S.M., Ziebis, W., Kuever, J., Sahm, K. Relative abundance of Archaea and Bacteria along a thermal gradient of a shallowwater hydrothermal vent quantified by rRNA slot-blot hybridization. *Microbiology*, 2000; **146**: 1287-1293.
 9. Lau, M.C., Aitchison, J.C., Pointing, S.B. Bacterial community composition in thermophilic microbial mats from five hot springs in central Tibet. *Extremophiles*, 2009; **13**: 139-149.
 10. Verma, A., Gupta, M., Shrikot, P. Isolation and characterization of thermophilic bacteria in natural hot water springs of Himachal Pradesh (India). *Bioscan*, 2014; **9**:947-952.
 11. Aanniz, T., Ouadghiri, M., Melloul, M., Swings, J., Elfahime, E., Ibijbijen, J., Amar, M. Thermophilic bacteria in Moroccan hot springs, salt marshes and desert soils. *Brazilian Journal of Microbiology*, 2015; **46**(2):443-453.
 12. Khiyami, M.A., Serour, E.A., Shehata, M.M., Bahkilia, A.H. Thermo-aerobic bacteria from geothermal springs in Saudi Arabia. *African Journal of Biotechnology*, 2012; **11**(17):4053-4062.
 13. Haki, G.D., Rakshit S.K. Developments in industrially important thermostable enzymes: a review. *Bioresour Technol*, 2003; **89**:17-34.
 14. Bouzas, T.M., Barros-Velázquez, J., Villa, T.G. Industrial applications of hyperthermophilic enzymes: a review. *Protein Pept Lett*, 2006; **13**:645-651.
 15. El-Gayar, K.E., Zaghloul, T. I., Haroun, M.A., Saeed, H.M. Purification of alkaline protease from hydrolyzed chicken feather waste using recombinant *B. subtilis* strain. *Scientific journal of King Faisal university, KSA*, 2012; **13**(1): 1433.
 16. Al Abboud, M.A. Isolation and Identification of protease producing bacterial strain from Jazan province, KSA. *Journal of Jazan University - Applied Sciences Branch*, 2014; **3**(1).
 17. Mienda, B.S., Yahya, A., Galadima, I.A., Shamsir, M.S. An overview of microbial proteases for industrial applications. *Res J Pharm Biol Chem Sci*, 2014; **5**: 388-396.
 18. Pednekar, P., Jain, R., Mahajan G. Anti-infective Potential of Hot-spring Bacteria. *J Glob Infect Dis*, 2011; **3**(3): 241-245.
 19. Yang, C.H., Liu, W.H. Purification and properties of a maltotriose-producing α -amylase from; *Thermobifidafusca*. *Enzyme Microb Technol*, 2004; **35**(2): 254-60.
 20. Paul, D. Microorganisms and α -amylase: A concise review. *Innovare journal of sciences*, 2016; **4**(4).
 21. Essa, A. M. M., Khallaf M. K. Antimicrobial potentiality of consolidation polymers impregnated with copper nanoparticles. *BMC Microbiology*, 2016; **16**:144.
 22. Agwa, O.K., Uzoigwe, C.I., Wokoma, E.C. Incidence and antibiotic sensitivity of *Bacillus cereus* isolated from ready to eat foods sold in some markets in Portharcourt, Rivers state, Nigeria. *Asian J. Microbiol. Biotech. Env. Sc*, 2012; **14** (1) : 13-18.
 23. Essa A.M.M. Macaskie L.E. and Brown N.L. 2005. A new method for mercury removal. *Biotechnology Letters* **27**(21): 1649-55.
 24. Essa, A.M.M. Effect of continuous mercury stress on mercury reducing community of some characterized bacterial strains. *Afr J Microbiol Res*, 2012; **6**: 1255-1261.
 25. Qadeer, M.A., Aurangzeb, M., Igbal, J. Production of raw starch hydrolyzing enzymes by mould cultures. Proceeding of the International Symposium on Biotechnology for Energy, Malik, K. A.; Nagvi, S.H. M. and Aleem, M.I. H. (eds.) *Faisalabad, Pakistan*, 1989; pp119-128.
 26. Ranjard, L., Poly, F., Nazaret, S. Monitoring complex bacterial community using culture-independent molecular techniques: application to soil environment. *Res Microbiol*, 2000; **151**: 167-177.
 27. Knapp, C.W., McCluskey, S.M., Singh, B.K., Campbell, C.D., Hudson, G., Graham, D.W. Antibiotic resistance gene abundances correlate with metal and geochemical conditions in archived Scottish soils. *PLoS ONE*, 2011; **6**: 27300.
 28. Zhu, Y.G., Johnson, T.A., Su, J.Q., Qiao, M., Guo, G.X., Stedtfeld, R.D., Hashsham, S.A., Tiedje, J.M. Diverse and abundant antibiotic resistance genes in Chinese swine farms. *Proc. Natl. Acad. Sci. USA*. 2013; **110**: 3435-3440.
 29. Martinez, J.L., Rojo, F. Metabolic regulation of antibiotic resistance. *FEMS Microbiol. Rev*, 2011; **35**: 768-789.
 30. Chen, S., Li, X., Sun, G., Zhang, Y., Su, J., Ye, J. Heavy metal induced antibiotic resistance in bacterium LSJC7. *International journal of molecular sciences*, 2015; **16**(10):23390-23404.
 31. Motta, A.S., Brandelli, A. Properties and antimicrobial activity of the smear surface cheese coryneform bacterium *Brevibacterium*

- linens*. *European Food Research and Technology*, 2008; **227**(5): 1299-1306.
32. Errington, J., Mountain, A. Is *Bacillus* an alternative expression system? In: *Protein Production by Biotechnology*. T.J.R. Harris (ed.), Elsevier Science Publishers Ltd., England, 2008; 1–14.
 33. Bakermans, C., Neelson, K.H. Relationship of critical temperature to macromolecular synthesis and growth yield in *Psychrobacter cryopegella*. *Journal of Bacteriology*, 2004; **186**: 2340-2345.
 34. Sepahy, A.A., Jabalameli, L. Effect of culture conditions on the production of an extracellular protease by *Bacillus sp.* isolated from soil sample of Lavizan Jungle Park. *Enzyme Research*, 2011; Article ID: 219628.
 35. Olajuyigbe, F.M. Optimized production and properties of thermostable alkaline protease from *Bacillus subtilis* SHS-04 grown on groundnut (*Arachis hypogaea*) meal. *Advances in Enzyme Research*, 2013; **1**(4):112.
 36. Gouda, M.K. Optimization and purification of alkaline proteases produced by marine *Bacillus sp.* MIG newly isolated from Eastern Harbour of Alexandria. *Polish Journal of Microbiology*, 2006; **55**: 119-126.
 37. Chu, W.H. Optimization of extracellular alkaline protease production from species of *Bacillus*. *Journal of industrial microbiology & biotechnology*, 2007; **34**(3): 241-245.
 38. Kunamneni, A., Poluri, E., Davuluri, S. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. *AAPS Pharm Sci Tech*, 2003; **4**:1-9.
 39. Sayem, S.A., Alam, M.J., Hoq, M.M. Effect of temperature, pH and metal ions on the activity and stability of alkaline protease from novel *Bacillus licheniformis* MZK03. *Proceedings-Pakistan Academy of Sciences*, 2006; **43**(4):257.
 40. Sharma, K.M., Kumar, R., Panwar, S., Kumar, A. Microbial alkaline proteases: Optimization of production parameters and their properties. *J Genetic Engineering and Biotechnology*, 2017.
 41. Nanganuru, H., Korrapati, N., Mutyala, S. Studies on the production of α -amylase by *Bacillus subtilis*. *IOSR J. Eng.* 2012; **2**(5): 1053-1055.
 42. Shabbiri, K., Adnan, A., Noor, B., Jamil, S. Optimized production, purification and characterization of alpha amylase by *Brevibacterium linens* DSM 20158, using biostatistical approach. *Annals of microbiology*, 2012; **62**(2):523-532.
 43. Prakasham, R.S., Rao, Ch.S., Rao, R.S. Sarma P.N. Enhancement of acid amylase production by an isolated *Aspergillus awamori*. *Journal of applied microbiology*, 2007; **102**(1): 204–211.
 44. Demirkan, E. Production, purification, and characterization of alpha-amylase by *Bacillus subtilis* and its mutant derivative. *Turkish Journal of Biology*, 2011; **35**(6): 705-712.
 45. Deb, P., Talukdar, S.A., Mohsina, K., Sarker, P.K., Sayem, S.A. Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. *SpringerPlus*, 2013; **2**(1):154.
 46. Gupta, R., Gigras, P., Mohapatra, H., Goswami, V.K., Chauhan, B. Microbial α -amylases: a biotechnological prospective. *Process Biochem*, 2003; **38**: 1599–1616.
 47. Vengadaramana, A. Industrial Important Microbial alpha-Amylase on Starch-Converting Process. *Scholars Academic Journal of Pharmacy (SAJP)*, 2013; **2**(3):209-221.