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Isolation, Identification and Molecular Characterization of Thermophilic Bacteria from Soil and Water of Khurmal Spring

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Abstract

Thermophilic bacteria are regarded as a key source of thermostable enzymes that are of great industrial importance. The present study was conducted to identify and characterize the thermophilic bacteria isolated from Khurmal spring in Sulaimaniyah, Kurdistan region of Iraq. Water and soil samples from different sites were collected used to isolate thermophilic bacteria. Seven different isolates of *Bacillus* (5 from water and 2 from soil) were characterized for their biochemical properties. Activity of different enzymes (catalase, amylase, cellulose, protease, and esterase) was measured for each isolate. The bacterium with the ability to tolerate high temperatures was identified as *Bacillus* sp. both by using phenotypic and genotypic methods including microscopic characterization, Gram staining and 16S rRNA sequencing. BLAST search analysis of the sequence showed maximum identity with different *Bacillus sp*. with similarities in the range of (95-98%). The study confirmed that the isolated *Bacillus* sp. to be a true thermophile and could be a source of thermostable enzyme which can be exploited for pharmaceutical and industrials applications.

Introduction

Bacteria are ubiquitous in most environments and they are extremely diverse as they can successfully colonize even from inhospitable places. Thermophilic microorganisms (an optimal thermal growth temperature of 50 ° C or more) have attracted great attention among extremophiles as they are the source of thermostable enzymes (e.g. amylases, catalase, cellulases, chitinases, pectinases, xylanases, proteases, lipase, and DNA polymerases) [1], these enzymes show unique features that can be suitable for performing biotechnological processes at elevated temperatures. These enzymes show a great stability against many solvents, acidic and alkaline pH [2]. The catalase enzyme, which is commonly, used in the biology, medicine, food sectors and it functions in decomposing H2O2 demonstrates high potential in the pulp and paper processing, ambient protection, and chemical industries and it is one of the most commercially significant enzymes [3]. In addition, cellulase enzymes showed great potential for the manufacturing of glucose feedstock from industrial cellulose and for bioethanol and value-added organic compounds from renewable residues [4]. On the other hand, Amylases are used together in many industries such as the food industry, detergent industries, and pharmaceuticals [5]. Another important enzyme is lipase which hydrolyzes triglycerides into diglycerides, monoglycerides, and glycerol. This enzyme is an option for potential applications in the food, pharmaceutical, leather, cosmetic, and paper industries [6].

Thermophiles can be categorized into hyperthermophiles (optimum growth temp, 80–110°C), extreme thermophiles (optimum growth temp, 60–80°C), and moderate thermophiles (optimum growth temp, 50–60°C) [2]. Most thermophilic bacteria are classified into Gram-positive or Gram-negative species, they exist

in aerobic or anaerobic conditions, and some are spores forming [7]. Many research groups focused on discovering new thermophilic bacterial genus and species due to increasing their importance, applications, and their roles in different fields [2].

This study was performed to isolate and characterize of thermophilic bacteria from Khurmal spring.

Materials and Methods

Study site & samples collection

The Khurmal spring is situated at the khurmal area, Sulaimaniyah, Iraq. Five Soil samples were collected from different sites of the spring in clean dry sterile containers, and four water samples collected in 50ml sterile falcon tubes and immediately brought into the laboratory and stored at 4˚C for further study. The samples of water were taken from different depth 10cm, 20 cm, and 30 cm. Two replica of each sample were used throughout the study.

Figure 1**:** Khurmal spring, Sulaymaniyah, Kurdistan region, Northern Iraq.

Isolation of bacteria

Following a serial dilution method, bacteria were isolated in nutrient agar media. The protocol was as follows: 10 grams of soil sample was diluted in 90 ml sterile saline water (0.85%) and kept in a 250 ml conical flask. The solution was put in an orbital shaker at 150 rpm and left to homogenize. The soil samples were serially diluted to 10^{-7} dilutions and 100 µL aliquots of each dilution were inoculated into nutrient agar plates. The plates were incubated for bacterial growth at 55ºC for 24 hrs. After incubation, the plates were observed for the bacterial colonies and the total number of colonies was counted and calculated (CFU) [8]. Different bacterial colonies were isolated on the nutrient agar from various diluted samples, then the bacterial colonies were picked up and streaked on a freshly prepared nutrient agar to get pure cultures from each bacterial isolate that sub cultured on nutrient agar slants and stored at 4°C for the study. The thermophilic properties of the pure bacterial isolates were detected by inoculation of each isolate into a test tube contains 5 ml of nutrient broth and incubated at 40°C for 12 h. Later on, each colony was streaked onto freshly prepared nutrient agar medium tested for their thermo-tolerance at higher temperature. Finally the bacteria that could tolerate temperature of 70°C were selected for further study [8].

Identification and characterization of the bacterial isolates

The selected isolates were examined and identified according to the bacterial morphology using Gram staining technique and the growth characteristics through colony shape and color, in addition to different biochemical assays [9].

Enzymes screening tests

The study bacterial isolates were screened for their enzymatic activities such as amylase, cellulase, lipase, catalase and protease activities.

Amylase test

The activity of amylase enzyme was measured in the starch agar (SA). Isolated colonies were picked up from each plate and streaked in a single line on nutrient starch agar plates with starch as the only carbon source medium and incubated at 37°C for 24-48 h. A positive test indicated by the formation of a clear zone around colonies and these were visualized after treating with Lugol's Iodine solution. In the zone of degradation no blue color forms, which is the basis of the detection and screening of an amylolytic strain and this was followed by measuring of the zone of clearance in millimeter (mm) [10].

Catalase test

Catalase activity was determined using slide method. Isolated colonies were picked up using a sterile loop from each plate containing pure culture and transfer to the surface of a clean, dry glass slide. A drop of 3% H_2O_2 was placed in the glass slide. Evolution of oxygen bubbles indicates the activity of the catalase enzyme [11].

Cellulase test

Fresh cultures of study bacterial isolates were inoculated on carboxymethyl cellulose agar (CMC); a medium has the following composition (g/L): carboxymethylcellulose, 10; peptone, 5; yeast, 5; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.2; NaCl, 10; and agar, 15. After inoculation on the CMC media the plates were incubated for 48 hours at a temperature of 30 °C for 2 days. Cellulose degrading potential was screened by flooding of 0.1% Congo red solution on the plates for 10 minutes and washing with 1.0M% NaCl. The positive results revealed by the formation of a clear zone in the area around the colony [12].

Lipase and esterase test

Bacterial colonies appeared on nutrient agar plates were subjected to screening for esterase/lipase activity on tributyrin agar (TBA) plate and incubated at 55^0 C. The medium contained 1% tributyrin (glycerol tributyrate). Formation of a zone of clearance (hydrolysis) around the colonies indicates the hydrolytic feature of the isolates. Clear and wide zone around the colonies were determined as as lipase producers.. The zone size was measured after 24 and 48 h of incubation [13].

Protease activity

Protease activity was determined in skim milk agar (SMA). Isolated colonies were streaked over skim milk agar plates and incubated at 37°C for 24-48 h. Protease activity indicated by the formation of hydrolysis zone around the culture [14].

Quantitative assays of the Bacterial Enzymes:

Amylase assay

The bacterial samples were grown in LB-medium overnight at 37°C. Then samples were centrifuged for 10 minutes using a centrifuge (Beckman counter, Germany) and the pellets were collected after discarding the supernatants. The cells were suspended in 1000µl of phosphate buffer (pH 7.0). The cells were subjected to lyses using a homogenizer (Bendelin electronic, Germany). Following cell lyses, the samples were further centrifuged for 10 minutes after which the supernatants were collected. Alpha-amylase activity of the resulted supernatant was measured according to Bernfeld method [15], by mixing 250 µl. of test supernatant with 250 µl of starch solution (at pH 6, 7 and 8) and incubated at different temperature (25°C, 37°C and 80°C) for 10 minutes, 500µl of DNS reagents was added to the mixture, The solutions were boiled in a boiling water bath for 5 minutes, then cooled in a running tap water, 1ml of deionized distilled water was added to each solution. A blank was prepared without enzyme. The absorbance was read at 540nm using UV-Vis spectrophotometer (Agilent Technologies, Cary 60 UV-Vis-U.S.A). One unit of α-amylase activity can be defined as the amount of enzyme needed to liberate 1 mg of in 30 min at 35°C. A standard curve was constructed to enable the calculation of the amount of the reducing sugar released during α-amylase assays.

Catalase Assay

The catalase activity was measured spectrophotometrically by monitoring the decrease in the absorbance at 240 nm of H_2O_2 by the hydrolysis to H_2O and O_2. This decrease in absorbance results from the decomposition of H_2O_2. The extinction coefficient (ε) for H_2O_2 at 240 nm set to be 0.036 mM. cm^{-1}. The enzyme activity was measured by adding 10 µl of the isolate to 40 mM H_2O_2 solution. The substrate solution was prepared in 50 mM potassium phosphate buffer, pH 7.0, and the blank containing the same buffer. The spectrophotometric assay was carried out at 1 mL cuvette and the reaction was performed at 25°C. Activity of the enzyme was measured by following this equation (Abs*dilution factor)/ ε240 = 0.036 $mM^{-1}cm^{-1}$ (dilution factor = 100). One unit of the enzyme can be defined as the amount of enzyme that decomposes 1 µmol of H_2O_2 per min [16].

Cellulase assay

Endo-β-1,4-glucanase of cellulose activity was measured by using 3,5-dinitrosalicylic acid (DNS) method through the amount of reducing sugars liberated from CMC by hydrolysis. The reaction mixture was prepared by mixing 50 µL of crude enzyme solution with 50 µL of 1% (w/v) CMC dissolved in 100 mM phosphate buffer, pH 7.0. The mixture was incubated at 50^0 C for 20 min. 0.3 mL of DNS reagent to stop the reaction. The sample was heated in boiling water at 100^0 C for 5 min, cooled is water for color development and stabilization and the absorbance was measured at 575 nm. A calibration curve of glucose was drawn to determine the enzyme activity by applying this equation Activity = (product concentration from glucose standard curve *1000* 8 {dilution factor})/M.wt glucose (180)*5 incubation time. One unit (U) of the enzyme activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 µmol of glucose within 1 minute of reaction [17].

Molecular Identification of bacterial isolates

The bacterial isolates were further identified by Amplification and sequencing of the 16S rRNA gene for each isolate. This was done by the following methods.

DNA extraction

The total genomic DNA for seven thermophilic bacteria was extracted using the Amplisens nucleic acid extraction Kit (USA).

Polymerase chain reaction and cloning of 16S rRNA gene fragments

The 16SrRNA genes were selectively amplified from purified genomic DNA using oligonucleotide primers designed to anneal to the conserved positions in the 3′ and 5′ regions of the bacterial 16S rRNA genes. The primers are shown in table 1 and the PCR reaction condition of the thermal cycler is shown in table 2 [18, 19].

From the extracted DNA, the 16S rRNA gene was amplified using Polymerase Chain Reaction.

Table 1: Primers used in the PCR reaction

	Sequence	Type
Forward: UNI16S-L	5′-ATTCTAGAGTTTGATCATGGCTCA-3′	Universal
Reverse: UNI16S-R	5′-ATGGTACCGTGTGACGGGCGGTGTGTA-3′	Universal

Table 2: PCR reaction condition

PCR condition	Temperature	Time
Initial denaturation	95°C	2 minutes
Denaturation	95°C	30 seconds
Annealing	60°C	1 minute
Extension	72°C	2 minutes
Final extension	72°C	5 minutes

Detection of DNA using agarose gel electrophoresis

Horizontal electrophoresis in 1.0% agarose slab gel in Tris–borate EDTA (TBE) buffer was used to check the amplification. Agarose was dissolved in 1X TBE to give a final concentration of 1.0% agarose and was heated to dissolve in a microwave oven for about 30 seconds. After that it was allowed to cool down to about 50^0C. To the cooled agarose, 2 µl Ethidium Bromide (EtBr) stain was added and mixed in order to stain the DNA bands. The agarose was then poured on the tray previously set with the comb and allowed to solidify. 6 µl aliquot of the PCR product mixed 2 µl of loading dye was loaded into the gel wells. A 1kb size ladder (Invitrogen, USA) was used to check amplification product and measure the exact product size which was estimated to be within 1000 bp. The DNA bands were observed on a UV transilluminator at 365 nm [20].

Measurement of DNA concentration and purity

The quantity and purity of the extracted DNA were determined by a Nano Drop instrument (Nanodrop 1000 Spectrophotometer Thermo Scientific) at 260 nm. To the NanoDrop, 1.5 µl of nuclease free water was used as blank. The blank was removed and 1.5 µl of sample was loaded. By using 10µl DNA extracted solution mixed with 90µl of distilled water. DNA concentration was measured in ng/µl [21].

DNA sequencing

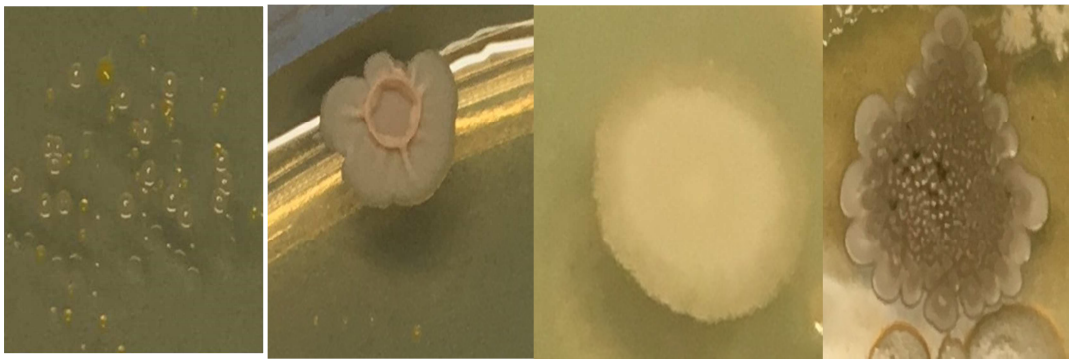
The DNA sample was sent to the '1st BASE Laboratories' in Malaysia via Invent Technologies Ltd. where it was sequenced using the Sanger method.

Bioinformatic analysis

The acquired gene sequence trace was trimmed and cleaned using Mega6 and Lasergene Seqman software. The cleaned genetic sequence was then compared to different 16S rRNA gene of different bacteria in the reference RNA sequences (16S ribosomal RNA) database of NCBI Nucleotide BLAST website using Blast tool in order to identify the genus of the selected isolate. The query sequence was converted to FASTA format using EMBOSS SEQRET website and was then used to create a phylogenetic tree using Mega6 software [22].

Results and Discussion

Microorganisms regarded as one of the most important sources for enzyme production. These enzymes are superior particularly for industrial applications on commercial scales. In the present study soil and water samples collected from Khurmal spring, sulaimaniyah, Iraq was investigated for thermophilic bacteria. Out of the total 18, only seven isolates were found to grow in temperatures up to 70°C (Figure 2). All 7 isolates were identified as *Bacillus* both by morphological and molecular characterization. The thermophilic *Bacillus* isolates were screened for lipase, cellulase, catalase, and protease and amylase activity.



W4 W5 W6 W7

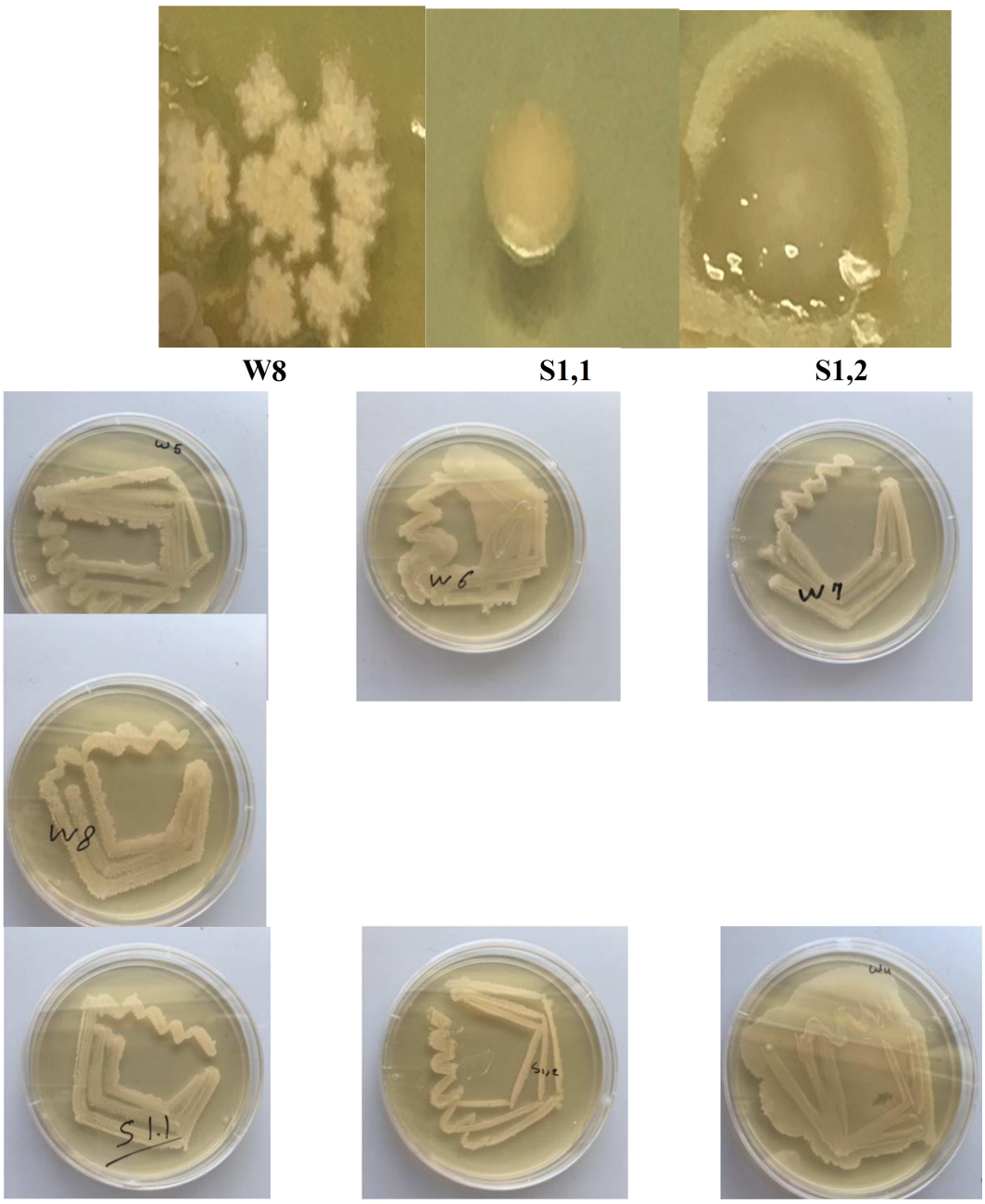


Figure 2: The Bacterial cultures on the nutrient agar after incubation at 55 ⁰ C for 24 hours.

The bacterial isolates: W4, W5, W6, W7, and W8 were isolated from water while S1 and S2 were isolated from soil.

Depending on the Gram's stain reaction and microscopic examination, the bacterial isolates appeared as Gram-positive bacilli whereas the macroscopic examination of the bacterial colonies revealed the following characters as shown in Table 3.

Table 3: Macroscopic and Microscopic characterization of the bacterial isolates.

Bacterial characterization	Isolate Code						
	Water before mix			Bottom water spring			
	W4	W5	W6	W7	W8	S1	S2
Shape of the colony	Circular	Circular	Circular	Circular	Circular	Circular	Circular
Surface of the colony	Convex	Flat	Flat	Convex	Convex	Flat	Flat
Texture of the colony	Shiny	Opaque	Opaque	Opaque	Shiny	Shiny	Shiny
Color of the colony	White Transparent	White	White	Grey	White	White	Grey
Edge of the colony	regular	Irregular	regular	Irregular	regular	regular	Regular
Size of the colony	Very small	large	large	Small	Very small	Large	Large
Grams' reaction	Positive	Positive	Positive	Positive	Positive	Positive	Positive

Regarding the bacterial enzymatic activity, all bacterial isolates revealed a positive amylase activity results in formation of a clear zone around the colonies when treated with Lugol's Iodine solution, this indicated to the amylase activity in degradation of the starch (Figure 3).

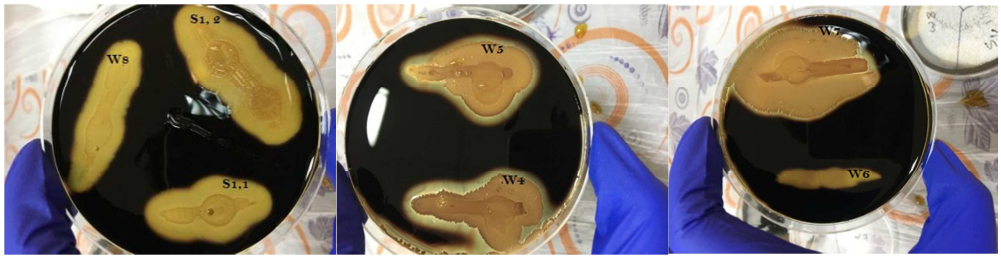


Figure 3: A starch agar showing amylase activity with clear white zone surrounding the colonies

Cellulase activity was determined on carboxymethyl cellulose agar (CMC), all bacterial samples produce clear zone when treated with congo red dye (Figure 4).

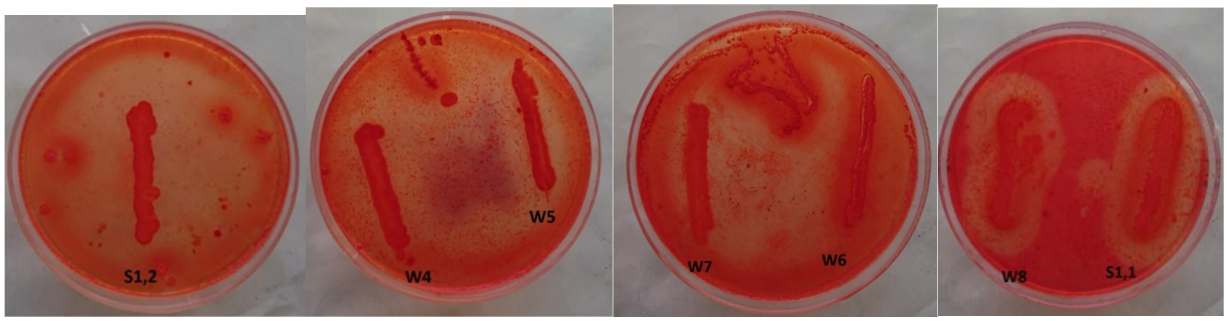


Figure 4: CMC agar cultured with cellulase-producing bacteria followed by staining with Congo red dye.

All seven bacterial isolates showed the lipase activity on Tributyrin agar (TBA) as shown in the Figure 5.

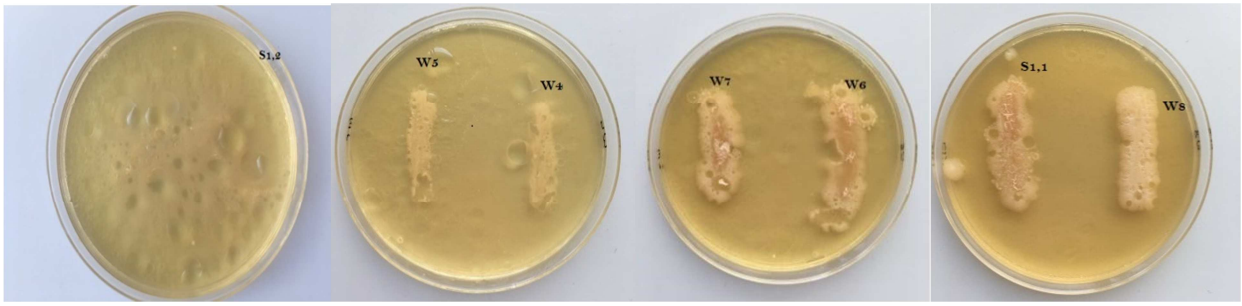


Figure 5: TBA plate showing bacterium with lipase activity (clear zone)

In the screening for the bacterial catalase activity, the isolates (W4,W8, W5 and S2) produced a stronger reaction than the isolates (W6,W7, and S1) as appeared in the Figure 6.

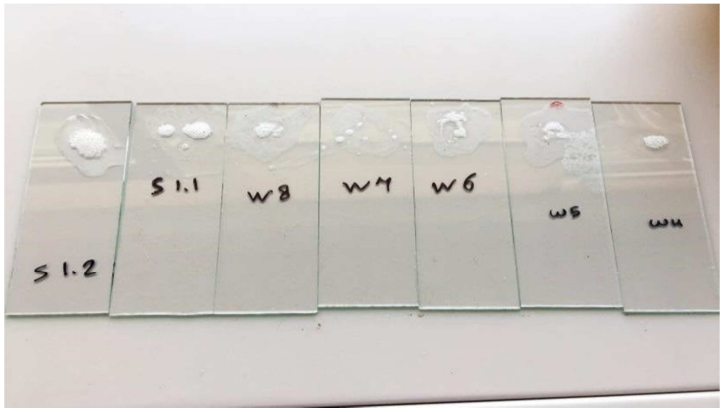


Figure 6: Catalase positive reaction (air bubble formation).

Protease activity of the bacterial isolates was also determined by culturing the isolates on the skim milk agar (SMA). Proteolytic bacteria hydrolyze casein using caseinase to form soluble compounds displayed as a clear zone around colonies (Figure 7).

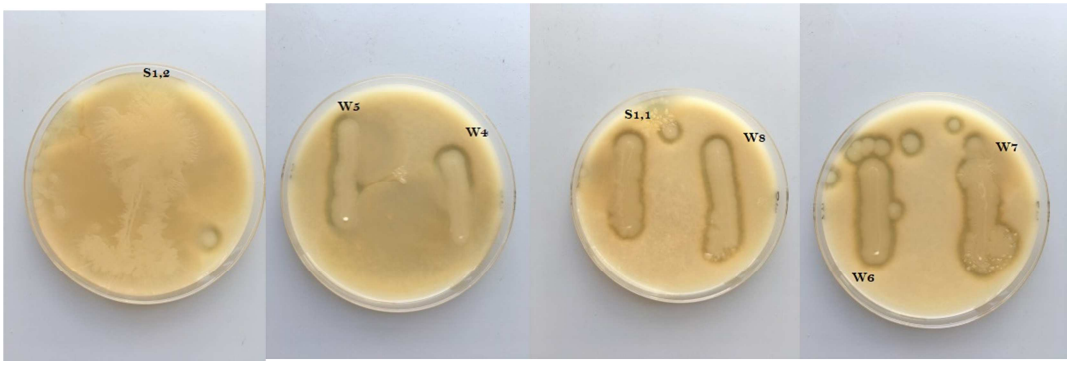


Figure 7: Qualitative assay for protease activity on skim milk agar.

Spectrophotometric method was used to quantify the enzymes released by the seven bacterial isolates. Dinitrosalicylic (DNS) method was employed for the amylase activity assay. Absorbance of the enzyme was measured spectrophotometrically and the effects of pH and temperature were determined. The enzymatic activities differ is respect to the bacterial isolates in relation to pH and temperature.

The results indicated that at pH 7.0, the highest amylase activity was obtained at 80 ^{o}C incubation for all samples, while at pH 6.0 the lowest activity was obtained at 80 ^{o}C. At pH 8.0, the results were different according to the bacterial isolates. The isolates W4, 5, and 6 showed highest activity at 80 ^{o}C, while W7, W8, S1, andS2 showed the lowest activity at 80 ^{o}C. Moreover, S2 has the highest amylase activity at pH 7.0 and 80 ^{o}C which is ca 15 U/min (table Sup1).

Hydrogen peroxide used as a substrate and potassium phosphate as buffer. Absorbance of the enzyme was measured sphectrophotometrically. The enzymatic activities differ in respect to the bacterial isolate. The isolate W5 showed the highest catalase activity while S2 showed the lowest catalase activity (Table 4).

Table 4: Catalase Assay Results for Bacterial isolates.

Bacterial isolates	Absorbance	Activity
W4	**0.4652**	1292.22
W5	**0.5119**	1421.944
W6	**0.4667**	1296.389
W7	**0.4832**	1342.222
W8	**0.4859**	1349.722
S1	**0.4886**	1357.222
S2	**0.4057**	1126.944

Cellulase activity was determined and estimated according to the carboxy-methyl cellulase method .One unit of CMCase activity is expressed as the quantity of enzyme, which is required to release 1µg of glucose per minute under standard assay conditions, absorbance read at 540nm. The values obtained were compared with glucose standard curve. W5 showed the highest cellulase activity compared to other samples while S2 showed the lowest activity (Table 5).

Table 5: Cellulase Assay Results for Bacterial isolates.

Bacterial samples	**Absorbance**	Activity
W4	**0.0061**	1.4652
W5	**0.0131**	1.648024
W6	**0.0027**	1.3764
W7	**0.0059**	1.459976
W8	**0.0011**	0.7448
S1,1	**0.0026**	1.373788
S1,2	**0**	0

The bacterial DNA extraction was conducted for all the isolates and the purity was determined. According to the results obtained, the quality of purification is close to 2.0 and DNA concentrations were between 150 and 185 ng/ ml (Table 7).

Table 6: The Quality and concentration of the extracted DNA in all bacterial isoltes.

Bacterial Isolates	DNA concentration (ng/ml)	Extraction quality
W4	185	1.9
W5	170.5	1.7
W6	153	2
W7	180	1.8
W8	175	1.9
S1	170	2
S2	160	1.6

Amplification of the DNA samples using the universal kit was performed and agarose gel electrophoresis was used to confirm the size of the DNA (Figure 8). The results showed that the DNA size of all samples was about 1 Kbp.

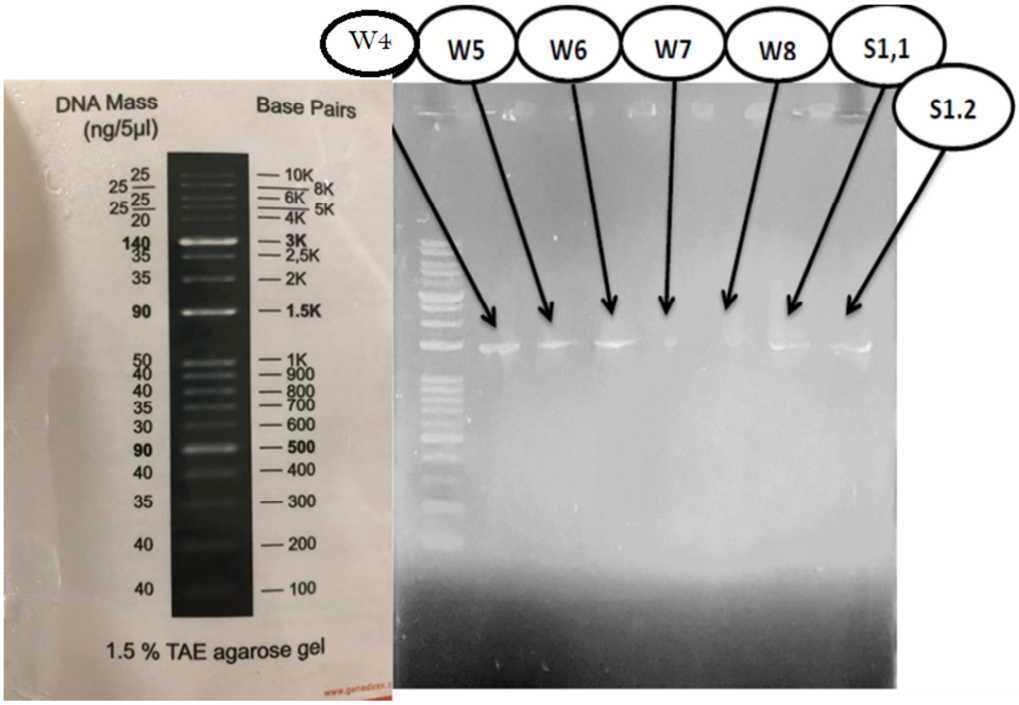


Figure 8: Agarose Gel Electrophoresis for PCR products.

Phylogenetic Analysis Results

Depending on the 16s ribosomal RNA sequence analysis, the seven bacterial isolates were belonged to the bacterial family *Bacillaceae*. Morphological characterization of the isolate indicated it to be *Bacillus* sp. Species level confirmation of the isolate was done by 16S rDNA sequencing. Based on BLAST search analysis of the sequence, the isolate (W4) showed maximum identity with *Bacillus sonorensis* (95.81%) having accession number MF977361.1, the isolate (W5) showed maximum identity with *Bacillus subtilis (97.04%*) having accession number MG836202.1, the isolate (W6) showed maximum identity with *Bacillus sp. CCMMB1023 (97.76%)* having accession number KF879328.1,the isolate (W7) showed maximum identity with *Bacillus sp. DV9-35 (97.77%)* having accession number GQ407180.1, the isolate (W8) showed maximum identity with *Bacillus sp. CCMMB848 (97.93%)* having accession number KF879230.1,the isolate (S1,1) showed maximum identity with Bacillus sp. CCMMB848 *(97.12%)* having accession number KF879230.1, the isolate (S1,2)showed maximum identity with *Bacillus* sp. BAB-643 *and Bacillus subtilis (95.39%)* having accession number KF913673.1 and MK574945.1, respectively. Phylogenetic study of the isolates with other bacterial isolates occurring as thermophiles showed a close affiliation with thermophilic *Bacillus* species and is grouped within the clade of thermophiles. This validates the isolate to be a thermophilic *Bacillus* species. The ability of this genus to tolerate harsh environment may be one of the reasons for the availability of the genus in all samples in that area. The phylogenetic trees for the bacterial species are as follows (Figure 9).

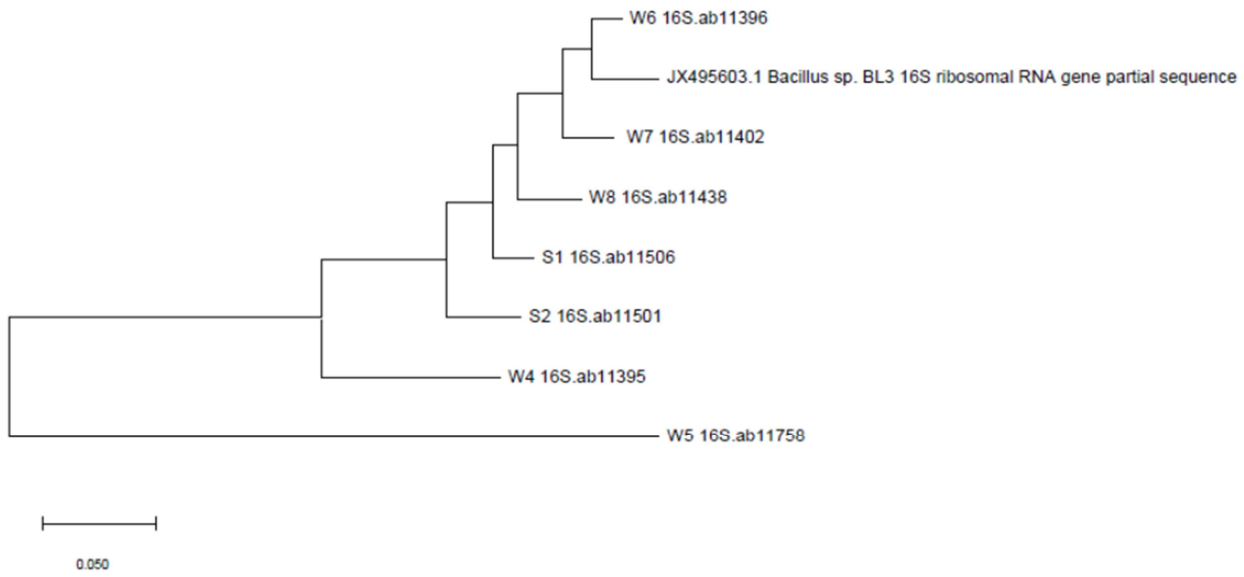


Figure 9: Phylogenetic tree representing thermophilic bacteria.

The The GC content of the isolate was also found to be relatively high (Figure 10). This might be the reason for its ability to survive in high temperature [23]. However, recent report of Wu *et al*. suggests that higher GC content could not only be the sole reason for an organism to survive in extreme temperature [24]. Similarly, Hurst and Merchant reported that high GC content is not an adaptation to high temperature among prokaryotes. Thus GC content cannot be taken into account to explain the adaptability of thermophiles in high temperature [25, 26]. Even this study is not the first report of thermophilic bacteria in springs, still it is the first reported in Khurmal area which may open the way to study these bacterial species in detail, expression of different enzymes in these isolates.

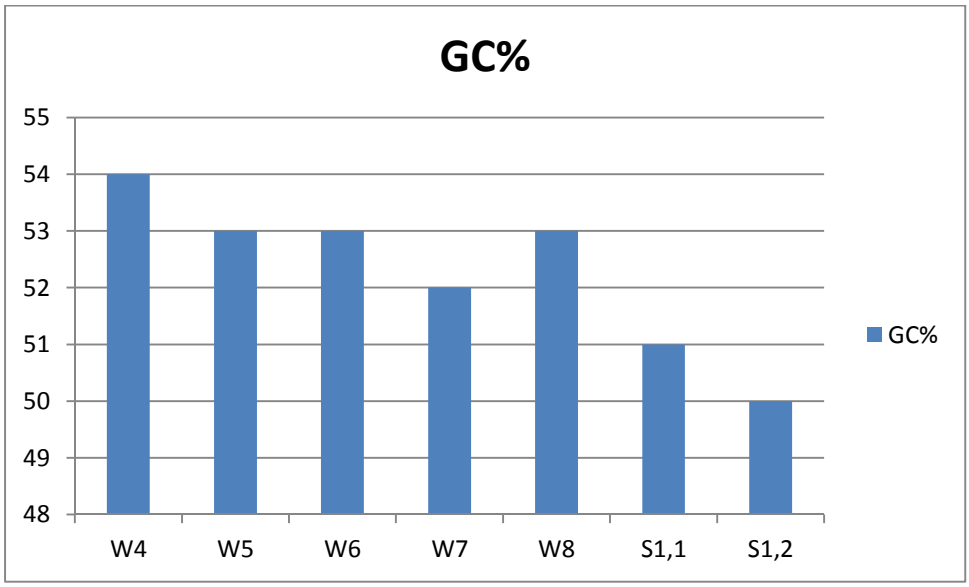


Figure 17: Show GC% for seven isolates.

Conclusion

Different thermophilic bacteria, *Bacillus sp.* were isolated and their enzymatic properties were characterized. This is the first ever report on thermophilic bacteria in the Khurmal spring in Kurdistan Region. The results are encouraging and providing an insight for the potential production of thermostable enzymes from the isolated bacterial strains. This study will provide the opportunity for further researches to

explore more specific strains. This study clearly revealed that *bacillus sp.* represents a potential source for some industrial enzymes that can be exploited in biotechnological purposes.

Acknowledgment

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Table Sup 1: Amylase Activity of the isolates at different pH and temperature

	W4			W5			W6			W7			W8			S1			S2		
pH	25 ˚C	37 ˚C	80 ˚C	25 ˚C	37 ˚C	80 ˚C	25 ˚C	37 ˚C	80 ˚C	25 ˚C	37 ˚C	80 ˚C	25 ˚C	37 ˚C	80 ˚C	25 ˚C	37 ˚C	80 ˚C	25 ˚C	37 ˚C	80 ˚C
6	7.85	8.16	3.14	9.69	9.84	1.26	10.27	7.30	5.32	9.95	6.07	0.88	6.74	3.86	2.61	10.04	5.95	1.59	13.84	12.55	0.56
7	5.61	0.98	7.53	6.85	2.10	9.80	9.69	0.73	9.71	9.17	0.48	8.88	6.31	0.63	5.50	9.49	0.78	7.26	12.91	0.87	14.95
8	0.58	0.80	1.16	0.66	1.21	1.76	1.14	0.57	2.44	0.98	0.60	0.49	0.76	0.43	0.42	9.49	0.58	0.53	0.55	0.89	0.47