JJBS

Volume 16, Number 3,September 2023
ISSN 1995-6673
Pages 413 – 424
https://doi.org/10.54319/jjbs/160304

Jordan Journal of Biological Sciences

Moderately Thermophilic Bacteria from Jordanian Hot Springs as Possible Sources of Thermostable Enzymes and Leukemia Cytotoxic Agents

Maher Obeidat[1,*], Belal Al-Shomali[2]

[1]Department of Medical Laboratory Sciences, Faculty of Science, Al-Balqa Applied University, Al-Salt, Jordan;[2]Department of Biotechnology, Faculty of Agricultural Sciences, Al-Balqa Applied University, Al-Salt, Jordan.

Received: December 8, 2022; Revised: January 22, 2023; Accepted: January 28, 2023

Abstract

This study was conducted to isolate and identify thermophilic and thermotolerant bacteria from Jordanian hot springs and to determine hydrolytic, antimicrobial, and anticancer activities of the isolates. Thirty bacterial isolates were recovered from water samples of five main local hot springs. Nineteen of the isolated colonies were light yellow and circular to rhizoid on nutrient agar; cells were Gram-positive, endospore-forming, and rod-shaped. Eleven isolates were Gram-negative non-spore forming rods. It was found that 21 isolates met the criteria of moderate thermophiles; all isolates were grown aerobically (JA5 was facultative anaerobes) at 40-60 °C, pH 6-9, and 0-4% salt concentration and most of these isolates were reacted positively with catalase and oxidase. The remaining nine isolates were thermotolerant. Depending on the 16S rRNA gene sequences of the isolates, it was found that 19 thermophilic isolates have 97-100% sequence homology to the genus *Bacillus*; eight isolates were closely related to the thermophilic genus *Geobacillus* showing 97-100% homology to *G. stearothermophilus* ATCC 7953. The isolate JM2 shares 99% sequence homology with *Thermomonas hydrothermalis*. Remarkably, it was found that the 16S rDNA sequence of isolate JZ9 were highly similar (99% identity) to the thermophilic bacterium *Caldimonas hydrothermale*. To our knowledge, this is the first record of *Caldimonas* isolation from Jordanian hot springs. A wide spectrum of hydrolytic activities for protease, lipase, xylanase, cellulase, amylase, and pectinase was detected from the obtained isolates. It was found that JM1, JS3, and JZ11 isolates produced all tested enzymatic activities. Antimicrobial activities were only exhibited by three isolates (thermophilic JH1 and JM11 and thermotolerant JS3). Results indicated that three thermophilic *Bacillus* isolates (JA2, JM11, and JM12) produced selective cytotoxicity against human leukemia cell line K562. Therefore, many of the obtained isolates in this study can be considered as a promising source of effective agents that may be used for medical, pharmaceutical, and industrial purposes.

Keywords: Hot spring; Thermophilic; Thermotolerant; *Caldimonas*; Enzymatic; Leukemia

1. Introduction

Thermophiles can live and reproduce in hot environments where they have been able to grow at high temperature and they have been isolated from many geothermal sites such as hot springs. They are classified into moderate (40 to 70 ºC), extreme (55 to 85 ºC), and hyperthermophiles (75 to 113 ºC) based on their growth temperatures (Baker *et al*., 2001). Generally, moderate thermophiles are primarily bacteria (Baker *et al*., 2001). Interest in thermophilic bacteria has come from their significant potential for production of valuable compounds such as thermostable enzymes, antibiotics, and hormones (Maugeri *et al*., 2001; Singh, 2006).

Hot springs are fairly distributed in Jordan and formed due to volcanic activity or movement of the Earth's crust which form a pressure leads to the upward mobilization of heated water (Simoneit *et al*., 2000). Different microbial communities have been successfully colonized in hot springs predominantly thermophilic bacteria such as *Bacillus* and thermophilic archaea, for example *Methanococcus* and *Sulfolobus* (Chen and Roberts, 1999; Lengeler *et al*., 1999; Saul *et al*., 1999). The genus *Bacillus* is highly diverse and includes many important thermophilic and thermotolerant species that have biotechnological significance as a sources of thermostable enzymes were industrially important such as proteases, lipases, xylanases, amylases, cellulases, pectinases, and DNA restriction endonucleases as well as DNA polymerases including *Taq* DNA polymerase that used for polymerase chain reaction (PCR) (Maugeri *et al*., 2001).

Thermophilic bacteria have been adapted to hot environments by often having high G+C content in their DNA, more H-bonds, and having reverse DNA gyrase (producing positive supercoils in the DNA) which rise the melting point of DNA (Galtier and Lobry, 1997). In addition, thermophilic bacteria adapt to high temperatures by increased electrostatic, disulphide, and hydrophobic interactions in their proteins. The membrane fatty acids of thermophilic bacteria are highly saturated to remain stable and functional at high temperatures (Galtier and

* Corresponding author. e-mail: obeidat@bau.edu.jo.

Lobry, 1997). Compared with their mesophilic counterparts, the structural and functional proteins of thermophiles are very heat stable due to dehydration and more hydrophobic amino acids and salt bridges (Karlin, *et al*., 2002).

Jordan has a unique ecological location with extreme environments including hot springs. There are about 200 thermal springs in Jordan distributed in three territories (Swarieh, 2000). Interest in isolation and identification of thermophilic organisms from hot springs in Jordan has been growing in recent years. Most of the isolated bacteria in such springs were found to belong to the genus *Bacillus* (Khalil, 2002; Malkawi and Al-Omari, 2010; Fandi *et al*., 2012; Obeidat *et al*. 2012; Mohammad *et al*. 2017). Moreover, the ability of the isolated thermophilic *Bacillus* species to produce thermostable enzymes that have importance in industrial and biotechnological applications was also investigated (Al-Qodah, 2006; Obeidat *et al*., 2012; Mohammad *et al.* 2017). Therefore, this study was conducted to isolate and identify thermophilic bacteria from Jordanian hot springs and to determine their extracellular enzymatic activity as well as their antimicrobial activity. Furthermore, because all current treatments for cancer have not until now adequate and have not been conducted on the level of medical community satisfaction, this research was also aimed to evaluate the possible anticancer effect of isolated bacteria from local hot springs.

2. Materials and Methods

2.1. Collection of samples and isolation of bacteria

Fifty water samples (10 from each selected hot spring) were collected during summer/ 2011 from five main Jordan hot springs (Hammat Afra, Jordan Himma, Shuna-North, Zara-Bani Hamida, and Ma`in-Roman Bath). The samples were collected in 500 ml sterile containers from 20-40 cm below the surface away from the margin. After filtration of water samples through 0.45 µm membrane filter, the remaining residues on membrane filter were resuspended in 10 ml of sterile water. A 100 µl aliquot from each sample was plated by spreading on nutrient agar (NA) plates (five replicates) and incubated, aerobically and anaerobically, for 48 hr at 40°C. The developing colonies were selected and subcultured on NA medium.

2.2. Phenotypic and physiological characterization of isolates

Phenotypic characterestics including colony and cell morphology, Gram and endospore stainings along with catalase and oxidase activity were performed for each isolate according to the standard protocols. To determine the cardinal temperatures and pH ranges for the growth of each isolate, the isolates were incubated in NB medium at 20°C to 80°C with an interval of 5 units and at pH in the range 4.0-12.0 in NB medium with an interval of 0.5 unit using hydrochloridric acid (HCl) or sodium hydroxide (NaOH). Growth was checked after 24 hr of incubation. The effect of sodium chloride (NaCl) concentration on the growth of isolates was studied by incubating the bacterial isolates at 40°C for 24 hr in 10 ml NB medium containing 0.0 to 10% NaCl with 0.5% interval. Growth was checked after 24 hr of incubation.

2.3. PCR amplification, sequencing, and phylogenetic analysis of the 16S rRNA gene

Luria Bertani (LB) broth cultures of bacterial isolates and the reference strain *Geobacillus stearothermophilus* ATCC 7953 were incubated overnight with shaking at 150 rpm at 40°C. After centrifugation of LB cultures at 14000 rpm for 5 min, cell pellets were washed two times with distilled water, then the genomic DNA was extracted, using Wizard Genomic DNA purification kit (Promega, USA, part no. A1120), according to the manufacturer's instructions.

The 16S rRNA gene from purified genomic DNA was amplified by PCR according to Belduz *et al*. (2003) using the forward primer UNI16S-L (5′-ATTCTAGAGTTTGATCATGGCTCA- 3′) and the reverse primer UNI16S-R (5′-ATGGTACCGTGTGACGGGCGGTGTGTA- 3′). The PCR products with predicted size of ~1400 bp were analyzed by 1.5% agarose gel electrophoresis using 10 µl of each PCR sample. The 1 kb DNA ladder marker (Genedirex, USA) was used and the generated bands were digitally photographed under UV light.

Using the ABI PRISM cycle sequencing kit (Macrogen, Korea), the 16S rRNA gene sequences from the isolates and the reference strain *G. stearothermophilus* ATCC 7953 were determined. They were then compared with those found in GenBank using BLAST search. Then, the phylogenetic tree was created by DNAMAN software for the closely related sequences that were retrieved from the database.

2.4. Extracellular enzymatic activity

Six enzymatic activities were assessed for each bacterial isolate; protease, lipase, xylanase, cellulase, amylase, and pectinase activities were detected using the diffusion agar method on skim milk agar medium, lipase test medium, xylanase test medium, carboxymethylcellulose (CMC) medium, starch medium, and polygalacturonase (PGase) test medium, respectively. Sterile cork borer (6 mm i.d.) was used to make wells in each medium and 50 µl from bacterial NB-culture (about 10^6 CFU / ml) was added into each well and left for 1 hr for proper diffusion (Gessner, 1980; Priest *et al.*, 1988; Bragger *et al*., 1989; Kobayashi *et al.*, 1999; Haba *et al.*, 2000; Ten *et al.*, 2004).

For protease and lipase tests, after incubation at 40°C for 48 hr, the development of clear zones around wells indicated the presence of proteolytic activity (Priest *et al*. 1988) and the appearance of opaque halos around the wells demonstrated a positive lipase activity (Haba *et al.*, 2000). For xylanase and cellullase activities, after incubation for 3 days at 40 ºC, 0.1% Congo red solution was poured onto the plates and left for 30 minutes at room temperature. Then, the plates were washed with 1 M NaCl solution. Clear zones around the wells on a red background were taken as the evidence for the xylanase and cellulase activities (Bragger *et al*., 1989; Ten *et al.*, 2004). After incubation for 48 hr at 40 ºC, starch plates were flooded with 1% iodine solution to determine amylase activity. The development of yellow clear zones around the wells against a blue background was interpreted as an indication of positive α-amylase activity (Gessner, 1980; Bragger *et al*., 1989). After incubation of PGase medium for 3 days at 40 ºC, pectinase activity was determined by pouring 1%

cetyltrimethylammoniumbromide (CTAB) solution onto the surface of the plates and left for 10 min at room temperature. The formation of clear zones around wells was taken as an indication of positive pectinase activity (Kobayashi *et al.*, 1999).

2.5. Preparation of bacteria for antimicrobial and anticancer activities

To examine the antimicrobial and anticancer activities, cultures of isolated bacteria from hot springs were grown in 25 ml NB at 40°C for one week and centrifuged at 13,000 rpm for 10 min. The supernatant was evaporated after being filtered through a 0.45 µm membrane filter. To get a concentration of 200 mg/ml, the leftover residues were resuspended in a suitable amount of phosphate buffer saline (PBS).

2.6. Antimicrobial activity

To research an isolate's antibacterial and antifungal properties, 11 reference bacterial species (*Staphylococcus aureus* ATCC 25923 and Methicillin resistant *S. aureus* ATCC 95047 (MRSA), *Escherichia coli* ATCC 8739 and ATCC 25922, *Klebsiella pneumonia* ATCC 7700 and *K. oxytoca* ATCC 13182, *Proteus vulgaris* ATCC 33420 and *P. mirabillis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 27253, *Salmonella typhimurium* ATCC 14028 and *Enterobacter aerogenes* ATCC 35029) were cultured in NB at 37°C for 24 hr to achieve $2x10^6$ CFU/ml and two fungal species (*Candida albicans* ATCC 10231 and *Aspergillus brasiliensis* ATCC 16404) were cultured in sabouraund dextrose broth (SDB) at 28 °C for 48 hr that adjusted to achieve $2x10^5$ spore/ml for fungi (Ceylan *et al.*, 2008).

Resuspended crudes of each bacterial isolate were screened for antimicrobial activities using the agar-well diffusion method. A 50 µl aliquot from each test microorganism was swabbed on NA medium for bacteria and sabouraund dextrose agar for fungi. A sterile cork borer (6 mm i.d.) was used to create three wells in the medium. Following that, 50 µl of the crude (10 mg) from each isolate was carefully poured to each well and incubated at 40°C for 48 hours after being let to stand on the bench for 1 hr. The antibacterial and the antifungal activities were measured by assessment of the diameter of generated inhibition zones in mm (Ceylan *et al.*, 2008). Data were expressed as the mean ± standard deviation (SD).

2.7. Hemolytic and anticancer activities

The type of hemolysis for bacterial crudes was verified by inoculating 50 µl of crude into each well (6 mm i.d.) prepared on blood agar plates and incubating the plates at 40°C for 48 h (Carillo *et al.*, 1996).

Normal mammalian Vero cells and human leukemia cancer cell line K562 were used to investigate the anticancer activities of non-hemolytic bacterial crudes. The K562 cells and Vero cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium and in Dulbecco's Modified Eagle Medium (DMEM) medium, respectively, and incubated at 37°C in a humidified 5% CO_2 incubator according to Freshney (1987). The cells were harvested, subcultured, and reseeded in fresh medium every 48 h.

To determine the selective cytotoxicity of non-hemolytic bacterial crudes, 200 µl of non-hemolytic bacterial crude (2 mg/well) was added to 200 µl of freshly harvested cells and 100 µl medium in each 96 well micro test plate. Then, the plates were incubated at 37°C in a humidified 5% CO_2 incubator for 48 hr. The viability of cells was determined by MTT assay as previously demonstrated by Mosmann (1983) and Heiss *et al.* (1997) using ELISA microplate reader at 450 nm with 630 nm reference wavelength. Each treatment was performed in triplicate and repeated five times.

2.8. Statistical Analysis

The cytotoxicity of non-hemolytic bacterial crudes was calculated according to Obeidat (2017), and the median inhibitory concentration (IC_{50}) was determined by comparing the average of mortality values with control by using the most fit non-linear regression analysis software.

3. Results

3.1. Isolation of bacteria from Jordanian hot springs

In the current study, the existence of thermophilic bacteria in hot water was investigated in five main hot springs distributed through the three territories of Jordan (Table 1), including Jordan-Himma and Shuna-North in North territory, Ma`in-Romman Bath and Zara-Bani Hamida in Middle territory, and Hammat Afra in South territory. The water temperatures and pH of thermal vents are approximately 40-65 °C and 6.0-7.0, respectively. Table 1 demonstrated that a total of 30 diverse bacterial isolates were obtained from the screened water samples.

Table 1. Diversity of moderately thermophilic and thermotolerant bacteria from five main Jordanian hot springs

Territory	Thermal Vent	Water Temperature (°C)	Water pH	No. of Selected Isolates
North	Jordan Himma	42.1	7.0	7
	Shuna-North	54.5	6.6	2
Middle	Ma`in-Roman Bath	62.3	6.0	8
	Zara-Bani Hamida	54.1	6.3	8
South	Hammat Afra	48.2	6.5	5
	Total			30

3.2. Phenotypic and physiological characterization of isolates

Regarding morphological, physiological, and some biochemical properties, 21 bacterial isolates obtained from hot springs were unable to grow below 40 °C and met the criteria of thermophilic bacteria (Table 2 and 3). Out of them, 19 were Gram-positive, rod-shaped, endospore-forming, and most of their developed colonies on nutrient agar were light yellow circular to rhizoid (Table 2), whereas the remaining thermophilic isolates JM2 and JZ9 were Gram-negative non-spore forming rods and produced light brown and transparent colonies, respectively (Table 3). On the other hand, nine isolates (JA4, JH7, JH8, JM13, JM14, JS3, JZ11, JZ12, JZ14) were found thermoltolerant (can grow below 40 °C) Gram-negative non-spore forming rods (Table 3). As shown in Tables 2 and 3, all isolates were able to grow aerobically (JA4, JA5, JH8, JM14 and

JS3 were facultative anaerobes) and exhibited positive-catalase and positive-oxidase for 13 thermophilic isolates (JA2, JA5, JH4, JM1, JM2, JM5, JM7, JM11, JM12, JS1, JZ1, JZ5, JZ9, and JZ13) and for two thermotolerant isolates (JH7 and JZ14).

All thermophilic isolates were capable to grow at 40 to 60 °C (optimum growth temperature at 50 °C; except JA5 has 55 °C optimum temperature). However, most thermotolerant isolates were able to grow between 30 to 60 °C and the optimum growth temperature was in the range of 30-40 °C (Table 2 and 3). Moreover, all isolates were able to grow at pH 6 to 9 and in 0-4% salt concentration; six thermophilic isolates (JA2, JA3, JH3, JH4, JH6, and JM5) and one thermotolerant isolate (JM13) were found tolerant to 10% NaCl concentration.

For further identification of Gram-negative non-spore forming rod-shaped bacteria, some biochemical tests were investigated (Table 3). All isolates were negative for indole and methyl red (MR). JZ9 was the only isolate that exhibited urease activity. It was found that four isolates (JM13, JM14, JZ11, and JZ12) were non-motile, catalase-positive, oxidase-negative, and reacted negatively for Voges-Proskauer (VP), citrate, nitrate, and triple sugar iron (TSI) tests (Table 3), three isolates (JA4, JH8, and JS3) were motile, catalase-positive, oxidase-negative, and were reacted positively for VP, citrate, nitrate, and TSI (ferment glucose, lactose and/or sucrose, and give gas bubbles). Isolates JH7 and JZ14 were found motile, catalase-positive, oxidase-positive, utilize citrate, do not reduce nitrate, and give negative TSI (Table 3). The thermophilic isolates JM2 and JZ9 were non-motile and positive for catalase, oxidase, and citrate tests. JM2 was negative for the other tests, while JZ9 was positive for the urease and nitrate tests.

Table 2. Phenotypic and growth characteristics of thermophilic Gram-positive bacterial isolates obtained from Jordanian hot springs

Hot Spring	Isolate	Colony Morphology	Catalase / Oxidase	Gram Stain	Cell Shape	Spore former	O_2[a]	Temperature (optimum)[b]	pH	NaCl %
Hammat Afra	JA1	Light yellow, rhizoid, rigid	+ / -	+	Rod	+	+	40-60 (50)	5-11	0-4
	JA2	Light yellow, Flat, circular	+ / +	+	Rod	+	+	40-60 (50)	4-10	0-10
	JA3	Light yellow, circular, convex	+ / -	+	Rod	+	+	40-60 (50)	4-10	0-10
	JA5	Light yellow, rhizoid, mucoid	+ / +	+	Rod	+	±	40-65 (55)	5-10	0-4
Jordan Himma	JH1	Light yellow, circular	+ / -	+	Rod	+	+	40-60 (50)	6-9	0-4
	JH3	Transparent, irregular	+ / -	+	Rod	+	+	40-60 (50)	4-10	0-10
	JH4	Light yellow, irregular	+ / +	+	Rod	+	+	40-60 (50)	4-10	0-10
	JH5	Light yellow, irregular, mucoid	+ / -	+	Rod	+	+	40-60 (50)	5-10	0-5
	JH6	Light yellow, flat, circular	+ / -	+	Rod	+	+	40-60 (50)	5-10	0-10
Ma`in-Roman Bath	JM1	Paige, rhizoid, mucoid	+ / +	+	Rod	+	+	40-60 (50)	6-9	0-4
	JM5	Paige, circular	+ / +	+	Rod	+	+	40-60 (50)	4-11	0-10
	JM7	Light yellow, rhizoid, rigid	+ / +	+	Rod	+	+	40-60 (50)	5-11	0-8
	JM11	Milky, undulate margins	+ / +	+	Rod	+	+	40-60 (50)	4-10	0-5
	JM12	Yellow, rhizoid	+ / +	+	Rod	+	+	40-60 (50)	4-10	0-5
Shuna-North	JS1	Light yellow, circular	+ / +	+	Rod	+	+	40-60 (50)	6-9	0-5
Zara-Bani Hamida	JZ1	Light yellow, rhizoid, rigid	+ / +	+	Rod	+	+	40-60 (50)	6-11	0-7
	JZ5	Light yellow, rhizoid, mucoid	+ / +	+	Rod	+	+	40-60 (50)	6-9	0-5
	JZ10	Dull gray, undulate margins	+ / -	+	Rod	+	+	40-60 (50)	4-10	0-5
	JZ13	Light yellow, Flat, circular with undulate margin	+ / +	+	Rod	+	+	40-60 (50)	4-10	0-5

[a]O_2 Requirement: +; aerobic, ±; facultative anaerobes. [b]Temperature is measured in ^oC.

Table 3. Phenotypic and growth characteristics of thermotolerant and thermophilic Gram-negative bacterial isolates obtained from Jordanian hot springs

Isolate	JA4	JH7	JH8	JM2	JM13	JM14	JS3	JZ9	JZ11	JZ12	JZ14
Colony Morphology	Gray, moist, smooth	Yellow, circular	Translucent, circular	Light brown, circular	Opaque yellow, mucoid	milky, muciod, smooth	Light yellow, circular	Transparent circular	Gray, domed, muciod, smooth	pale, mucoid	Yellow, circle
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	+	-	+	-	-	-	+	-	-	+
Gram Stain	-	-	-	-	-	-	-	-	-	-	-
Cell Shape	Rod	Rod	Rod	Rod	Coccobacilli	Rod	Rod	Rod	Coccobacilli	Coccobacilli	Rod
Sporulation	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	-	-	-	+	+	-	-	+
O_2[a]	±	+	±	+	+	±	±	+	+	+	+
Temperature (optimum)[b]	20-65 (35)	20-60 (40)	20-65 (35)	40-60 (50)	30-60 (40)	30-60 (40)	20-55 (40)	40-65 (50)	30-60 (40)	35-60 (40)	30-60 (40)
pH	5-10	5-10	5-11	6-9	4-10	5-10	5-9	8-9	4-10	4-10	5-10
NaCl %	0-4	0-5	0-4	0-4	0-10	0-4	0-4	0-6	0-5	0-5	0-5
Indole	-	-	-	-	-	-	-	-	-	-	-
Methyl red	-	-	-	-	-	-	-	-	-	-	-
Voges Proskauer	+	-	+	-	-	-	+	-	-	-	-
Citrate	+	+	+	-	-	-	+	+	-	-	+
Urease	-	-	-	-	-	-	-	+	-	-	-
Nitrate	+	-	+	-	-	-	+	+	-	-	-
TSI[c]	A/A,+,-	K/K,-,-	A/A,+,-	K/K,-,-	K/K,-,-	K/K,-,-	A/A,+,-	ND	K/K,-,-	K/K,-,-	K/K,-,-

[a]O_2 Requirement: +; aerobic, ±; facultative anaerobes.

[b]Temperature is measured in ^oC.

[c]TSI: Triple Sugar Iron; A/A,+,-: glucose, lactose and/or sucrose fermentation, gas bubbles production but no H_2S production; K/K,-,-: no fermentation, no gas bubbles and no H_2S production.

3.3. Molecular characterization and sequence alignment

To confirm the conventional methods of classification of the bacterial isolates, the 16S rRNA gene sequence of the isolates and *G. stearothermophilus* ATCC 7953 was investigated by amplification with UNI16S-L and UNI16S-R primers and production of PCR band with about 1400 bp in size (Figure 1). Based on the 16S rDNA sequences' BLAST matching to GenBank sequences, 19 thermophilic isolates from all examined hot springs were found to belong to the genus *Bacillus*/*Geobacillus* with 97-100% identity and the thermophilic Gram-negative bacterial isolates JM2 and JZ9 showed 99% homology to the genera *Thermomonas* and *Caldimonas*, respectively (Table 4). For thermotolerant Gram-negative bacterial isolates, BLAST alignment of GenBank sequences demonstrated that four isolates (JM13, JM14, JZ11 and JZ12) were closely related to the genus *Acinetobacter* with 96-99% identity. Isolates JA4 and JH8 were shown 98% identity to the genus *Enterobacter*. Isolates JH7 and JZ14 were found closely related, with 97% and 99% identity respectively, to the genus *Pseudomonas*. The remaining isolate JS3 was found closely related, with 96% identity, to the genus *Cronobacter* (Table 4).

Based on the obtained sequences, a homology matrix and a phylogeny tree were created as shown in Figure 2. The phylogenetic analysis of the 16S rDNA sequences illustrated that Gram-positive and Gram-negative isolates were allocated into two separate clades and reflected the affiliation of eight thermophilic isolates (JA1, JH1, JH5, JM1, JM5, JS1, JZ1, and JZ5) to *Geobacillus* with 97-100% sequence homology to the reference strain *G. stearothermophilus* ATCC 7953; the 16S rRNA gene sequence of isolates JA1, JH1, and JM1 had 100% identity to *G. stearothermophilus* ATCC 7953. Moreover, it was observed that the 16S rDNA sequence of nine thermophilic isolates had 97 to 100% identity to the thermophilic bacteria *Bacillus licheniformis* (Table 4).

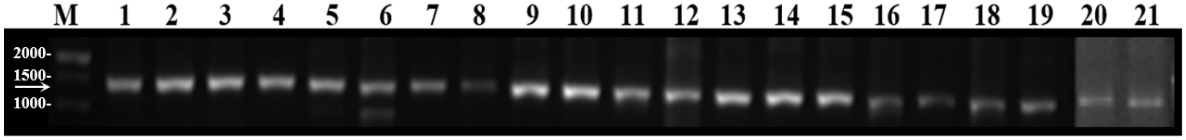

Figure 1. Electrophoresis of PCR amplification of 16S rRNA gene fragments with forward primer UNI16S_L and reverse primer UNI16S_L of thermophilic isolates on 1.5% agarose gel. Lanes 1-21: isolates JA1, JA2, JA3, JA5, JH1, JH3, JH4, JH5, JH6, JM1, JM2, JM5, JM7, JM11, JM12, JS1, JZ1, JZ5, JZ9, JZ10, and JZ13, respectively. Lane M: 1 kb DNA ladder marker (Genedirex, USA); the molecular size of DNA bands is in base pairs (bp). The left arrow indicated 1400 bp band.

Table 4. Comparing the 16S rRNA gene sequences of the 30 isolates to those at GenBank

Thermal Vent	Isolate	Sequence		
		No. of aligned nucleotides	Closest phylogenetic relative (GenBank accession No.)	% identity[a]
Thermophilic Isolates				
Hammat Afra	JA1	1308	*Geobacillus stearothermophilus* (HQ143640)	99
	JA2	1359	*Bacillus licheniformis* (CP022477)	98
	JA3	1356	*Bacillus licheniformis* (DQ071568)	97
	JA5	1394	*Bacillus licheniformis* (JX847115)	97
Jordan Himma	JH1	1369	*Geobacillus stearothermophilus* (HQ143640)	100
	JH3	1237	*Bacillus* sp.(C4KC310834)	98
	JH4	1252	*Bacillus licheniformis* (FJ614258)	97
	JH5	1381	*Geobacillus* sp. RSNPB7 (HM588147)	97
	JH6	1311	*Bacillus licheniformis* (KP050497)	97
Ma`in-Roman Bath	JM1	1237	*Geobacillus stearothermophilus* (HQ143640)	99
	JM2	1284	*Thermomona hydrothermalis* (AF542054)	99
	JM5	1271	*Geobacillus* sp. RSNPB7 (HM588147)	99
	JM7	1082	*Bacillus licheniformis* (DQ071560)	99
	JM11	1341	*Bacillus* sp.(C4KC310834)	97
	JM12	1370	*Bacillus licheniformis* (FJ614258)	100
Shuna-North	JS1	1134	*Geobacillus* sp. RSNPB7 (HM588147)	97
Zara-Bani Hamida	JZ1	1240	*Geobacillus kaustophilus* (NC006510)	99
	JZ5	1373	*Geobacillus* sp. RSNPB7 (HM588147)	97
	JZ9	1291	*Caldimonas hydrothermale* (HE798193)	99
	JZ10	1391	*Bacillus licheniformis* (KC443100)	97
	JZ13	1398	*Bacillus licheniformis* (JQ411812)	100
Thermotolerant Gram-negative Isolates				
Hammat Afra	JA4	1438	*Enterobacter* sp. (GQ418085)	98
Jordan Himma	JH7	1291	*Pseudomonas* sp. (DQ205301)	97
	JH8	1300	*Enterobacter* sp. (JN697628)	98
Ma`in-Roman Bath	JM13	1365	*Acinetobacter calcoaceticus* (JN700142)	99
	JM14	1356	*Acinetobacter baumannii* (JN668579)	97
Shuna-North	JS3	1099	*Cronobacter sakazakii* (HQ880366)	96
Zara-Bani Hamida	JZ11	1372	*Acinetobacter baumannii* (JX966428)	97
	JZ12	1399	*Acinetobacter baumannii* (JN669235)	96
	JZ14	1345	*Pseudomonas* sp. (DQ205301)	99

[a]The percentage identity with the 16S rRNA gene sequence of the closest phylogenetic relative of bacteria.

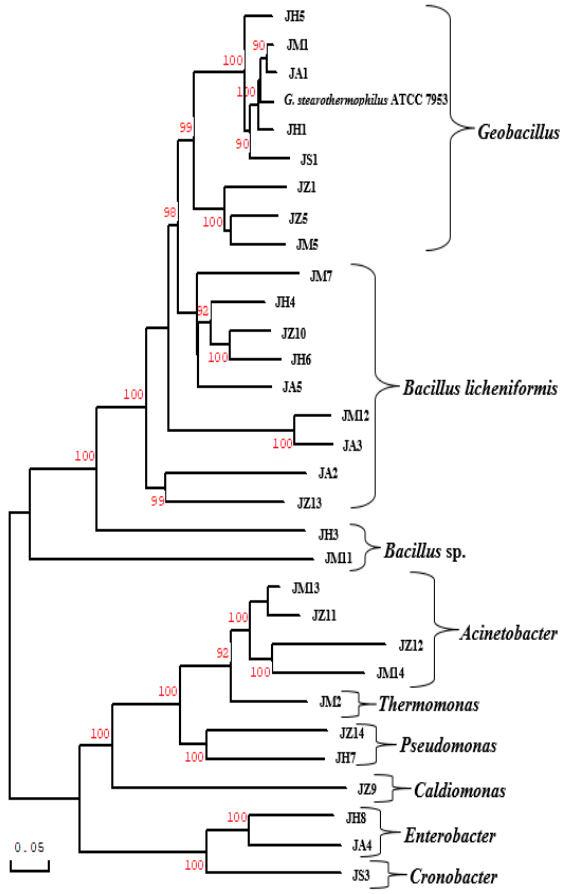

Figure 2. Phylogenetic analysis of 16S rDNA sequences of 21 thermophilic isolates, 9 thermotolerant isolates, and *G. stearothermophilus* ATCC 7953. The phylogenetic tree is based on the maximum likelihood parameter analysis. The bootstrap confidence values are shown at the nodes and expressed as percentages of 1000 replications.

3.4. Hydrolytic Activities

Figure 3 illustrates the distribution of hydrolytic activities among 30 bacterial isolates obtained from thermal water of five hot springs investigated in this study. It was found that Jordanian hot springs were rich in bacteria producing protease (27 isolates), lipase (20 isolates), xylanase (22 isolates), cellulase (18 isolates), amylase (20 isolates), and pectinolytic/polygalacturonase (16 isolates).

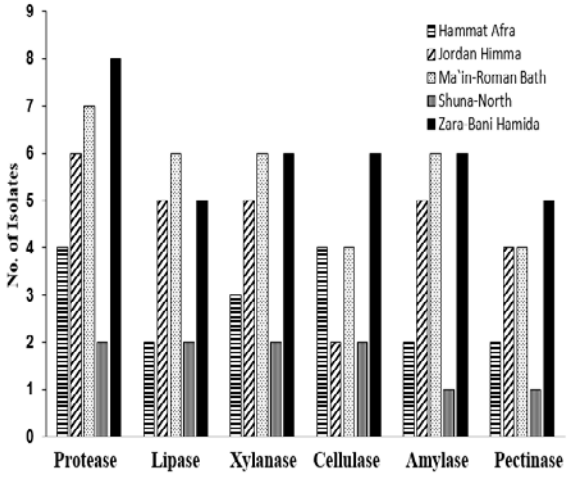

Figure 3. Hydrolytic activities of the obtained 30 bacterial isolates from five Jordanian hot springs.

Results of hydrolytic activities produced from tested thermophilic and thermotolerant bacterial isolates obtained from local hot springs indicated that most isolates produced wide spectrum of enzymes (Table 5). Interestingly, it was found that one thermophilic isolate (JM1) and two thermotolerant isolates (JS3 and JZ11) produced all enzymatic activities tested in this study. All isolates produced protease activity except JA4, JH6, and JM14. The degree of protease activity in most isolates was high. Lipase and amylase activities, ranged from low to very high, were observed in two-third of the isolates. More than 50% of the isolates had polygalacturonase activity ranged from low to high. It was noticed that the degree of enzymatic activities was low to moderate in all isolates producing xylanase or cellulase.

Table 5. Enzymatic activities of bacterial isolates obtained from Jordanian hot springs

Hot Spring	Isolate	Protease	Lipase	Xylanase	Cellulase	Amylase	Polygalacturonase
Hammat Afra	JA1	+	++	++	++	-	+++
	JA2	++++	-	-	+	++	+
	JA3	++	-	+	++	-	-
	JA4	-	-	+	+	-	-
	JA5	++++	++	-	-	++	-
Jordan Himma	JH1	++++	+	+	-	-	+++
	JH3	++	+++	++	-	+++	-
	JH4	+++	++	-	-	+++	+
	JH5	+++	-	-	-	-	++
	JH6	-	+++	++	-	+	-
	JH7	+++	+++	+	++	+++	-
	JH8	++	-	+	+	++	++
Ma`in-Roman Bath	JM1	++	+++	++	+	++++	+
	JM2	+++	+	-	+	+++	-
	JM5	+++	++	++	+	+++	-
	JM7	++	++	+	-	++++	-
	JM11	+++	++	+	-	-	+
	JM12	++	++	+	-	++	+
	JM13	++++	-	+	-	++	+
	JM14	-	-	-	+	-	-
Shuna-North	JS1	+++	++	++	+	-	-
	JS3	++	+	+	+	+++	+
Zara-Bani Hamida	JZ1	++	++	-	+	+++	++
	JZ5	+	++	+	-	++++	-
	JZ9	+	++	+	+	-	+
	JZ10	++++	-	++	++	+++	-
	JZ11	++	++	+	+	+++	++
	JZ12	+++	++	+	+	-	-
	JZ13	+++	-	-	-	+++	+
	JZ14	+++	-	++	+	++++	+

The degree of enzymatic activity was graded on the basis of the inhibition zone diameter (millimeter): ++++, very high (≥31); +++, high (21 to 30); ++, moderate (11 to 20); +, low (7 to 10); ±, very low (1 to 6); -, no inhibition

3.5. Antimicrobial activities

The inhibitory effects of thermophilic and thermotolerant bacterial crudes were screened against 11 reference bacterial species and two fungal species. It was found that only three isolates (two thermophilic (JH1 and JM11) and one thermotolerant (JS3) isolated from Jordan Himma, Ma`in-Roman Bath, and Shuna-North, respectively) produced antimicrobial effect (Figure 4). Isolates JH1 and JS3 (allocated to genera *Bacillus* and *Coronobacter*, respectively) exhibited antibacterial activity against *K. pneumonia* ATCC 7700, whereas the *Bacillus* isolate JM11 showed anticandidal activity against *C. albicans* ATCC 10231. The remaining isolates exhibited neither antibacterial activity nor antifungal activity.

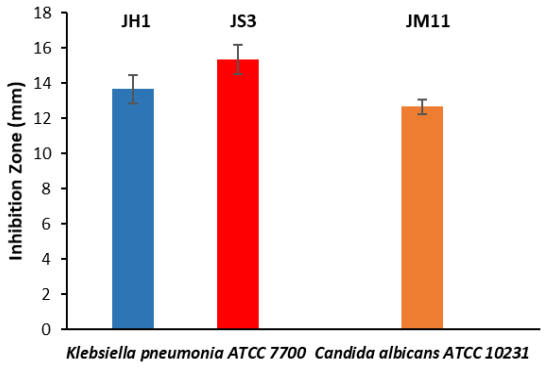

Figure 4. Antimicrobial activity of thermophilic and thermotolerant isolates against test microorganisms. Inhibition zone diameters are expressed as Means±SD of triplicate.

3.6. Hemolytic and anticancer activities

The crudes of six thermophilic *Bacillus* isolates (JA1, JA2, JA3, JA5, JM11, and JM12) and five thermotolerant Gram-negatve isolates (JA4, JM13, JM14, JZ12, and JZ14) were non-hemolytic (γ-type), whereas the remaining isolates displayed either α- or β-hemolysis against human erythrocytes (Data are not shown).

Non-hemolytic bacterial crudes were selected and screened for their ability to induce cytotoxic effect against normal Vero cells and leukemic K562 cell line (Figure 5). The viability of cells was determined by MTT assay. A total of three non-hemolytic thermophilic isolates obtained from Hammat Afra and Ma`in-Roman Bath (JA2, JM11, and JM12) exhibited very low to low cytotoxicity against Vero cells and very high (Inhibition is greater than 90%) selective cytotoxicity against K562 leukemic cells (Figure 5); selective cytotoxicity of an isolate is when the isolate crude had no to low cytotoxicity against Vero cells and had moderate to very high cytotoxicity against K562 leukemia cells. These cytotoxic isolates JA2, JM11, and JM12 were found to belong to the genus *Bacillus*. Isolate JM11 was the only selective cytotoxic isolate which had antimicrobial activity. As shown in Table 6, the IC_{50} values of the three cytotoxic bacterial crudes against K562 cells ranged from 1.48 to 1.93 mg, and the highest cytotoxic effect was obtained from JM12.

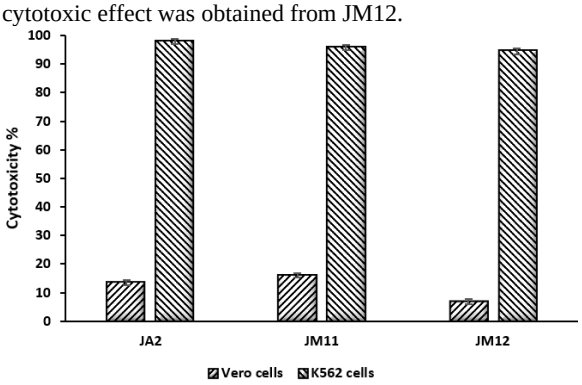

Figure 5. Cytotoxicity screening of non-hemolytic thermophilic and thermotolerant isolates against Vero cells and leukemic cell line K562**.** The degree of cytotoxicity was expressed as Means±SD and graded on the basis of the relative value of absorbance to the vehicle (Absorbance; Inhibition%): "very high (<0.1; >90%); high (0.1 to <0.4; >60% to 90%); moderate (0.4 to <0.7; >30% to 60%); low (0.7 to <0.9; >10% to 30%); very low (0.9 to <0.95; >5% to 10%); non-toxic (≥0.95, ≤5%)".

Table 6. Cytotoxicity of non-hemolytic thermophilic isolates against leukemic K562 cells

Thermal Vent	Isolate	IC_{50}[a] (mg)	R^2[b]	Regression equation[c]
Hammat Afra	JA2	1.93	0.99	y = 25.713Ln(x) + 33.138
Ma`in-Roman Bath	JM11	1.66	0.98	y = 29.518Ln(x) + 34.988
	JM12	1.48	0.99	y = 32.465Ln(x) + 37.186

[a]IC_{50}: the median inhibitory concentration.

[b]R^2: correlation coefficient.

[c]Y; inhibition percentage, X; inhibitory concentration at Y (X = IC_Y).

4. Discussion

Due to their potential value in biotechnological and industrial applications, extremophiles have been the focus of substantial and intensive research efforts over the past few decades. The discovery of new chemicals and pathways, the isolation of novel microbial strains, and the molecular and biochemical characterisation of cellular components has been raising these research efforts tremendously. Furthermore, the importance of thermostable biomolecules in the growing field of biotechnology has encouraged research into organisms capable of growth at high temperatures. Thus, the isolation of novel thermophilic organisms, from both the domains archaea and bacteria, has received attention for their potential in the production of thermostable enzymes. For example, DNA polymerases have been obtained from thermophilic bacteria for application in PCR technology. Therefore, the main goals of this study were to isolate thermophilic bacteria from local hot springs and to identify them by phenotypic and molecular tools. Furthermore, this study was initiated to determine the hydrolytic, antimicrobial, and anticancer activities of isolated bacteria in attempt to determine isolates with unique or promising activity. The present study was the first to examine the anticancer activity of moderately thermophilic bacteria detected in water from Jordan hot springs.

Thirty bacterial isolates were isolated from five hot springs in Jordan. Out of them, 19 isolates appeared to belong to moderate thermophiles of the genus *Bacillus* or *Geobacillus* in terms of phenotypic and physiological properties. This result, based on morphological and physiological characteristics, is in agreement with the findings of several preceding studies (Ezeji *et al*., 2005; Fortina *et al*., 2001; Nazina *et al*., 2001, 2004; Romano *et al*., 2005), whereas the remaining 11 isolates were not related to *Bacillus* because they were Gram-negative, did not produce endospores, and their 16S rRNA gene sequences were allocated into other genera.

To confirm the conventional identification of the bacterial isolates, 16S rDNAs of the 30 isolates along with the *G. stearothermophilus* ATCC 7953 were sequenced and compared with those in the GenBank. Consequently, the sequences of isolates, which were phenotypically and physiologically *Bacillus*, revealed 97-100% sequence homology to the genus *Bacillus*. Eight *Bacillus* isolates were found closely related to the genus *Geobacillus* (Figure 2) and showed 97-100% identity to *G. stearothermophilus* ATCC 7953. Nazina *et al*. (2001) reported that different *Geobacillus* species have more than 96% sequence homology. As a result, these isolates can be allocated into the genus *Geobacillus*. Furthermore, the phylogenetic analysis of the 16S rDNA sequences of three *Geobacillus* isolates (JA1, JH1, and JM1) illustrated that these isolates can be grouped in the species *stearothermophilus* with 100% homology. However, further analysis is required, it was clearly noticed that the investigated Jordanian hot springs were rich in *Geobacillus*. The results of this study were consistent with findings of Obeidat *et al*. (2012).

In conclusion, the results presented in this study indicated that thermophilic bacilli were ubiquitous and diverse in thermal water of Jordanian hot springs. The

dominance of thermophilic *Bacillus* species in waters obtained from hot springs of Jordan was reported previously by several Jordanian researchers (Khalil, 2002; Malkawi and Al-Omari, 2010; Fandi *et al*., 2012; Obeidat *et al*., 2012; Mohammad *et al*., 2017). In several previous studies (Al-Qodah, 2006; Elnasser *et al*., 2007; Obeidat *et al*., 2012), it was reported that thermal waters of Jordanian hot springs were rich in the thermophilic bacillus *G. stearothermophilus*. This study showed that thermophilic *B. licheniformis* seems to be abundant in Jordanian hot springs. This is in agreement with Mohammad *et al*. (2017) who demonstrated that thermophilic *B. licheniformis* were prevalent in the Jordanian hot springs.

Geobacillus isolates were found to exhibit a wide array of enzymatic activities. All isolates were protease and lipase producers. Isolate JA1 was produced high pectinolytic activity. Isolates JH1 and JM5 produced very high and high protease activity, respectively, while isolates JM1, JM5, JZ1, and JZ5 were able to give high to very high amylolytic activity. Only one *Geobacillus* isolate (JZ1) did not give activity of xylanase enzyme. Four isolates (JA1, JM1, JM5, and JZ1) showed cellulase activity. These findings were consistent with that reported previously (Obeidat *et al*., 2012). Al-Qodah (2006) isolated amylolytic *Geobacillus* isolate from Ma`in-Roman Bath. In consistence, two amylolytic *Geobacillus* isolates (JM1 and JM5) were obtained from Ma`in-Romman Bath in the present study.

Interestingly, it was found that the 16S rDNA sequence of Zara isolate JZ9 was highly related (99% identity) to that of the thermophilic bacterium *Caldimonas hydrothermale*. This isolate was thermophilic (grew between 40 to 60 ^{o}C, at pH 8-9, and at 0-6% NaCl) with rod cells, strictly aerobic, stained Gram-negative, non-spore forming, and positive to catalase and oxidase tests. Moreover, isolate JZ9 produced different important enzymatic activities which have been used in biotechnological and industrial applications, including; proteolytic, lipolytic, xylanolytic, celluolytic, and pectinolytic activities. To our knowledge, no previous work reported the isolation of *Caldimonas* from Jordanian hot springs. Moreover, this is the second study after Bouraoui *et al*., (2010) which demonstrated the isolation of *Caldimonas* that may be allocated to the species *hydrothermale*.

The 16S rDNA sequence of JM2, which was isolated in 2011 from Ma`in-Roman Bath, shares 99% sequence similarity with the thermophilic bacterium *Thermomonas hydrothermalis*. This bacterium species had been repeatedly detected in 2017 and isolated from different Jordanian hot spring called Jordan Himma or Al- Hemma (Mohammad *et al*., 2017) and furtherly analyzed for biotechnological and medical purposes by Al-Daghistani *et al*., 2021. The enzyme profiles detected for *Thermomonas* by Mohammad *et al*. (2017) were positive for cellulase and amylase activity which is in agreement with the finding of this study, but it is negative for lipase activity which is contrary to the result obtained in this study.

It was found that a total of nine Gram-negative thermotolerant bacterial isolates were allocated, based on 16S rDNA sequences, into *Acinetobacter* (JM13, JM14, JZ11, and JZ12), *Cronobacter* (JS3), *Enterobacter* (JA4 and JH8), and *Pseudomonas* (JH7 and JZ14). Since these isolates are human pathogens, they might be present as contaminants from patients who visited hot springs for physiotherapy purposes.

While these results are important for further taxonomic work, positive results on several enzymes including protease, lipase, xylanase, cellulase, amylase, and pectinase (polygalacturonase) of most isolates are indicative of probable applications in industry and biotechnology. It was found that three isolates (thermophilic JM1, thermotolerant JS3 and JZ11) produced all hydrolytic activities which tested in the current study. Therefore, those isolates might draw a lot of attention largely because they produce vital enzymes for industry.

In the beginning of this century, many serious bacterial infections have developed resistance to commonly used antibiotics and become a major worldwide healthcare issue (Alanis, 2005). On the other hand, fungal pathogens cause serious problems worldwide in agriculture and food industry and many fungal pathogens produce mycotoxins, which are harmful to humans and livestock (Augustine *et al*., 2005). Finding new natural sources, such as thermophilic bacteria, of antibacterial and antifungal drugs is therefore urgently needed. Only three isolates (JH1 and JM11 were thermophilic and JS3 was thermotolerant) produced a narrow range of antimicrobial activity against test microorganisms (Figure 4). Results indicated that two isolates JH1 and JS3 exhibited antibacterial activity against *K. pneumonia* and isolate JM11 produced antifungal activity against *C. albicans*. This result is in agreement with preceding studies (Venugopalan *et al*., 2008; Muhammad *et al*., 2009; Sethy and Behera 2012). Crudes of these three isolates can be developed for the use in drugs industry. Remarkably, these isolates were protease, lipase, xylanase, and polgalacturonase producers. As a result, the antimicrobial activity of those isolates toward Gram-negative bacteria might be correlated to their ability to produce protease activity. This is in agreement with Farouk (1982) who demonstrated that proteolytic enzymes such as protease exhibited higher killing effect against Gram-negative bacteria than Gram-positive bacteria. Furthermore, this result implies that the produced antimicrobial agents are cationic and effectively interacting with the negatively charged surface of the outer lipopolysaccharide (LPS) of Gram-negative cell wall to cause membrane instability and rupturing, which ultimately leads to cell death.

After cardiovascular illnesses, cancer was the second leading cause of death worldwide and in Jordan (Heron *et al*., 2009; Al-Tarawneh *et al*., 2010). So, finding new sources of anticancer agents, such as bacterial byproducts, is urgently needed. No previous studies demonstrated the ability of thermophilic bacteria to produce anticancer agents. The results showed that crudes of three *Bacillus* isolates (JA2, JM11, and JM12) showed non-hemolytic activity against human erythrocytes and displayed selective *in vitro* cytotoxicity against human leukemic cell line K562 (Figure 5). Theses cytotoxic isolates were found to produce low pectinolytic activity and moderate to very high proteolytic activity. So, protease activity could be responsible for cytotoxic effect against leukemic cells. Given that the cytotoxic isolates exhibited no hemolytic activity against human erythrocytes, the anticancer activity in such isolates was not attributed to the induced

hemolysis. Because of their ability to discriminate between cancer cells (K562) and healthy Vero cells by killing cancer cells only, these isolates are considered to produce promising bioactive chemicals with selective *in vitro* cytotoxicity against leukemia cells. This finding clearly suggests that thermophilic bacteria, which naturally make selective substances against cancer, could be used for medical and pharmaceutical therapies of some cancer types.

5. Conclusion

In terms of phenotypic and physiological properties as well as 16S rDNA sequences, the majority of the thermophilic isolates obtained from thermal water of local hot springs belonged to the genus *Bacillus*/*Geobacillus*. This study was considered the first that described the isolation of *Caldimonas* bacterium from local hot springs in addition to the isolation of *Thermomonas*. Most of the isolates were found to produce protease, lipase, xylanase, cellulase, amylase, and pectinase. As an achievement of this study, three isolates exhibited selective *in vitro* cytotoxicity against human leukemia cells. To our knowledge, this is the first study that examined the anticancer activity of thermophilic bacteria crudes against leukemia cancer cells. Therefore, hot springs of Jordan are rich sources for the isolation of different thermophilic bacterial species producing hydrolyzing enzymes and anticancer agents that may be used in medical, biotechnological, and industrial applications.

Acknowledgment

The authors are grateful to “Ministry of Higher Education of Jordan; grant no. M-Ph/2/14/2008” for financial support and to “Dr. Saeid Ismaeil, Faculty of Medicine, University of Jordan” for providing cell lines used in this work.

References

Alanis, AJ. 2005. Resistance to antibiotics: are we in the post-antibiotic era? *Arch Med Res*, **36 (6):** 697-705.

Al-Daghistani, HI, Mohammad, BT, Kurniawan, TA, Singh, D, Rabadi, AD, Xue, W, Avtar, R, Othman, MHD and Shirazian S. 2021. Characterization and applications of *Thermomonas hydrothermalis* isolated from Jordan's hot springs for biotechnological and medical purposes. *Process Biochem*, **104:** 171-181.

Al-Qodah, Z. 2006. Production and characterization of thermostable α-amylase by thermophilic *Geobacillus stearothermophilus*. *Biotechnol J*, **1:** 850–857.

Al-Tarawneh, M, Khatib, S and Arqub, K. 2010. Cancer incidence in Jordan, 1996–2005. *East Mediterr Health J*, **16(8):** 837-845.

Augustine, SK, Bhavsar, SP and Kapadnis, BP. 2005. A non-polyene antifungal antibiotic from Streptomyces albidoflavus PU 23. *J. Biosci*, **30(2):** 201-11.

Baker, G, Gaffar, S, Cowan, DA and Suharto, ARI. 2001. Bacterial Community Analysis of Indonesian Hotsprings. *FEMS Microbiol Lett*, **200 (1):** 103-109.

Belduz, AO, Dulger, S and Demirbag, Z. 2003. *Anoxybacillus gonensis* sp. nov., a moderately thermophilic, xylose-utilizing, endospore-forming bacterium. *Int J Syst Evol Microbiol*, **53:** 1315-1320.

Bouraoui, H, Boukari, I, Touzel, JP, O'Donohue, M and Manai, M. 2010. *Caldimonas hydrothermale* sp. nov., a novel thermophilic bacterium isolated from Roman hot bath in south Tunisia. *Arch Microbiol*, **192(6):** 485-491.

Bragger, J.M, Daniel, RM, Coolbear, T and Morgan, HW. 1989. Very stable enzymes from extremely thermophilic archaebacteria and eubacteria. *Appl Microbiol Biotechnol*, **31(5-6):** 556-561.

Carrillo, PG, C Mardaraz, SI, Alvarez, P and Giulietti, AM. 1996. Isolation and selection of biosurfactant producing bacteria. *World J Microbiol Biotechnol*, **12 (1):** 82-84.

Ceylan, O, Okmen, G and Ugur, A. 2008. Isolation of soil Streptomyces as source antibiotics active against antibiotic-resistant bacteria. *Eurasia J Biosci*, **2:** 73-82.

Chen, L and Roberts, MF. 1999. Characterization of a tetrameric inositol monophosphatase from the hyperthermophilic bacterium *Thermotoga maritima*. *Appl Environ Microbiol*, **65 (10):** 4559-4567.

Elnasser, Z, Maraqa, A, Owais, W and Khraisat, A. 2007: Isolation and Characterization of New Thermophilic Bacteria in Jordan. *Internet J Microbiol*, **3(2)**: 1-10.

Ezeji TC, Wolf A and Bahl H. 2005. Isolation, characterization, and identification of *Geobacillus thermodenitrificans* HRO10, an α-amylase and α-glucosidase producing thermophile. *Can J Microbiol*, **51(8):** 685-693.

Fandi K, Massadeh M and Laatsch, H. 2012. LC-MS/MS Profiling-based metabolite screening of thermophilic bacteria from Jordanian hot springs. International Conference on Applied Chemistry and Pharmaceutical Sciences (ICACPS'2012), May 19-20, 2012 Penang (Malaysia).

Farouk, A. 1982. Antibacterial activity of proteolytic enzymes. *Int J Pharm*, **12(4):** 295-298.

Fortina, MG, Mora, D, Schumann, P, Parini, C, Manachini, PL and Stackebrandt, E. 2001. Reclassification of *Saccharococcus caldoxylosilyticus* as *Geobacillus caldoxylosilyticus*. *Int J Syst Evol Microbiol*, **5:** 2063-2071.

Freshney, RI. 1987. Culture of Animal Cells: A Manual of Basic Techniques, 2a ed., Alan R. Liss, Inc., New York.

Galtier, N and Lobry, JR. 1997. Relationships between genomic G+C content, RNA secondary structures, and optimal growth temperature in prokaryotes. *J Mol* **Evol, 44(6):** 632-636.

Gessner, RV. 1980. Degradative enzyme production by salt march fungi. *Bot Mar*, **23:** 133-139.

Haba, E, Bresco, O, Ferrer, C, Marques, A, Basquets, M and Manresa, A. 2000. Isolation of lipase secreting bacteria by deploying used frying oil as selective substrate. *Enzyme Microb Technol*, **26:** 40-44.

Heiss, P, Bernatz, S, Bruchelt, G and Schmidtke, SR. 1997. Cytotoxic effect of immunoconjugate composed of glucose-oxidase coupled to an anti-ganglioside (GD2) antibody on spheroids. *Anticancer Res*, **17 (4B):** 3177-3178.

Heron, M, Hoyert, DL, Murphy, SL, Xu, J, Kochanek KD and Tejada-Vera, B. 2009. Deaths: Final Data for 2006. *Natl Vital Stat Rep*, **57(14):** 1-135.

Karlin, S, Brocchieri, L, Trent, J, Blaisdell, BD and Mra´zek, J. 2002. Heterogeneity of Genome and Proteome Content in Bacteria, Archaea, and Eukaryotes. *Theor Popul Biol*, **61 (4):** 367–390.

Khalil, AB. 2002. Isolation and characterization of thermophilic Bacillus species from thermal ponds in Jordan. *Pak J Biol Sci*, **5(11):** 1272-1273.

Kobayashi, T, Koike, K, Yoshimatsu, T, Higaki N, Suzumatsu A and Ozawa, T. 1999. Purification and properties of a low-molecular weight, high-alkaline pectate lyase from an alkaliphilic strain of Bacillus. *Biosci Biotechnol Biochem*, **63 (1):** 72-75.

Lengeler, JW, Drews, G and Schlegel, HG. 1999. Biology of the Prokaryotes: Blackwell Science Inc., pp. 652-915.

Malkawi, HI and Al-Omari, MN. 2010. Culture-dependent and culture-independent approaches to study the bacterial and archaeal diversity from Jordanian hot springs. *Afr J Microbiol Res*, **4(10):** 923-932.

Maugeri, LT, Gugliandolo, C, Caccamo, D and Stackebrandt, E. 2001. A polyphasic taxonomic study of thermophilic Bacilli from shallow, marine vents. *Syst Appl Microbiol*, **24 (4):** 572-587.

Mohammad BT, Al Daghistani HI, Jaouani A, Abdel-Latif S and Kennes C. 2017. Isolation and characterization of thermophilic bacteria from Jordanian hot springs: *Bacillus licheniformis* and *Thermomonas hydrothermalis* isolates as potential producers of thermostable enzymes. *Int J Microbiol*, **2017:** 6943952.

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*, **65 (1-2):** 55-63.

Muhammad, SA, Ahmad, S and Hameed, A. 2009. Antibiotic production by thermophilic *Bacillus specie* SAT-4. *Pak J Pharm Sci*, **22(3):** 339-345.

Nazina, TN, Lebedeva, EV, Poltaraus, AB, Tourova, TP, Grigoryan, AA, Sokolova, DS, Lysenko, AM and Osipov, GA. 2004. *Geobacillus gargensis* sp. nov., a novel thermophile from a hot spring, and the reclassification of *Bacillus vulcani* as *Geobacillus vulcani* comb. Nov. *Int J Syst Evol Microbiol*, **54 (6):** 2019-2024.

Nazina, TN, Tourova, TP, Poltaraus, AB, Novikova, EV, Grigoryan, AA, Ivanova, AE, Lysenko, AM, Petrunyaka, VV, Osipov GA, Belyaev, SS and Ivanov, MV. 2001. Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov., and *Geobacillus uzenensis* sp. nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus, G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermoglucosidasius* and *G. thermodenitrificans*. *Int J Syst Evol Microbiol*, **51 (2):** 433-446.

Obeidat, M. 2017. Cytotoxicity of n-butanol extracts of *Streptomyces* against human breast cancer cells. *Int J Pharm*, **13(8):** 969-979.

Obeidat, M, Khyami-Horani, H, Al-Zoubi A and Otri, I. 2012. Isolation, characterization, and hydrolytic activities of *Geobacillus* species from Jordanian hot springs. *Afr J Biotechnol*, **11(25):** 6763-6768.

Priest, GF, Goodfellow, M and Todd, CA. 1988. Numerical Classification of the genus *Bacillus*. *J Gen Microbiol*, **134:** 1847-1882.

Romano, I, Poli, A, Lama, L, Gambacorta, A and Nicolaus B. 2005. *Geobacillus thermoleovorans* subsp. *stromboliensis* subsp. nov., isolated from the geothermal volcanic environment. *J Gen Appl Microbiol*, **51(3):** 183-189.

Saul, DJ, Reeves, RA, Morgan, HW and Bergqquist, PL. 1999. *Thermus* diversity and strain loss during enrichment. *FEMS Microbiol Ecol*, **30(2):** 157-162.

Sethy, K and Behera, N. 2012. Antimicrobial activity of thermotolerant bacterial isolate from coal mine spoil. African Journal of Microbiology Research, **6(26):** 5459-5463.

Simoneit, TRB, Aboul-Kassim, TAT and Tiercelin, JJ. 2000. Hydrothermal petroleum from lacustrine sedimentary organic matter in the East African Rift. *Appl Geochem*, **15:** 355-368.

Singh, SP. 2006. Extreme Environments and Extremophiles. National Science Digital Library (CSIR): E- Book on Environmental Microbioogy, pp. 1-35.

Swarieh, A. 2000. Geothermal energy resources in Jordan, country update report. Proceedings World Geothermal Congress. pp. 469-474.

Ten, LN, Im, WT, Kim, MK, Kang, MS and Lee, ST. 2004. Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates. *J Microbiol Methods*, **56(3):** 375–382.

Venugopalan, V, Singh, B, Verma, N, Nahar, P, Bora, TC, Das, RH and Gautam, HK. 2008. Screening of thermophiles from municipal solid waste and their selective antimicrobial profile. *Curr Res Bacteriol*, **1(1):** 17-22.