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Biochemical and Molecular Characterization of Thermophilic Enzymes of Bacterial Strains Isolated from Jordanian Hot Springs

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Abstract: Twenty four local thermophilic, endospore forming bacterial isolates belonging to the genus Bacillus were biochemically analyzed for the following enzymes: phosphatase, proteinase, lecithinase and amylase. The enzymes were found to be active at temperatures 28°C, 40°C, 50°C, 60°C but not at 70°C, with optimum enzyme activity at 50°C. Furthermore, the genes of some enzymes were amplified by polymerase chain reaction to show whether these enzymes are plasmid and/or chromosomally encoded. Most of the tested bacterial isolates were found to contain plasmids ranging in size from 4.0Kb to 28.0Kb. Lecithinase gene was found in some isolates to be encoded by both plasmid and chromosome, while lipase gene was found to be only chromosomally encoded in all bacterial isolates.

Key words: Enzymes · Thermophilic Bacteria · Hot springs · Jordan.

INTRODUCTION

Natural environments for thermophilic microorganisms are widespread on earth surface. The most common and accessible thermal habitats are hot springs, sulfatara and geothermally heated soils which have a limited species composition [1, 2]. Thermophilic microorganisms are adapted to thrive at temperatures above 60°C. They are a source of interesting enzymes that are both thermoactive and thermostable [3]. As a consequence of growth at high temperature and unique macromolecular properties, thermophilic bacteria can possess high metabolic rates, physically and chemically stable enzymes and lower growth but higher end product yields than similar mesophilic species. Thermophilic processes appear more stable, rapid and less expensive and facilitate reactant activity and product recovery [4].

Thermophiles were reported to contain proteins which are thermostable and resist denaturation and proteolysis [5, 1]. Specialized proteins known as chaperonins are produced by these organisms which help, after their denaturation, to refold the proteins to their native form and restore their functions [6]. The cell membranes of thermophiles are made up of saturated fatty acids. The fatty acid provides a hydrophobic environment for the cell and keeps the cell rigid enough to live at elevated temperatures [7].

With better knowledge and purification of enzymes, the number of applications has increased many folds and with the availability of thermostable enzymes, a number of new possibilities for industrial processes have emerged [8].

Thermozymes offer several biotechnological advantages over mesophilic enzymes: they are easier to purify by heat treatment, have a higher resistance to chemical denaturants such as solvents and guanidinium hydrochloride and withstand higher substrate concentrations. Because of their stability at elevated temperature, thermozyme reactions are less susceptible to microbial contamination and often display higher reaction rates than mesozyme catalyzed reactions. In view of these important advantages, thermozymes are attracting much industrial interest [9].

One of the most important advances in molecular biology during the past twenty years is the development of polymerase chain reaction (PCR) [10] using thermostable Taq DNA polymerase enzyme. Taq DNA Polymerase is a thermostable enzyme derived from the thermophilic bacterium *Thermus aquaticus*. It is able to withstand repeated heating to 95°C without significant loss of activity. It was suggested that plasmids play a role in promoting thermophilic growth in which plasmids often contain genes or gene cassettes that confer a selective advantage to the bacterium harboring them, such as the ability to make the bacterium antibiotic

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resistant [11]. Although many native plasmids have been isolated from members of the genus *Thermus*, their functions still remain unclear [12]. Furthermore, genome comparison of *Thermus thermophilus* strains HB27 and HB8 indicated that two megaplasmids were implicated in promoting thermophilic growth [6].

Recently, 132 thermophilic bacterial isolates were isolated in our laboratory [13]. The isolates belong mainly to the genus *Bacillus* and originated from Jordanian hot springs. 24 thermophilic bacterial isolates were selected for this study to investigate their enzyme activities using both biochemical and molecular approaches. The targeted thermophilic enzymes are, phosphatases, proteinases, amylases and lecithinases. These enzymes were choosen for their importanc in biotechnology and industrial microbiology.

MATERIALS AND METHODS

Bacterial Isolates and Biochemical Tests: Twenty four bacterial isolates belonging mainly to thr genus Bacillus were previously isolated [13] and subcultured in our laborartory since their isolation. The isolates were isolated from different Jordanian hot springs, the bacterial isolates were checked for the presence of endospores by spore staining procedure [14]. Additionally, the following biochemical tests were performed according to Sharmin and Rhaman [14]; Catalase, Methyl Red, Voges Proskauer, Indole production, Nitrate reduction, Citrate utilization tests.

Assay of Thermophilic Enzymes Activities: Activities of the following enzymes were assayed according to Bel'kova *et al.* [15].

Phosphatase activity was determined using alkaline phosphatase kit (Biocon, Germany) according to the manufacturer's instructions.

Proteinase activity was assayed on milk agar containing defatted milk. The bacterial samples of each of the 24 isolates were inoculated in this medium and incubated for 24 hour at 28°C, 50°C, 60°C and 70°C. Then, the presence and diameter of clearance zone of each test was checked and measured indicating enzyme activity by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain.

Lecithinase (phospholipase C) activity was determined according to Bel'kova *et al.* [15].

Amylase activity was also determined according to Bel'kova *et al.* [15].

Molecular Characterization of Thermophilic Enzymes

Extraction of Genomic DNA: Genomic DNA was isolated from each bacterial isolate as follows: One separate colony from each bacterial isolate culture was inoculated into 10 ml nutrient broth and incubated overnight at the same temperature that the isolate was initially grown. One ml from each of the nutrient broth culture was centrifuged at 15000 rpm for 15 min. The pellet was suspended in 0.5 ml lysis buffer (100 mM Tris-HCl, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and 200 μg/ml proteinase K. pH 8.5) and incubated at 55°C for 2 hours. Samples were then subjected to RNase treatment (1.5 mg/ml) for 30 min at 37°C. DNA was precipitated with equal volume of ice cold isopropanol and the pellet was washed with 70% ethanol, then dried and suspended in TE buffer. This DNA was used as template for PCR reactions.

Extraction of Plasmid DNA: The plasmids of thermophilic bacterial isolates were isolated and purified with Qiagen plasmid Midi kit according to the manufacturer's instructions and following the procedures described by Ruan and Xu [12].

Lecithinase and Lipase Genes Amplification from Genomic and Plasmid DNA: Polymerase chain reaction amplification for gene sequences of each lipase [16] and lecithinase [17] enzymes was performed on each genomic and plasmid DNA separately isolated from each bacterial isolate as follows: 50 μl PCR reaction containing 25 μl master mix, 0.3 μl of (0.247) μg/μl of each primer for each gene (reverse and forward primers listed in Table 1) and 2 μl of DNA template.

For lecithinase gene, Reaction mixtures were incubated in DNA thermal cycler (Perkin Elmer 480) for 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72C°for 1 min. While for lipase gene amplification the reaction mixtures were incubated for 35 cycles of denaturation at 95°C for 1 min, annealing at 45°C for 2 min and extension at 72°C for 1 min. All reaction mixtures were stored at 4°C until analyzed.

Plasmid DNA, Genomic DNA and the PCR amplification products were separated on 1% w/v agarose gels in 1X TBE buffer (0.089 M Tris-borate, 0.089 M boric acid, 0.002 M EDTA, pH 8.0) at 100 V for I hour using horizontal gel electrophoresis apparatus. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed by BioDocAnalyze. 1 kb DNA ladder was used as reference standard.

Table 1: Sequences of primers used for detection of lecithinase and lipase genes in *Bacillus* sps.

Primer sequence (5'¬3')	Gene specificity	Amplicon size (bp)	Reference
Forward:			
GAG TTA GAG AAC GGT ATT TAT GCT GC	Lecithinase	411	[24]
Reverse:			
CTA CTG CCG CTC CAT GAA TCC			
Forward:			
GA(G/T)A(A/G)(C/G)ATGATGAAA(T/G)GCTG.	lipase	1300	[5]
Reverse: C(C/T)(C/T)(T/G)(C/T)(A/T)TTAAAGGCCGCAAACTC			

RESULTS

Biochemical Tests: All 24 bacterial isolates are endospore-formers (Table 2), some endospores were terminal, some were central and some are free out side the cells.

Twenty one out of 24 isolates (87.5 %) were catalase positive, 9 out of 24 isolates (37.5 %) were strong positive for methyl red test and 9 isolates (37.5 %) were weak positive, the remaining 6 isolates (25 %) were negative for indole production test all 24 bacterial isolates (100%) gave negative results. For nitrate reduction test, 15 out of 24 isolates (62.5 %) were positive and 5 isolates (20.8 %) were negative while 4 isolates (16.6 %) were positive after addition of Zink. For Voges Proskauer test all 24 bacterial isolates (100 %) were negative; and for Citrate utilization test 14 out 24 isolates (58.3 %) were positive while the other 10 isolates (41.6 %) were negative. The results of the biochemical tests are summarized in (Table 2).

Enzyme Activities of the Thermophilic Bacterial Isolates: Phosphatase activity was determined for all 24 bacterial isolates after incubation at 50°C, the activity of the enzyme was measured and the results were recorded as shown in (Figure 1a, 1b, 1c, 1d, 1e and 1f): 2 out of 24 bacterial isolates (MAJ 37, MAJ 38) showed enzyme activity more than 100 U/l (8.3 %) and 15 isolates (MAJ 1, MAJ 3, MAJ 4, MAJ 10, MAJ 11, MAJ 25, MAJ 33, MAJ 41, MAJ 52, MAJ 56, MAJ 68, MAJ 77, MAJ 80, MAJ 87, MAJ 90) showed enzyme activity between 10-100 U/l (54.1 %), while 7 isolates (MAJ 8, MAJ 48, MAJ 60, MAJ 70, MAJ 81, MAJ 84, MAJ 110) showed enzyme activity less than 10 U/l (37.5 %). The highest Phosphatase activity was determined in the isolates MAJ 37 and MAJ 38 while the lowest activity was determined in isolates MAJ 70, MAJ 81 and MAJ 84.

Starch-hydrolyzing (amylase) activity was determined after incubation at the temperatures 28°C, 40°C, 50°C, 60°C and 70°C; the diameter of the clearance zones varied from

Table 2: Biochemical tests for the 24 bacterial isolates*

Bacterial isolates codes	Catalase test	Methyl red test	Nitrate reduction test	Citrate utilization test
MAJ 1	+	++	+	+
MAJ 3	+	+	-	+
MAJ 4	+	++	+ (zinc)	+
MAJ 8	+	+	-	+
MAJ 10	+	++	+	-
MAJ 11	+	++	+	+
MAJ 25	+	+	+ (zinc)	+
MAJ 33	+	-	+	-
MAJ 37	-	-	+ (zinc)	+
MAJ 38	+	-	-	-
MAJ 41	+	++	+	+
MAJ 48	+	++	+	-
MAJ 52	+	+	+	+
MAJ 56	+	+	+	+
MAJ 60	+	++	+	-
MAJ 68	+	++	+	-
MAJ 70	-	+	+	-
MAJ 77	+	+	-	+
MAJ 80	+	-	+	-
MAJ 81	+	-	+	-
MAJ 84	+	+	-	+
MAJ 87	-	-	+	+
MAJ 90	+	+	+ (zinc)	+
MAJ 110	+	++	+	-

(-): negative result; (+): weak positive result, (++): strong positive result, (+zinc): give positive result after addition of zinc.
* All bacterial isolates spore-formers and were negative for both Voges Proskauer test and indole production test.

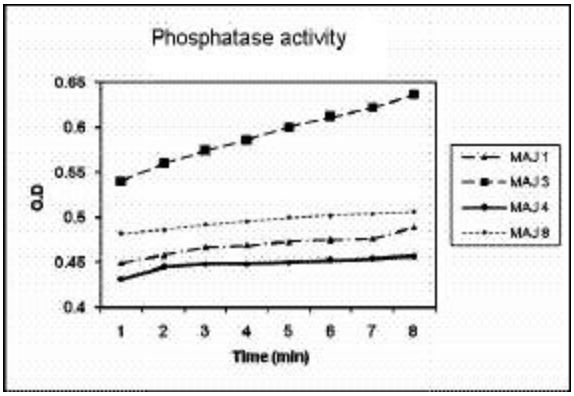


Fig. 1a: Phosphatase activity of bacterial isolates (MAJ 1, MAJ 3, MAJ 4, and MAJ 8).

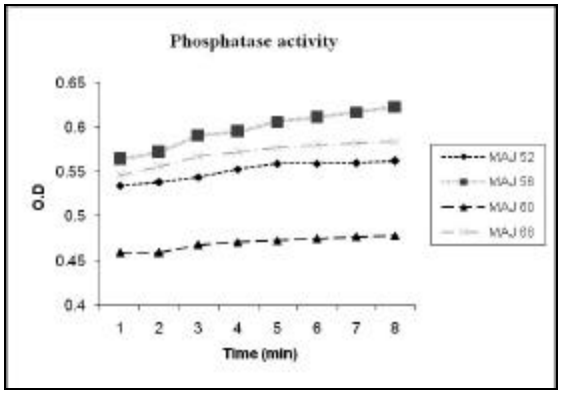


Fig. 1d: Phosphatase activity of bacterial isolates (MAJ 52, MAJ 56, MAJ 60, and MAJ 68).

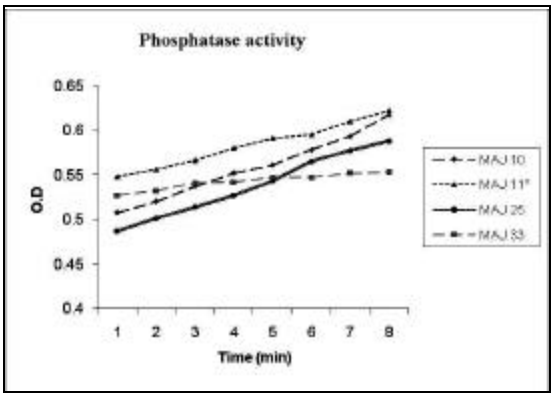


Fig. 1b: Phosphatase activity of bacterial isolates (MAJ 10, MAJ 11, MAJ 25, and MAJ 33)

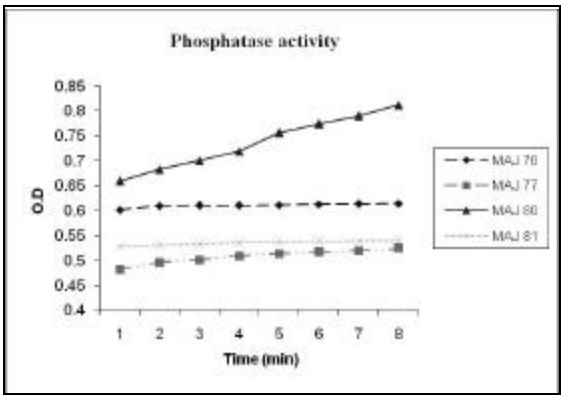


Fig. 1e: Phosphatase activity of bacterial isolates (MAJ 70, MAJ 77, MAJ 80, and MAJ 81)

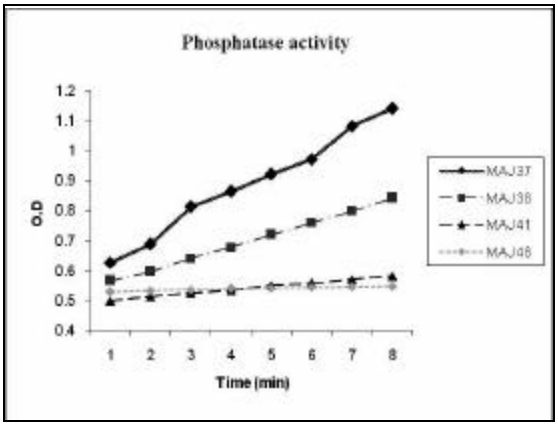


Fig. 1c: Phosphatase activity of bacterial isolates (MAJ 37, MAJ 38, MAJ 41, and MAJ 48).

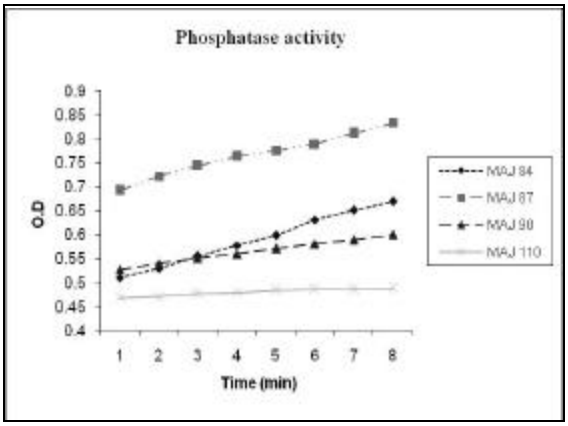


Fig. 1f: Phosphatase activity of bacterial isolates (MAJ 84, MAJ 87, MAJ 90, and MAJ 110)

Fig. 1: Phospatase activity of the 24 bacterial isolates

Table 3: The diameter of clearance zone (mm) for each bacterial isolate investigated for amylase activity on starch agar at different incubation temperatures*

Bacterial isolate	Diameter of C.Z at 28°C (mm)	Diameter of C.Z at 40°C (mm)	Diameter of C.Z at 50°C (mm)	Diameter of C.Z at 60°C (mm)
MAJ 1	10	23	33	No growth
MAJ 3	No growth	No growth	14	No activity
MAJ 4	No growth	18	8	No activity
MAJ 8	No growth	10	38	25
MAJ 10	No growth	12	14	15
MAJ 11	15	24	31	No growth
MAJ 25	10	22	27	No growth
MAJ 33	14	22	44	No activity
MAJ 37	14	24	34	No activity
MAJ 38	14	24	36	No activity
MAJ 41	13	18	23	6
MAJ 48	10	18	24	18
MAJ 52	15	22	24	No activity
MAJ 56	12	22	24	No activity
MAJ 60	7	20	32	No growth
MAJ 68	14	14	33	No growth
MAJ 70	14	21	29	No growth
MAJ 77	15	23	40	No growth
MAJ 80	15	22	29	No activity
MAJ 81	12	23	29	No activity
MAJ 84	12	25	30	No growth
MAJ 87	12	19	23	No activity
MAJ 90	12	21	22	No activity
MAJ 110	12	22	23	10

C.Z: Clearance zone

* No growth was observed at 70°C

Table 4: The diameter of clearance zone (mm) for each bacterial isolates investigated for proteinase activity on milk agar at different incubation temperatures

Bacterial isolates code	Diameter of C.Z at 28°C (mm)	Diameter of C.Z at 40°C (mm)	Diameter of C.Z at 50°C (mm)	Diameter of C.Z at 60°C (mm)	Diameter of C.Z at 70 °C (mm)
MAJ 1	14	20	61	22	No growth
MAJ 3	No growth	No growth	No growth	No growth	No activity
MAJ 4	No growth	No growth	No activity	No growth	No growth
MAJ 8	8	20	33	5	No growth
MAJ 10	No growth	No growth	No activity	No activity	No growth
MAJ 11	3	15	29	9	No growth
MAJ 25	9	18	71	21	No growth
MAJ 33	9	21	49	9	No growth
MAJ 37	12	20	75	11	No growth
MAJ 38	9	21	37	5	No growth
MAJ 41	8	15	75	16	No growth
MAJ 48	5	18	56	8	No growth
MAJ 52	4	18	61	13	No growth
MAJ 56	8	18	64	13	No growth
MAJ 60	4	20	43	8	No growth
MAJ 68	10	20	55	11	No growth
MAJ 70	10	16	79	19	No activity
MAJ 77	6	17	40	9	No growth
MAJ 80	11	18	60	15	No growth
MAJ 81	14	20	64	24	10
MAJ 84	7	18	33	4	No growth
MAJ 87	8	18	37	8	No growth
MAJ 90	9	18	42	6	No growth
MAJ 110	8	16	63	13	No growth

C.Z: Clearance zone

Table 5: The diameter of clearance zone (mm) for each bacterial isolate for investigated lecithinase activity on milk agar at different incubation temperatures*

Bacterial isolate	Diameter of C.Z at 40 °C (mm)	Diameter of C.Z at 50 °C (mm)	Diameter of C.Z at 60 °C (mm)
MAJ 1	18	28	No activity
MAJ 3	20	31	No activity
MAJ 4	22	71	No activity
MAJ 8	No growth	No activity	No activity
MAJ 10	20	66	No activity
MAJ 11	14	19	No activity
MAJ 25	20	67	No activity
MAJ 33	13	58	No activity
MAJ 37	35	55	No activity
MAJ 38	28	11	No activity
MAJ 41	24	36	No activity
MAJ 48	16	32	25
MAJ 52	15	36	No activity
MAJ 56	11	29	9
MAJ 60	16	31	No activity
MAJ 68	31	56	No activity
MAJ 70	20	58	24
MAJ 77	26	73	No activity
MAJ 80	No activity	No activity	No activity
MAJ 81	47	66	No activity
MAJ 84	11	12	No activity
MAJ 87	20	43	No activity
MAJ 90	45	56	22
MAJ 110	15	39	No activity

C.Z: Clearance zone

* No growth was observed was observed at either 28 °C or 70 °C

7 to 15 mm at 28°C, 14 to 25 mm at 40°C, 8 to 44 mm at 50°C, 6 to 25 at 60°C, while at 70°C, all 24 isolates did not demonstrate any growth (Table 3). Twenty isolates (83.3 %) (All isolates except MAJ 3, MAJ 4, MAJ 8 and MAJ 10) demonstrated the amylase activity at 28°C, while 23 isolates (95.8 %) (except MAJ 3) demonstrated the amylase activity at 40°C. The best amylase activity was observed at 50°C in which all 24 isolates (100 %) demonstrated amylase activity at high range, while at 70°C no activity was observed and 5 out 24 isolates (20.8 %) (MAJ 8, MAJ 10, MAJ 41, MAJ 48, MAJ 110) were positive for amylase activity at 60°C.

Proteinase activity as indicated in (Table 4) was determined after incubation at 28°C, 40°C, 50°C, 60°C and 70°C; the diameter of the clearance zones ranged from 3 to 14 mm at 28°C, 15 to 21 mm at 40°C, 29 to 79 mm at 50°C, 4 to 21 at 60°C, while at 70°C all 24 bacterial isolate did not demonstrate any growth except one bacterial isolate (MAJ 81). Twenty one bacterial isolates (87.5 %) (all except MAJ 3, MAJ 4 and MAJ 10) demonstrated proteinase activity at 28°C and 21 bacterial isolates (87.5 %) (all except MAJ 3, MAJ 4 and MAJ 10) demonstrated proteinase activity at 40°C, the highest proteinase activity was observed at 50°C and 21 isolates (87.5%) (except MAJ 3, MAJ 4 and MAJ 10) demonstrated proteinase activity at high range, while at 70°C no activity was observed except one bacterial isolates (MAJ 81) (4 %) which showed enzyme activity and 21 out 24 bacterial isolates (87.5 %) (except MAJ 3, MAJ 4 and MAJ 10) had low proteinase activity at 60°C.

The only bacterial isolate that showed proteinase activity at 70°C was MAJ 81 with a 10 mm diameter of clearance zone.

lecithinase activity, as indicated in (Table 5), was demonstrated after incubation at 28°C, 40°C, 50°C, 60°C and 70°C; the diameter of the clearance zones varied from 13 to 47 mm at 40°C, 13 to 73 mm at 50°C, 9 to 25 at 60°C, while all 24 isolates did not show any growth at 28°C and at 70°C. Twenty two isolates (91.6 %) (all except MAJ 8 and MAJ 80) demonstrated lecithinase activity at 40°C and at 50°C 22 isolates (91.6 %) (Except MAJ 8 and MAJ 80) demonstrated lecithinase activity, 4 out 24 isolates (16.6 %) (MAJ 48, MAJ 56, MAJ 70 and MAJ 90) had the enzyme activity at 60°C. All 24 bacterial isolates demonstrated lecithinase activity except 2 isolates (MAJ 8 and MAJ 80) where no activity was observed at any incubation temperature.

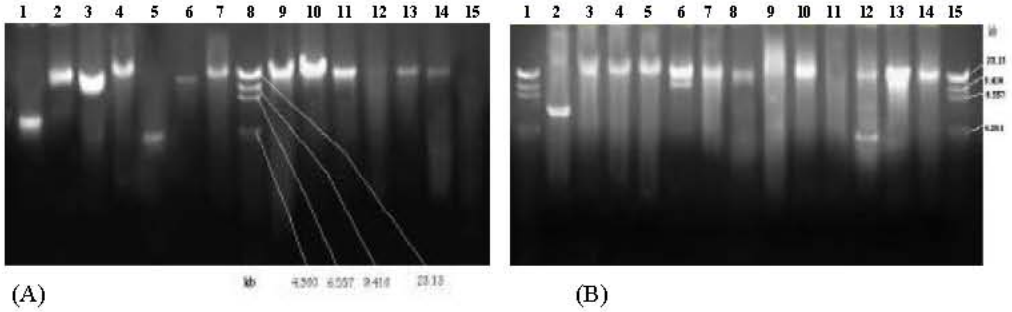


Fig. 2: A: Agarose gel electrophoresis of plasmid pattern of bacterial isolates. Lane 1, *E.*coli (TOP 10^{R}) (positive control); lane 2, MAJ 1; lane 3, MAJ 3; lane 4, MAJ 4; lane 5, MAJ 8; lane 6, MAJ 10; lane 7, MAJ 11; lane 8, Lambda Hind Ø molecular weight DNA marker; lane 9, MAJ 25; lane 10, MAJ 33; lane 11, MAJ 37; lane 12, MAJ 38; lane 13, MAJ 41; lane 14, MAJ 48; lane 15, *Bacillus cereus* (negative control).
B: Lane 1 and lane 15, Lambda Hind Ø molecular weight DNA marker; lane 2, *E.coli* (TOP 10^{R}; lane 3, MAJ 52; lane 4, MAJ 56; lane 5, MAJ 60; lane 6, MAJ 68; lane 7, MAJ 70; lane 8, MAJ 77; lane 9, MAJ 80; lane 10, MAJ 81; lane 11, MAJ 84; lane 12, MAJ 87; lane 13, MAJ 90; lane 14, MAJ 110.

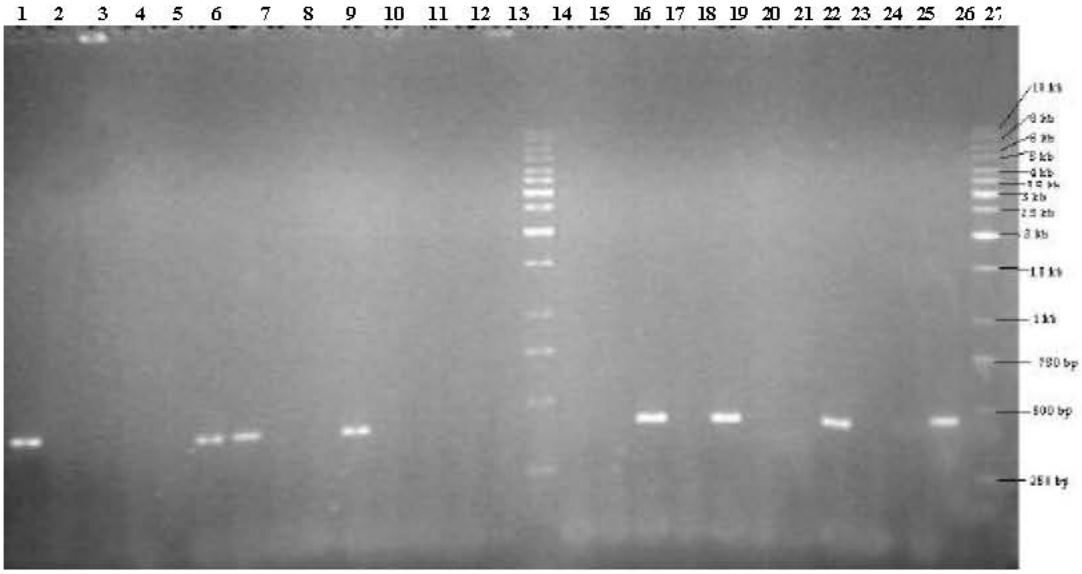
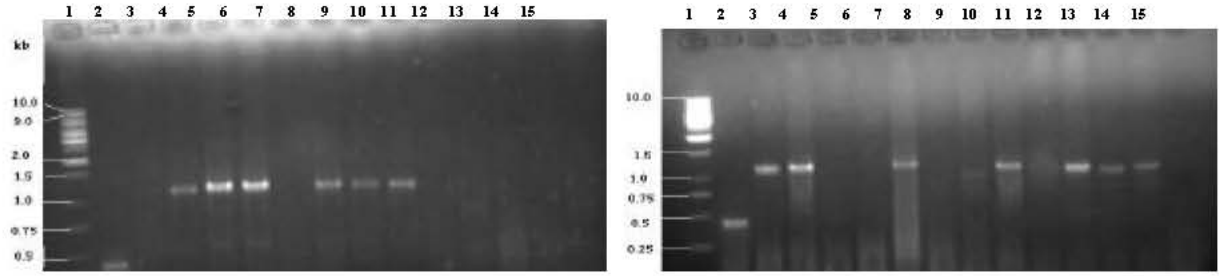


Fig. 3: Agarose gel electrophoresis of PCR amplification products of bacterial isolates genomic DNA using specific primers pair of lecithinase gene fragment. Lane 1, MAJ 1; lane 2, MAJ 3; lane 3, MAJ 4; lane 4, MAJ 8; lane 5, MAJ 10; lane 6, MAJ 11; lane 7, MAJ 25; lane 8, MAJ 33; lane 9, MAJ 37; lane 10, MAJ 38; lane 11, MAJ 41; lane 12, MAJ 48; lane 13, MAJ 52; lane 14, MAJ 56; lane 15 and lane 27, 1 kb molecular weight DNA marker; lane 16, MAJ 60; lane 17, MAJ 68; lane 18, MAJ 70; lane 19, MAJ 77; lane 20, MAJ 80; lane 21, MAJ 81; lane 22, MAJ 84; lane 23, MAJ 87; lane 24, MAJ 90; lane 25, MAJ 110; lane 26, *Bacillus cereus* (positive control).

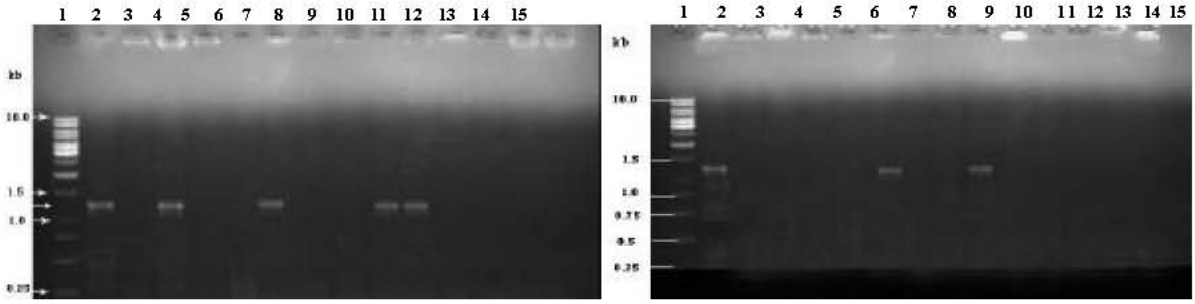
Isolation of Genomic and Plasmid DNA from Bacterial Isolates: Genomic DNA was extracted from bacterial isolates using lysis method, good quality and quantity of genomic DNA was obtained form each bacterial isolates.

Plasmid analysis was done for all 24 bacterial isolates; 2 out of 24 isolates (MAJ 38 and MAJ 84) did not contain plasmid DNA, while 2 isolates (MAJ 68 and MAJ 87) have two different plasmids in each one (Figure 2a and b). The size of plasmids among all 24 isolates ranged from 4.0 kbp to 28.0 kbp and plasmid size of 20.0 to 28.0 kb were the most frequently detected (MAJ 1, MAJ 4, MAJ 11, MAJ 25, MAJ 33, MAJ 37, MAJ 41, MAJ 48, MAJ 52, MAJ 56, MAJ 60, MAJ 68, MAJ 70, MAJ 77, MAJ 80, MAJ 81, MAJ 87, MAJ 90 and MAJ 110) and were seen in about 79.1 % of the isolates.



(A) (B)

Fig. 4A: A: Agarose gel electrophoresis of PCR amplification products of bacterial of plasmid DNA using specific primers pair of lecithinase gene fragment. Lane 1, 1 kb molecular weight DNA marker; lane 2, *Bacillus cereus* (genomic encoded) (positive control); lane 3, MAJ 1; lane 4, MAJ 3; lane 5, MAJ 4; lane 6, MAJ 8; lane 7, MAJ 10; lane 8, MAJ 11; lane 9, MAJ 25; lane 10, MAJ 33; lane 11, MAJ 37; lane 12, MAJ 38; lane 13, MAJ 41; lane 14, MAJ 48; lane 15, *Escherichia* coli (TOP 10R) (negative control).
B: Lane 1, 1 kb molecular weight DNA marker; lane 2, *Bacillus cereus* (positive control) (genomic encoded); lane 3, MAJ 52; lane 4, MAJ 56; lane 5, MAJ 60; lane 6, MAJ 68; lane 7, MAJ 70; lane 8, MAJ 77; lane 9, MAJ 80; lane 10, MAJ 81; lane 11, MAJ 84; lane 12, MAJ 87; lane 13, MAJ 90; lane 14, MAJ 110; lane 15, *Escherichia coli* (TOP 10R).



(A) (B)

Fig. 5A: A: Agarose gel electrophoresis of PCR amplification products of bacterial genomic DNA using specific primers pair of lipase gene fragment. Lane 1, 1 kb molecular weight DNA marker; lane 2, positive control (*Bacillus sterothermophilus*); lane 3, MAJ 1; lane 4, MAJ 3; lane 5, MAJ 4; lane 6, MAJ 8; lane 7, MAJ 10; lane 8, MAJ 11; lane 9, MAJ 25; lane 10, MAJ 33; lane 11, MAJ 37; lane 12, MAJ 38; lane 13, MAJ 41; lane 14, MAJ 48; lane 15, negative control (*Escherichia coli*).
B: Lane 1, 1 kb molecular weight DNA marker; lane 2, *Bacillus sterothermophilus* (positive control); lane 3, MAJ 52; lane 4, MAJ 56; lane 5, MAJ 60; lane 6, MAJ 68; lane 7, MAJ 70; lane 8, MAJ 77; lane 9, MAJ 80; lane 10, MAJ 81; lane 11, MAJ 84; lane 12, MAJ 87; lane 13, MAJ 90; lane 14, MAJ 110; lane 15, *Escherichia coli* (negative control).

The smallest size of plasmid 4.0 kbp was found in the isolates (MAJ 8 and MAJ 87) while the largest plasmid size 28 kbp was found in the isolate (MAJ 80). Strain of *Escherichia coli* (TOP 10^R) was used as a positive control which contains plasmid of the size of 5.3 kb while *bacillus cereus* was used as a negative control (containing no plasmid DNA).

Amplification of Lipase and Lecithenase Genes: Using primer pair specific to lecithinase gene sequence for thermophilic bacillus species, genomic DNA of all 24 isolates were examined, *Bacillus cereus* was used as a positive control while *Escherichia coli* (TOP 10^R) was used as a negative control, seven bacterial isolates (MAJ 1, MAJ 11, MAJ 25, MAJ 38, MAJ 70, MAJ 80 and

MAJ 87) showed positive results (presence of the 411 bp product specific for lecithinase gene as shown in (Figure 3) which suggests that lecithinase gene in those isolates is chromosomally encoded, while the other isolates (MAJ 3, MAJ 4, MAJ 8, MAJ 10, MAJ 33, MAJ 37, MAJ 41, MAJ 48, MAJ 52, MAJ 56, MAJ 60, MAJ 68, MAJ 77, MAJ 81, MAJ 84, MAJ 90 and MAJ 110) showed negative results (no amplification bands).

Using another primer pair specific to lecithinase gene sequence for thermophilic bacillus species, plasmid DNA of each 24 isolates were examined with this PCR reaction, *Bacillus cereus* (genomic encoded) was used as a positive control while *Escherichia coli* (TOP 10^R) was used as a negative control, 13 bacterial isolates (MAJ 3, MAJ 4, MAJ 8, MAJ 11, MAJ 25, MAJ 33, MAJ 52, MAJ 56, MAJ 70, MAJ 81, MAJ 87, MAJ 90 and MAJ 110) showed positive PCR amplification results but with different amplicon size compared with our results using genomic DNA, the size of the amplification product was 1.2 kb as shown in (Figure 4a and b). According to these results, four bacterial isolates (MAJ 11, MAJ 25, MAJ 70, MAJ 87) showed positive results for both genomic (with 411 bp amplicon size) and plasmid DNA (with 1.2 kb amplicon size) suggesting that the lecithinase gene in those isolates is chromosomally and plasmid encoded.

Using primer pair specific to lipase gene sequence for thermophilic bacillus species, genomic DNA of each of the 24 isolates was examined with this PCR reaction (Figure 5a and b), *Bacillus stearothermophilus* (Local isolate in our laboratory) was used as a positive control while *Escherichia coli* (TOP 10^R) was used as a negative control, six bacterial isolates (MAJ 3, MAJ 10, MAJ 33, MAJ 37, MAJ 70 and MAJ 81) showed positive results (presence of the lipase gene specific amplification product of the size 1.3 kbp) suggesting the lipase gene to be chromosomally encoded, while the others showed negative results (no amplification bands).

Using primer pair specific to lipase gene fragment for thermophilic bacillus species, plasmid DNA from each bacterial isolate was examined with this primer pair, but no positive amplification fragment for lipase gene was observed, which suggest that some of the bacterial isolates (MAJ 3, MAJ 10, MAJ 33, MAJ 37, MAJ 70 and MAJ 81) have the lipase gene encoded on their chromosomes and not plasmid. *Bacillus sterothermophilus* (local isolate) was used as a positive control for the amplification of lipase gene fragment (its genomic DNA and not plasmid) while *Escherichia coli* (TOP 10^R) was used as a negative control (its plasmid DNA).

DISCUSSION

Thermophilic bacteria are of major interest for novel scientific knowledge and potential industrial applications. Thermophilic bacteria are able to produce enzymes with unique characteristics such as unusual temperature, chemical and pH stability. Subsequently, they can be used in several industrial processes to replace mesophilic enzymes or chemicals. In this study 24 local thermophilic bacterial isolates were investigated, they were all gram positive and belonging to the genus *Bacillus* [13].

Endospores were detected in all bacterial isolates indicating their natural adaptation to live in harsh environments. Similar results were reported by Bel'kova *et al.* [15] when they demonstrated that most of their isolated thermophilic bacteria were gram positive spore-forming rods and were assigned to the genus *Bacillus*. Biochemical tests further verified that these bacterial isolates under our current study belong to the genus *Bacillus*.

Regarding activities of thermophilic enzymes (phosphatase, proteinase, lecithinase and amylase), phosphatase activity was observed at 50°C and other lower and higher temperatures with optimum phosphatase activity at 50°C, It has been indicated that proteins from thermophilic organisms will retain their native structures under extreme conditions, where their homologues from mesophilic organisms denature [18]. Bel'kova *et al.* [15] demonstrated phosphatase activity in 79% (31 isolates) of total number (39) of analyzed isolates after incubation at 50°C, which indicated that 50°C was the optimal temperature for most of their thermophilic isolates.

With regard to the enzyme amylase, most of our bacterial isolates showed amylase activity with different clearance zones at different temperatures with an increase in amylase enzyme activity with increasing temperature within the range of 28°C to 60°C. An absence in enzyme activity was observed at values of 70°C. Similar study was described for *Bacillus* α-amylases by Cordeiro *et al.* [19]. While Bel'kova *et al.* [15], indicated in their study that their strains (11 out of 39) demonstrated the lowest starch-hydlolizing amylase activity. Themostable amylases have been isolated long time ago from several species of *Bacillus* and are widely used in starch industry and other applications.

All bacterial isolates under the current study have Proteolytic (proteinase) activity when tested at different incubation temperatures except at 70°C, with the optimum proteinase activity at 50°C. Guangrong *et al.* [20] had done a similar study in which proteinases from

thermophilic bacterial isolates had a relatively broad temperature adaptability ranging from 28°C to 60°C with an optimal temperature of 50°C. Another study by Razak *et al.* [21] found that *Bacillus* sp. which produced a thermostable protease has an optimum activity at 60°C. While different *Bacillus stearothermophilus* strains produced an alkaline and thermostable protease which was optimally active at 85°C [4, 22, 23]. Among the several proteases, bacterial proteases are the most significant, compared with animal and fungal proteases and specifically, Bacillus species produces extra-cellular proteases. Thermostable proteases are preferred in some applications because higher processing temperature can be employed, resulting in faster reaction rate and reduction in microbial contamination of mesophilic microorganisms.

The majority of our bacterial isolates (22 out of 24) showed lecithinase activity at temperatures ranging from 40°C to 60°C with the optimal lecithinase activity observed at 50°C. Bel'kova *et al.* [15] used chicken egg yolk agar medium to demonstrate lecithinase activity in bacterial strains at 20°C and 50°C, the clearance zones varied from 4 to 12 mm for 67 % of their isolates, while the clearance zone for lecithinase activity ranged from 6 to 17 mm and 69% of total isolates showed lecithinase activity.

Plasmid analysis of our bacterial isolates indicated that 22 out of 24 contained plasmids with sizes ranged from 4.0 to 28.0 kb. The presence of plasmids suggests an evolutionary adaptation of bacteria to live in extreme environments as indicated also by the presence of large sizes of plasmids. Aslim and Beyatli [24] isolated thirty four *Streptococcus thermophilus* strains from yogurt samples and demonstrated that 7 strains did not carry any plasmid DNA, while the rest of their bacterial strains included plasmid DNA ranging in number from 1 to 5 plasmids. Another study was done by Bruggemann and Chen [11], in which they reported that *Thermus thermophilus* strains HB27 and HB8 have two megaplasmids which implicated in promoting thermophilic growth.

According to our results using PCR amplifications studies, seven bacterial isolates revealed the presence of lecithinase gene to be chromosomally encoded. Similar results were obtained by Schraft and Griffiths [17] when they used the same primer pair to detect lecithinase gene in several *Bacillus* spp. While 13 bacterial isolates showed positive PCR amplification when using plasmid DNA as template with a different amplicon size compared to genomic DNA, this difference may be due to amplification of larger fragment of the lecithinase gene, different isoform of this gene or a nonspecific PCR amplification reaction, but BLAST alignment for the primer pair indicated that this primer is specific only to lecithinase gene of the indicated species. However, in order to verify the nature of these PCR amplification results, DNA sequencing is recommended. The bacterial isolates with positive amplification products for both genomic and plasmid DNA suggest that the lecithinase gene in those bacterial isolates is both chromosomally and plasmid encoded. Further more, this may indicate the presence.

PCR results using primer pair specific to certain thermophilic *bacillus* spp. to detect if lipase gene sequence is encoded by the chromosome or/ and plasmid DNA, revealed 6 bacterial isolates with positive amplification product using genomic DNA as template. Similar study was done by Bell *et al.* [25] where they used the same primer pair to detect lipase gene in some thermophilic *bacillus* spp. and obtained similar results. When plasmid DNA was used as template in PCR, no positive amplification product for lipase gene was observed, indicating that the above bacterial isolates have lipase gene chromosomally encoded. Lipases of microbial origin are the most versatile enzymes and are known to bring about a range of bioconversion reactions, most of the industrial processes in which lipases are employed are required to exhibit an optimum temperature of around 50°C [26].

In conclusion, physiological and molecular investigation of all 24 bacterial isolates indicated the presence of thermophilic enzyme activities (amylase, lecithinase, phosphatase and proteinase) with an optimal at 50°C. Genes encoding those different enzymes were chomosomally and/ or plasmid encoded, they could be further studied in a gene library of our unique thermophilic *Bacillus* isolates.

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