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Research article

Determination of heat shock proteins in certain *Bacillus* species

Elcin Yenidunya Konuk*[1]

[1] *Izmir Bakircay University School of Medicine, Department of Medical Biology, 35665, Seyrek, Menemen, Izmir, Türkiye*

Abstract

In this study, the effects of four different temperatures on the sporulation and development of 6 *Bacillus* species, 2 native and 4 reference were investigated. The SDS PAGE analysis emerged that two different proteins, 40 and 39 kilodalton (kDa), were produced by *Bacillus sphaericus* ATCC 2362 after 24 and 48, respectively hours at 48°C, by local isolate 4 after 24 and 48 hours at 42°C and by local isolate 31 after 24 and 48 hours at 48°C. Additionally *Bacillus firmus* (ATCC 14573) produced a 40 kDa protein after 48 hours at 52°C and *Bacillus thuringiensis* var. *israelensis* produced a 42 kDa protein after 48 hours at 42°C. At temperatures of 48 and 52°C, after 12, 24 and 48 hours incubation, vegetative and heat resistant spore counts were determined to reduce by 10^4-10^6 fold according to bacterial counts. As a result, the data revealed that at 48 and 52°C spore vitality fell by a significant degree. Additionally, SDS PAGE analysis results showed that high temperature resistance was provided by different heat shock proteins a 40 kDa protein produced by *B. firmus* ATCC (14573), 40-39 kDa proteins produced by *B. sphaericus* (ATCC 2362), and local isolates 4 and 31 and 42 kDa protein produced by *B. thuringiensis* var. *israelensis*.

Keywords: *Bacillus spp.; electrophoretic; SDS-PAGE; sporulation; heat shock protein*

1. Introduction

Bacteria develop different types of adaptation for varying environmental conditions. The response of microorganisms to stress occurs through a number of regulatory mechanisms (Segal and Ron, 1998). A range of stress studies using ethanol, heat, hydrogen peroxide, salt, low-high pH, and heavy metals were completed for species in the *Bacillus* genus. It was found that the bacteria produce specific proteins depending on the stress factors to protect themselves under induced stress conditions (Browne and Dowds, 2001; Melly et al., 2002; Periago et al., 2002; Gomes and Simao, 2014). Heat stress is a significant stress factor with negative effects on bacterial development. According to Segal and Ron (1998), first studies on heat shock in bacteria were performed on *Escherichia coli* K-12. This species has σ^{32} factor with the ability to recognize the promoter region of the specific heat shock operon. Together with the transcriptional activator of this factor, half-life of the transcript in cytoplasm is very short. In addition to temperature, production of many other proteins are induced due to changes in other conditions. However, within these proteins one group is only induced by temperature which is called heat shock proteins. Different research groups completed many studies on the effects of different stress conditions on species in the *Bacillus* genus (Burke Jr et al., 1983; Hecker et al., 1989; Elcin et al., 1995; Antelmann et al., 1997; Browne and Dowds, 2001; Berber et al., 2003; Berber et al., 2004; Beladjal et al., 2018; Hantke et al., 2019; Vahdani et al., 2019; Xie et al., 2019). In these studies bacteria from *Bacillus* genus that includes gene groups producing different special proteins against stress factors were generally chosen (Hantke et al., 2019; Schafer et al., 2019). Additionally, as members of this genus can live in high alkali environments and includes alkalophilic and facultative alkalophilic species with industrial importance they are enriched in stress mechanisms. Facultative alkalophilic *Bacillus* species can grow at pH 10 in addition at neutral pH values. However, obligate alkalophilic *Bacillus* cannot grow at pH below 8.5 (Horikosi and Akiba, 1982). Alkalophilic *Bacillus* species are

* Corresponding author.
E-mail address: elcin.yenidunya@bakircay.edu.tr (E. Yenidunya Konuk).
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known to be industrially important due to the enzymes and metabolites they produce (Krulwich and Guaffanti, 1989).

The aim of this study was SDS PAGE identification of heat shock protein of 6 *Bacillus* bacteria species, four of which were reference and 2 were native, all grown at high temperatures.

2. Materials and methods

In this study, two local *Bacillus* isolates previously defined as facultative alkalophilic (unpublished data), in project no. TBAG-2103(101T146) *B. thuringiensis* var. *israelensis and B. sphaericus* ATCC 2362 obtained from Prof. Dr. Cumhur Cokmus (Ankara University, Faculty of Science, Biology Dept.), and *B. firmus* (ATCC 14573) and *B. alcalophilus* (ATCC 27647) references species obtained from Dr. Arthur A. Guffanti and Dr. Terry A. Krulwich (Department of Biochemistry, Mount Sinai School of Medicine) were used.

2.1. Preparation of bacterial spore suspensions and creation of stress conditions

B. thuringiensis var. *israelensis* and *B. sphaericus* (ATCC 2362) strains were prepared from synchronized cultures on Nutrient broth, after which were linearly seeded on NYSM agar and left for five days until they fully sporulated. The *B. firmus* (ATCC 14573), *B. alcalophilus* (ATCC 27647) reference species and facultative alkalophilic local *Bacillus* isolates 4 and 31 were prepared from synchronized cultures in alkali broth medium (starch 10g, peptone 5g, yeast extract 5g, NaCl 5g, KH_2PO_4 1g, $MgSO_4.SO_4$ 0.2g, 1000 ml distilled water, pH 9.5-10) Then, these cultures were linearly seeded on alkali agar media and left for five days for full sporulation. Samples were taken at different time point during sporulation and visualized under a phase-contrast microscope. Then well-sporulated, cultures were gathered on the petri surface with sterile distilled water and stock spore suspension with a concentration of 4.4 x 10^{10} ($9.7x10^8$-$1.33x10^{11}$) spores/ml was prepared. The prepared spore stock solution was stored in a refrigerator at +4°C.

From the spore stock solutions prepared for each bacteria. 100 µl samples were taken and seeded on 50 ml broth (alkali or nutrient) in 100 ml Erlenmeyer flasks. Then, the seeded media were shaken at 150 rpm and left to grow for 48 hours in incubators at four different temperatures. Samples were taken from the bacteria cultures at 12^{th}, 24^{th} and 48^{th} hours and transferred to sterile Eppendorf tubes. The samples were stored in a deep freeze at -70°C until bacterial counts, protein extraction and electrophoresis procedure.

2.2. Total spore counts

To identify total bacteria counts after 12, 24 and 48 hours at four different temperatures, 100 µl of bacterial samples stored at -70°C were transferred to sterile Eppendorfs containing 900 µl sterile physiologic serum and diluted in series to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} rates. Before each dilution, 100 µl bacteria suspension was added to sterile petri dishes containing sufficient NYSM agar or alkali agar and bacterial suspensions were homogeneously mixed with the medium. Later sterile petri dishes were left for 24 hours in 30°C incubators for spore colony development. At the end of this time petri dishes containing from 30-300 colonies were taken and counted and the total bacteria count per ml was determined. Counts were made as triplicates on 4 parallels.

2.3. Electrophoresis and sample preparation

Electrophoresis procedure was completed according to Laemmli (1970). Briefly, bacterial samples stored in a deep freezer were transferred to 10 ml sterile glass tubes, centrifuged at 5000 rpm for 5 minutes at +4°C and the supernatant was removed. After washing the pellet three times in sterile serum physiologic, it was transferred into a porcelain crucible containing 5 ml sterile serum physiologic. Later it was sonicated for 5 minutes at 48 W. The total cell protein extract was lyophilized for 12 hours and concentrated. Then the resulting protein extracts were treated with 40-50 µl SDS sample buffer (0.06 M Tris, 2.5% glycerol, 0.5% SDS, 1.25% β-mercaptoethanol and bromophenol blue) and boiled in a hot water bath for 5 minutes to have dissolved proteins. Then it was centrifuged for 5 minutes at 16,000 rpm.

2.3.1. Preparation of gels and electrophoresis

Acrylamide bisacrylamide stock solution contents are given Table 1. The prepared solution was filtered with Whatman No: 1 paper. It was stored at +4°C.

Table 1
Acrylamide bisacrylamide stock solution.

Component	Amount
Acrylamide	28.8 g
Bisacrylamide	0.12 g
Distilled water	100 ml

Separation gel tampon (1.5 M Tris-HCl, pH 8.6); The required amount of Trisma Base and SDS was weighed and taken into a clean flask, dissolved in some distilled water, adjusted to pH 8.6 with 6 N HCl and completed to the final volume. The solution was filtered through Whatman No.1 filter paper and sterilized in an autoclave for 15 minutes and stored at +4°C.

Stacking gel buffer (0.5 M Tris-HCl, pH 6.8) The required amount of Trisma Base and SDS was weighed and taken into a clean flask, dissolved in some distilled water, adjusted to pH 6.8 with 6 N HCl and completed to the final volume. The solution was filtered through Whatman No.1 filter paper and sterilized in an autoclave for 15 minutes and stored at +4°C.

Running buffer contents are given Table 2. The prepared solution was filtered with Whatman no:1 paper. It was stored at +4°C.

Table 2
Running buffer.

Component	Amount
Trisma Base	1.21 g
Glycine	5.76 g
SDS	1.00 g
Distilled water	1000 ml

Sample Buffer contents are given Table 3. Sample in resolving gel were stained with Coomassie Brillant Blue stain. Staining buffer content are given Table 4. The prepared solution was filtered with Whatman No: 1 paper. It was stored at room temperature %12 resolving gel prepared and poured into the lower. %5 stacking gel prepared and poured upp. After the electrophoresis process was finished, the molecular weights of the proteins were calculated using the software program Lab.

Image version 2.6 (Halle, Germany).

Table 3
Sample buffer.

Component	Amount
0.5 M Tris-HCl pH 6.8	5.12 ml
Glycerol	8.00 ml
Distilled water	3.00 ml
SDS	2.00 ml
2-β-mercaptoethanol	4.00 ml
Bromophenol Blue	0.025 g

Table 4
Staining buffer

Component	Amount
Coomassie Brillant Blue	1.50 g
Isopropyl Alcohol	250.0 ml
Glacial Acetic Acid	100.0 ml
Distilled water	650.0 ml

2.4. Statistical analysis

To determine differences between the temperature groups in terms of spore counts, the variance analysis technique was used. The difference between temperature groups was tested at the p=0.05 significance level. The Duncan Multiple Comparison test was used for two-way comparisons of the temperature groups. All analyses were performed with the SAS packet program.

3. Results

3.1. Electrophoretic results

The protein profiles belonging to *B. firmus* (ATCC 14573) species at 12, 24 and 48 hour time points at four different temperatures (35, 42, 48, 52°C) are given in Fig. 1.

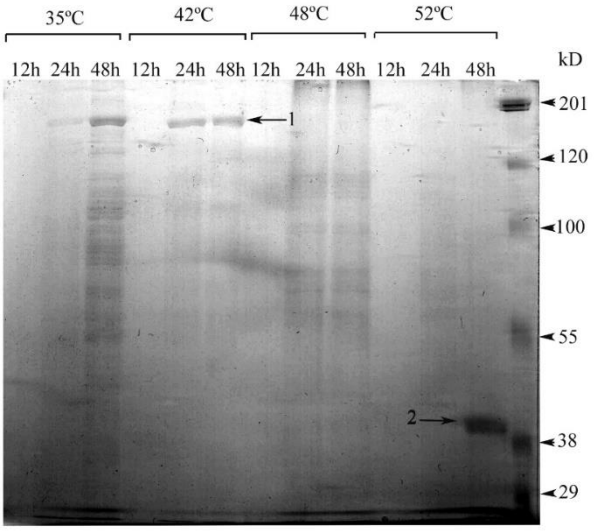

Fig. 1. Protein profiles from *B. firmus* (ATCC 14573) species developed at different temperatures, obtained with SDS PAGE.

As observed in Fig. 1, very significant differences observed between the 35, 42 and 48°C protein profiles at 12, 24 and 48 hours. The molecular weight of protein no. 1 is 177 kDa and it disappears at 48 and 52°C. At temperatures 35, 42 and 48°C, the protein profiles appear very weak after 12 hours. At 52°C, the 40 kDa molecular weight protein, labeled as, number 2, was produced after 48 hours.

The protein profiles of *B. alcalophilus* (ATCC 27647) species over 12, 24 and 48 hour durations at four different temperatures (35, 42, 48, 52°C) are given in Fig. 2. Protein profiles at 35, 42, and 48°C appear similar. However, the 116 kDa molecular weight protein band labeled number 1 was synthesized at 35 and 42°C, but the amount reduced at 48°C. The molecular weight band shown by number 2 is 79 kDa, and it was synthesized after 48 hours at 42°C. At 52°C, all bands synthesized at other temperatures were lost.

The protein profiles obtained after 12, 24 and 48 hour at temperatures of 35, 42, 48, and 52°C from *B. sphaericus* ATCC 2362 species are given in Fig. 3. At 35 and 42°C, *B. sphaericus* ATCC 2362 protein profiles at 12, 24 and 48 hours are very similar. When 48 and 52°C are examined, the protein amounts reduced, and at 52°C it appears there was no band. The 179 kDa and 149 kDa molecular weight proteins together shown by number 1 were protected at 35 and 42°C but were lost at 48 and 52°C. The number 2 band at molecular weight 109 kDa appeared to be only synthesized at 35°C after 12 and 24 hours. The molecular weight of the band at number 3 was calculated as 90 kDa and this band was synthesized at 42°C temperature after 12, 24 and 48 hours, while it disappeared at 48 and 52°C. The bacteria grown at 48°C only synthesized the double band with molecular weight 40 and 39 kDa shown by number 4 after 24 and 48 hours incubation.

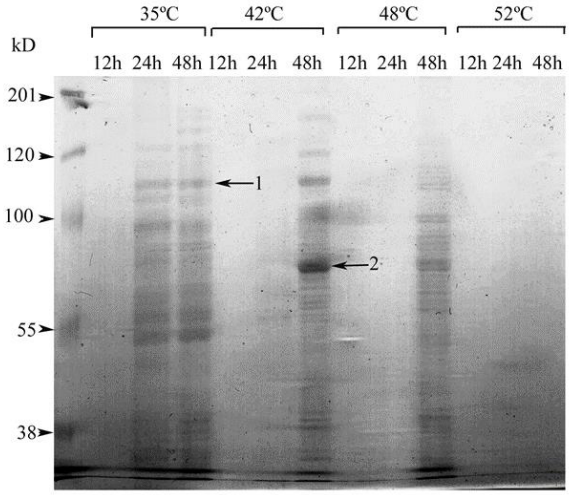

Fig. 2. Protein profiles from *B. alcalophilus* (ATCC 27647) species developed at different temperatures, obtained with SDS PAGE.

The protein profiles obtained after 12, 24 and 48 hours at temperatures of 35, 42, 48, and 52°C from *B. thurinrgiensis* var. *israelensis* are given in Fig. 4. At 35°C, there was no band formation after 12 hours, however after 24 and 48 hours, intense band profiles were observed. At 35°C, after 48 hours the density of protein labeled as number 3 with molecular weight 98 kDa was observed to increase. At 42°C, there was no band observed after 12 hours; however after 24 and 48 hours the band profile was densely observed. The band with molecular weight 178 kDa shown by number 1 and the 152 kDa band shown with number 2 were identified to intensely increased after 24 and 48 hours at 42°C. At 48°C, the bacteria did not grow well after 12 and 24 hours, while after 48 hours apart from the 42 kDa molecular weight band labeled number 4, no band formed. At 52°C, after

12, 24 and 48 hours no band formation was observed.

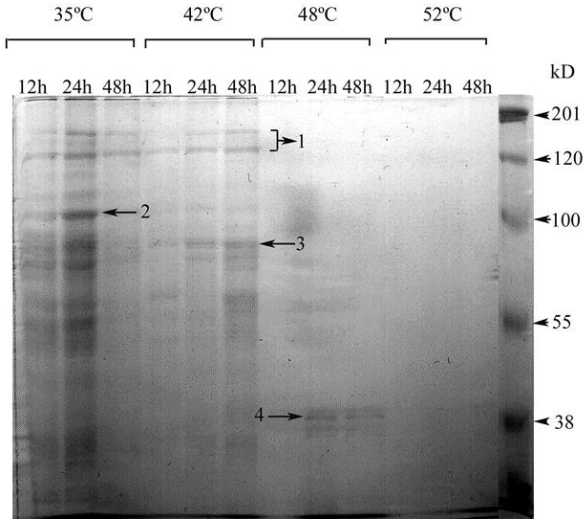

Fig. 3. Protein profiles from *B. sphaericus* (ATCC 2362) species developed at different temperatures, obtained with SDS PAGE.

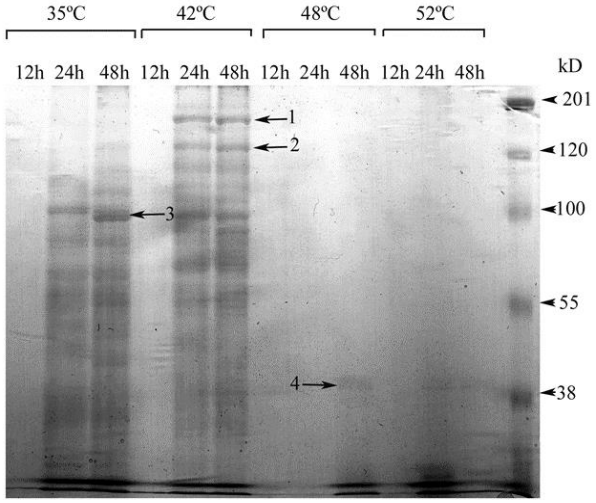

Fig. 4. Protein profiles from *B. thurinrgiensis* var. *israelensis* developed at different temperatures, obtained with SDS PAGE.

The protein profiles obtained after 12, 24 and 48 hour at temperatures of 35, 42, 48, and 52°C from local isolate 4 are given in Fig. 5. When protein synthesis at 35 and 42°C temperatures from local isolate 4 are examined, the band profiles appear very similar. The protein shown with number 1 with molecular weight 154 kDa, appeared after 12 and 24 hours at 35°C and 42°C. The amount of this protein synthesized at 42°C was identified to be higher than the amount synthesized at 35°C. The protein with molecular weight 97 kDa shown with number 2 was observed at 35°C and 42°C and this band also was observed after 48 hours. The amount of this protein synthesized at 35°C was higher than the amount synthesized at 42°C. At 48°C and 52°C, the bacteria did show good development after 12 hours. However, the twin band shown by number three with molecular weight 40 and 39 kDa was observed to develop at 42°C after 24 and 48 hours, at 48°C after 24 and 48 hours and at 52°C after 48 hours.

The protein profiles obtained after 12, 24 and 48 hour at temperatures of 35°C, 42°C, 48°C, and 52°C from local isolate 31 are given in Fig. 6. At the first two temperatures of 35°C and 42°C the band profiles appear similar. The band shown at number 1 with molecular weight 154 kDa and the band shown at number 2 with molecular weight 100 kDa were observed to be synthesized after 24 and 48 hours at 35°C and 42°C. These bands were lost at 48°C and 52°C. Band number 3 with molecular weight 97 kDa was produced at 35°C, 42°C and 48°C after 24 and 48 hours, but was lost at temperature 52°C. Band number 4 with molecular weight 52 kDa was produced after 24 and 48 hours at temperatures 35°C and 42°C; however it appeared to increase at 42°C. At 48°C, the band was not observed after 12 hours, but an increase was observed after 24 and 48 hours. The twin band shown by number 5 with molecular weight 40 and 39 kDa appeared to increase in amount at 48°C after 24 and 48 hours. Finally, at 52°C, no band formation was observed after 12, 24 and 48 hours.

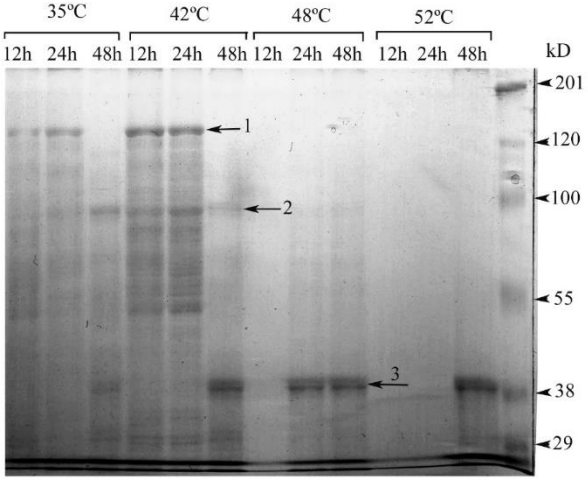

Fig. 5. Protein profiles from local isolate 4 developed at different temperatures, obtained with SDS PAGE.

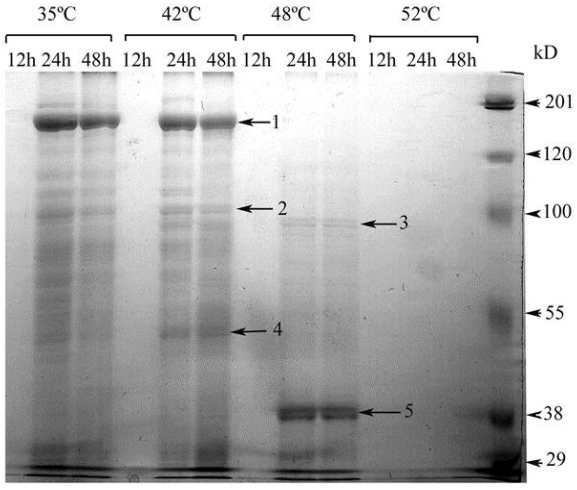

Fig. 6. SDS PAGE photograph showing development of isolate 31 at 35ºC, 42ºC, 48ºC, and 52ºC.

3.2. Bacterial development and sporulation results

The six different *Bacillus* species used in our study were found to have varying vegetative or spore state in a temperature (35ºC, 42ºC, 48ºC, 52ºC) and time-dependent manner. The time-dependent variation in counting results of these six bacteria species against temperature are shown in Table 5.

When the correlation between the temperatures and counting results for the 6 different bacteria species used in the study are examined, temperature was found to be very effective

Table 5
Count results for 6 bacteria used in the study (s: spore count, v: vegetative count, h: hour)

	35°C							42°C					
	12. h		24. h		48. h		12. h		24. h		48. h	
	*	V	S	V	S	V	S	V	S	V	S	V	S
B. firmus	$5.9x10^{10}$	$7.3x10^{4}$	$1.4x10^{4}$	$9.3x10^{6}$	$1.9x10^{6}$	$3.9x10^{6}$	$3.97x10^{7}$	$4.9x10^{4}$	$7.5x10^{4}$	$3.1x10^{5}$	$3.10x10^{5}$	$3.75x10^{5}$	$4.8x10^{4}$
B. alcalophilus	$5.48x10^{10}$	$3.6x10^{7}$	$3.69x10^{7}$	$1.63x10^{6}$	$1.4x10^{7}$	$3.15x10^{10}$	$3x10^{9}$	$1.70x10^{4}$	$1.45x10^{5}$	$4.98x10^{5}$	$3x10^{4}$	$1.41x10^{6}$	$1.9x10^{6}$
B. sphaericus	$8.7x10^{3}$	$8.33x10^{6}$	$1.83x10^{8}$	$4.11x10^{9}$	$3.36x10^{9}$	$1.3x10^{9}$	$1.43x10^{9}$	$1.36x10^{6}$	$4.9x10^{6}$	$4.5x10^{9}$	$3.99x10^{9}$	$1.64x10^{6}$	$5.91x10^{6}$
B. thuringiensis	$3.3x10^{9}$	$2.11x10^{6}$	$3.11x10^{6}$	$1.25x10^{7}$	$1.77x10^{6}$	$8x10^{6}$	$3x10^{9}$	$8.9x10^{5}$	$4x10^{4}$	$4.77x10^{5}$	$1.6x10^{5}$	$1.1x10^{5}$	$8.9x10^{5}$
4 Local isolate	$1.33x10^{11}$	$4.03x10^{7}$	$1.02x10^{7}$	$1.8x10^{6}$	$3.14x10^{6}$	$1.3x10^{9}$	$3.6x10^{6}$	$3.3x10^{7}$	$3.4x10^{6}$	$1.1x10^{7}$	$3.5x10^{7}$	$9x10^{7}$	$3.45x10^{6}$
31 Local isolate	$5.38x10^{9}$	$1.36x10^{5}$	$1.96x10^{5}$	$1.12x10^{8}$	$1x10^{6}$	$1.35x10^{9}$	$9x10^{6}$	$4.4x10^{4}$	$8x10^{4}$	$4.5x10^{6}$	$5.7x10^{6}$	$6.6x10^{7}$	$6.4x10^{7}$
	48°C							52°C					
	12. h		24. h		48. h		12. h		24. h		48. h	
	V	S	V	S	V	S	V	S	V	S	V	S
B. firmus	$4x10^{2}$	$1.9x10^{3}$	$7.9x10^{4}$	$1.6x10^{5}$	$1.64x10^{4}$	$7.1x10^{3}$	$8x10^{2}$	$3x10^{2}$	$2x10^{4}$	$1.81x10^{4}$	$1.37x10^{2}$	$3.1x10^{2}$
B. alcalophilus	$4.5x10^{3}$	$6.5x10^{3}$	$6.8x10^{3}$	$4.3x10^{3}$	$5.3x10^{5}$	$3.1x10^{5}$	$1.27x10^{4}$	$1.31x10^{4}$	$9.9x10^{3}$	$8x10^{2}$	$8.7x10^{3}$	$1.08x10^{4}$
B. sphaericus	$3.68x10^{4}$	$1.57x10^{4}$	$1.7x10^{5}$	$3.13x10^{6}$	$3.43x10^{5}$	$4.6x10^{5}$	$4.6x10^{3}$	$4.5x10^{3}$	$3.8x10^{3}$	$8.5x10^{3}$	$1.79x10^{4}$	$3.4x10^{4}$
B. thuringiensis	1.48x10	$3x10^{3}$	$1.55x10^{4}$	$1.12x10^{4}$	$3.1x10^{3}$	5.91x10	$3.33x10^{3}$	$1.5x10^{2}$	$6.54x10^{3}$	$1.8x10^{2}$	$3.1x10^{3}$	$5.91x10^{2}$
4 Local isolate	$1.13x10^{6}$	$5x10^{3}$	$3.1x10^{6}$	$1.35x10^{6}$	$1.13x10^{5}$	$3.35x10^{5}$	$3.5x10^{3}$	$5x10^{3}$	$3.5x10^{3}$	$8x10^{2}$	$1.54x10^{4}$	$2.97x10^{4}$
31 Local isolate	$1.6x10^{3}$	$3.9x10^{3}$	$1.5x10^{3}$	$1.72x10^{4}$	$3.73x10^{5}$	$3.34x10^{5}$	$6x10^{2}$	$3.3x10^{3}$	$1.3x10^{3}$	$7x10^{2}$	$1.16x10^{4}$	$1.01x10^{4}$

on heat resistant spore counts and this is shown in Table 6.

Table 6
Effect of temperature on heat resistant spore counts for six bacteria used in the study.

B. firmus (ATCC 14573)	p<0.0026
B. alcalophilus (ATCC 27647)	p <0.0012
B. sphaericus (ATCC 2362)	p<0.0001
B. thuringiensis var. *israelensis*	p<0.0001
Local isolate 4	p<0.0001
Local isolate 31	p<0.0012

4. Discussion

In our research, the development of 6 different *Bacillus* species was shown to reduce significantly when temperatures increased from 35ºC to 52ºC. Richter and Hecker (1986) in a study of rel A and rel A^+ species of *Bacillus subtilis*, determined that heat shock increased the synthesis of various proteins. At 52ºC cellular proteins were inhibited, while heat specific (heat shock) proteins were preserved. When temperatures rose from 37ºC to 52ºC synthesis of protein with 66 kDa weight was identified. In our study in *B. firmus* (ATCC 14573) 40 kDa weight protein and in local isolates 4 and 31- 40 kDa and 39 kDa weight protein synthesis was determined. Additionally, *B. cereus* was found to synthesize six different proteins at 43ºC; 76 and 66 kDa within 0-10 minutes, 57 and 39 kDa in 10 to 20 minutes and 98 and 43 kDa within 30-40 minutes (Browne and Dowds, 2001). When heat shock is applied to live cells, the synthesis rates of heat shock proteins are observed to increase (Gomes and Simao, 2014). Another study using heat shock stimulation of *B. subtilis* identified 97 and 66 kDa molecular weight proteins in membrane and 40 and 23 kDa molecular weight proteins in the cytosol (Qoronfleh and Streips, 1987). In our study at 35ºC and 42ºC temperatures, in local isolates 4 and 31, 97 kDa weight protein was synthesized and these results are in accordance with Qoronfleh and Streips (1987). We found that *B. thuringiensis* var. *israelensis* at 35ºC and 42ºC synthesized 98 kDa protein. At 52ºC *B. firmus* (ATCC 14573) reference bacteria synthesized a 40 kDa protein. After 48ºC temperature, *B. thuringiensis* synthesized a 42 kDa weight protein. Additionally, local isolates 4 and 31 and *B. sphaericus* (ATCC 2362) bacteria were observed to synthesize 40 kDa weight protein. A different study by Todd et al. (1985) applied heat shock to *B. subtilis* and found four heat shock proteins with molecular weights 84, 69, 32 and 22 kDa were produced. This study found that the 177 kDa molecular weight protein synthesized at 35ºC and 42ºC by *B. firmus* (ATCC 14573) and shown as band number 1 disappeared at higher temperatures (Fig. 1).
However the 40 kDa molecular weight at 52ºC is a heat shock protein. When *B. alcalophilus* (ATCC 27647) species was exposed to 35ºC, 42ºC and 48ºC, the amount of 166 kDa molecular weight protein shown by number 1 decreased at 48ºC and disappeared at 52ºC. After 42ºC temperature application, band number 2 was intensely synthesized and it reduced at 48ºC and was not observed at all at 52ºC (Fig. 2). At 35ºC and 42ºC, *B. sphaericus* (ATCC 2362) species developed a doublet band with 179 and 149 kDa molecular weight shown with number 1, which then disappeared at 48 and 52ºC. Stated differently, these proteins are susceptible to high temperature with the same situation present for bands shown with numbers 2 and 3. The doublet protein band shown by number 4 with molecular weight 40 and 39 kDa was not synthesized after 52ºC temperature application (Fig. 3). For *B. thuringiensis* var. *israelensis* a similar situation exists as for *B. sphaericus* (ATCC 2362). The bands shown by 1, 2 and 3 were intensely synthesized after 35 and 42ºC applications. The band shown by number 4 with molecular weight 42 kDa was synthesized at 48ºC but was lost at 52ºC (Fig. 4).

When local isolates 4 and 31 are examined, they synthesized a protein with molecular weight 154 kDa in band number 1 at 35ºC and 42ºC. The 97 kDa molecular weight protein shown with band number 2 produced by local isolate 4 and the 100 kDa molecular weight bands in local isolate 31 were not synthesized at 48 and 52ºC. The twin bands, shown by band 5 in local isolate 31 and by band 3 in local isolate 4 with 40 and 39 kDa molecular weight, were intensely synthesized at 48ºC and 52ºC. This band is thought to be a heat shock protein that can be synthesized at high temperature.

Heat shock studies of *Bacillus* species generally comprise of two stages preliminary shock and lethal shock. In our study we obtained results by applying four different temperatures over a 48 hour period. We identified two local isolates used in our study as being heat resistant. If the genes coding for the special proteins synthesized can be identified, we believe they may be

used in industry and for other applications.

Detailed studies have been carried out on genes and the control mechanism of these genes which code for heat shock proteins created specifically after heat shock of bacteria (Connors et al., 1986; Ron et al., 1999; Melly and Setlow, 2001; Versteeg et al., 2003). Many researchers have used high resolution gel electrophoresis (2D) to describe heat stress proteins in heat stress studies (Richter and Hecker, 1986; Connors et al., 1986; Qoronfleh and Streips, 1987; Antelmann et al., 1997; Browne and Dowds, 2001; Periago et al., 2002; Rosen and Ron, 2002; Han et al., 2008). In our study when high resolution gel electrophoresis is used the number of proteins may increase. A study by Elcin et al. (1995) encapsulated *B. sphaericus* (ATCC 2362) species in CMC (carboxymethylcellulose). The results of the study showed that at 50ºC the unencapsulated spores reduced 10^2-10^3 fold after 60 days, while the number of encapsulated spores did not change. In our study we noted that at 50ºC the number of heat resistant spores of *B. sphaericus* (ATCC 2362) species reduced 10^4 times. The data obtained in both studies show that *B. sphaericus* (ATCC 2362) is not resistant to heat.

When the counts at 35ºC for *B. firmus* (ATCC 14573), *B. alcalophilus* (ATCC 27647), *B. thuringiensis* var. *israelensis.* and *B sphaericus* (ATCC 2362) are compared with the counts at 52ºC, a severe reduction is observed. For local isolates 4 and 31, the results again identified a severe reduction from 35ºC to 52ºC. However, compared with the results from other species at 52ºC, the number of heat resistant spores appears to be higher. A variety of stress studies have been performed for bacteria in the *Bacillus* genus, used in a variety of areas. Studies of the alkalophilic *Bacillus* within the genus have generally focused on characterization of the microorganisms and the enzymes they produce, but studies related to stress were not found. It is possible that alkalophilic *Bacillus* may include gene groups that can produce different proteins depending on stress factors compared to other *Bacillus* species. Enzymes and proteins from alkalophilic bacteria are used in a variety of industrial fields such as detergent production and the paint industry. The gene groups of these bacteria, which can produce proteins and enzymes resistant to heat, can be determined and cloning studies can be made into different bacteria. After identifying heat shock proteins in our study, description of proteins with different techniques like 2D electrophoresis will be performed and later micro sequencing analysis will identify genes coding for these proteins. Additionally, the identification of gene groups coding for heat shock proteins determined in this study and the σ factor regulating transcription of these genes will contribute to studies in this area.

Recent research has determined that 4 out of 20 isolates from *B. sphaericus* were moderately toxic to larvae of the mosquito pathogen (Suryadi et al., 2016). We believe the use of heat shock proteins from two reference species that are mosquito pathogens used in our study will be a new area of use in biological intervention.

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