Current Research in Bacteriology

Morphological Changes Induced by Wet-heat in *Bacillus cereus* Endospores

[1]N.E.M. Mustafa, [2]U. Keller, [2]U. Malkus, [3]D. Harmsen, [2]R. Reichelt,
[4]A.A. Hussein and [1]S.M. El-Sanousi

[1]Department of Food Hygiene and Safety,
Faculty of Public and Environmental Heath, University of Khartoum,
P.O. Box 205, Post Code 11111, Khartoum, Sudan
[2]Institut Fuer Medizinische Physik und Biophysik, Universitaetsklinikum,
Westfaelische Wilhelms-Universitaet, D-48149 Muenster, Germany
[3]Institut Fuer Hygiene, Westfaelische Wilhelms-Universitaet,
D-48149 Muenster, Germany
[4]Department of Botany, Faculty of Science, University of Khartoum, Sudan

Abstract: Seven *Bacillus cereus* food isolates were subjected to microscopic investigation by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM) before and after wet-heat treatment at 100°C for 30 min using a heating device designed for rapid development of heat . Endospores suspensions were also autoclaved four times for 15 min each. Scanning electron microscopy showed complete destruction of exosporia in the majority of the strains studied while slight damages in endospore coats were observed. TEM showed destructive thermal treatment changes in cores and slight changes in cortices. Conventional autoclaving preserved endospores intact-structures. The clear morphological damage of the cores and the relatively slight damage to the cortex and coats support previous views suggesting that core contents might play more role/s in endospore resistance than expected. Hydrophobicity of endospores due to heat denatured proteinus exosporium of *Bacillus cereus* has been suggested as one of endospore wet-heat resistance factors.

Key words: *Bacillus cereus*, electron microscopy, endospore, heat, resistance, thermal treatment

INTRODUCTION

Spore former bacteria belonging to *Bacillus* and *Clostridium* genera are among the most important organisms considered in food contamination. Substantial problems of food contamination by spore former bacteria are food spoilage, lower food quality and potential risk of food poisoning outbreaks among consumers. Several species of *Bacillus* cause food poisoning and other infections. Emetic and diarrhoeal types of food poisoning are caused by *Bacillus cereus*. The emetic type is effective by a small cyclic heat-stable peptide, which causes vomiting a few hours after ingestion. Diarrhoeal types are attributed to enterotoxins (Hansen and Hendriksen, 2001; Granum and Lund, 1997).

Corresponding Author: Dr. Nazik Eltayeb Musa Mustafa, Department of Food Hygiene and Safety, Faculty of Public and Environmental Heath, P.O. Box 205, Post Code 11111, Khartoum, Sudan Tel: +249912133986

The endospores of *Bacillus* species are known for their extreme resistance to different parameters including heat. Food industries still rely largely on thermal treatment processes, such as pasteurization, ultra heat treatment and canning to produce safe food. These processes depend on both temperature of exposure and the time required to accomplish the required destruction rate.

Heat treatment effects on *Bacillus* and *Clostridium* endospores were extensively studied through thermal destruction time measurement, in which D and z values were recorded. D value is the time needed to reduce the size of the treated population by a factor of 10 where z value is the temperature interval at which D will decrease or increase by a factor of 10 (Scheldeman *et al.*, 2006). In spite the long history of wide use of electron microscopy techniques in investigating the ultrastructure of endospores, to the best of our knowledge, they are underutilized in studying the effect of heat treatment on endospores.

Electron microscopy data provided by several studies are still conflicting when come to study heat resistance. Kulikovskii (1979) evaluated the effect of heat on *Bacillus cereus* endospores using TEM and stated that no lyses or destruction occured in the main structural components of the spore when heated in distilled water at 99°C although 99% of the endospore population were destroyed after specific time. Molina-Garcia *et al.* (1989) used dynamic light scattering and SEM to study the effect of heat and disinfectants on endospores of *Bacillus cereus* and showed that some strains lost their integrity after 20 min of autoclaving where other strains retained their structural features even after 80 min of autoclaving. Leuschner and Lillford (2001) stated that heat activation of *Bacillus subtilis* endospores resulted in structural changes, which were revealed by TEM. Novak *et al.* (2003) showed that ultrastructural analysis of *Clostridium perfringens* endospores did provide additional evidence to support the dehydrated core mechanism of spore heat resistance; they argued that endospore core component SASP (small acid soluble proteins) in addition to dipicolinic acid (DPA) may be required for the attainment of heat resistance. Hyun *et al.* (1983) suggested that higher degree of endospore heat resistance is associated with endospore architecture displaying a thicker cortex layer and that heat resistance of endospores is proportional to the ratio of endospore cortex volume to cytoplasmic volume. Mallidis and Scholefield (1987) suggested that the amount and the organization of the cortex play an important role in the heat resistance mechanism.

The objective of this study was to observe and evaluate *Bacillus cereus* endospores morphological changes in each spore layer by both SEM and TEM before and after fast and slow wet-heat treatment and to correlate the structural changes with heat resistance capabilities of endospores.

MATERIALS AND METHODS

Source and Preparation of *Bacillus* Endospores

Bacillus cereus strains MK31, MK51, MK81, MK131, CH72, CH52 and MS21 were isolated from food of animal origin (milk, cheese and meat) in Khartoum State, Sudan during the year 2003. The isolates recovered aerobically on plate count agar and identified using conventional biochemical tests (Sneath, 1986). The isolates were further identified using Partial 16S rDNA sequencing. The partial 16S rRNA gene sequence (corresponding to primers for *E. coli* 16S rRNA positions 54 to 510) was determined using standard 16S rRNA gene primers for PCR and sequencing. Both forward and reverse strands were sequenced using standard procedures of cycle sequencing with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems). The generated 16S rRNA gene sequences were analyzed using the Ridom TraceEdit Pro (version 0.8 beta) software (Harmsen *et al.*, 2002; Blackwood *et al.*, 2004).

Spore Suspension Production

Brain Heart Infusion broth tubes were inoculated with *Bacillus cereus* strains required for spore production and incubated over night at 37°C. From the growth, aliquots of 0.1 mL were spread over plates of modified nutrient agar containing 0.3% MnSO$_4$. The plates were then enclosed in wide polyethylene bags to avoid medium dryness during the relatively long incubation time (7-9 days). When microscopic examination showed more than 95% spores, the plates were harvested by flooding the plates with sterile cold distilled water and scraping the surfaces of the plate with a bent glass rod. The obtained endospore suspensions were washed with sterile cold water using centrifugation and pelleting of endospores for 10 min each. This process was repeated 5 to 6 time until the supernatant was clear and the pellet was then dispersed in sterile distilled water then pasteurized at 80 °C for 10 min and rapidly cooled in running tap water and kept at 4°C until needed.

Endospore Thermal Resistance Determination

Thermal resistance was measured according to Harrigan (1998), using 100 mm length sealed tubes of 4 mm external diameter and 2 mm internal diameter. The tubes were flame sealed at one end to give a hemispherical end plugged with cotton wool and autoclaved. Endospore suspensions were enclosed in the tubes and flamed sealed. The tubes were subjected to heat treatment by immersion in heated oil bath. D-values of endospores were obtained from the equation:

$$D = \frac{t}{\log N - \log N0}$$

where, N is the final endospore population, t is the time at logN and No is the initial population of the straight line portion in the destruction curve produced by plotting the log10 number of survivors against time at 100°C (Haas *et al.*, 1996).

Heat Treatment of Endospores for Electron Microscopy Investigation of Heat Effect
Fast Heating Method

Hundred microliter of vortexed spore suspension were enclosed in glass capillaries (10×4) mm then flame sealed. The tubes were subjected to heat treatment at 100°C using a simple home-made thermal treatment device constructed for this study. The device reached the required stationary heating temperature in less than 30 sec and less than 10 sec were needed to reach 85 to 90% of the final temperature. The main component of the device was a solid copper block with a vertical drill hole (110 mm in length and 8 mm diameter) to house the 100 mm long and 4 mm outside diameter thermal treatment glass tube. The 2 mm space around the glass tube was filled with thermally stable heat conductive diffusion pump oil (SANTOVAC, Varian Vacuum Technologies, MA 02421, USA), which can be used several times without losing its heat conductivity efficiency. The device was calibrated before each heating experiment using a thermocouple inserted in a tube containing distilled water and was identical to the tubes containing spore suspensions. The thermocouple capillary tube was closed with heat resistant glue to provide the same environment of capillary tubes containing spore suspension. To record digitally the temperature-time characteristics the thermocouple was connected to amplifier, which interfaced to a computer and controlled through data logger software. Before each heating session data were calibrated and then analyzed with Excel software (Microsoft Corporation) by plotting heating curves to ensure proper and unified heating temperature.

Slow Heating Method

Conventional autoclaving of capillaries containing spore suspension was executed for four turns 15 min each, separated by cooling of autoclave content to room temperature.

Electron Microscopy
SEM

The spore suspension of the selected species was taken aseptically from its vial after brief vortexing and pelleted in 1.5 mL eppendorf tubes for 5 min. Six hundred microliter of 2.5% gluteraldehyde in phosphate buffer (PBS according to Sörensen (Romeis, 1968), pH 7.3) was added to each pellet and left at room temperature for one and half hour, after which the samples were preserved at 4°C till specimen preparation. Ten microliter of fixed spore suspension were placed onto small glass slides, which were coated before with a 2% solution of Poly-L-Lysine Hydrobromide (molecular weight 99.500, Sigma Aldrich, Hamburg, Germany). The spore suspension was left on the slides to settle for 3 h in a humidified chamber (wetted with PBS) to avoid drying of specimen. Subsequently, spores were post-fixed with osmium tetroxide (1% in phosphate buffer, pH 7.3) for 1 h at room temperature. Then the slides washed three times with phosphate buffer (pH 7.3) and dehydrated in a graded series of ethanol (30, 50, 70 and 90 and absolute ethanol). Each change solution was left for 15 to 20 min on the samples. The 70% and absolute ethanol changes were done twice. At this stage, the specimens were left in the absolute ethanol until Critical Point Drying (CPD) was performed. The small Petri dishes were sealed with Para film to avoid evaporation of the ethanol. After the CPD the slides were glued to aluminum stabs using conductive carbon cement (Neubauer Chemikalien, Muenster, Germany).

Since, spore surfaces are not conductive they have to be coated with electrically conductive material. For high resolution SEM work a very fine-grainy coating film is required. All samples were rotary shadowed with approximately 2.5 nm platinum/carbon (Pt/C) at an elevation angle of 55°. Another coating layer with the same thickness 166 was applied at an elevation angle of 15° to improve the electrical contact of the samples to the ground.

TEM

The spore suspensions were taken out of their vials separately and aseptically. After brief vortexing each suspension was pelleted in 1.5 mL eppendorf tubes with a microcentrifuge for 5 min. Six hundred microliter of 2.5% gluteraldehyde in PBS (pH 7.3) were added to each pellet and left at room temperature for 1.5 h. If storage of the specimens was required for some reasons the samples were kept at 4°C till further specimen preparation. After the fixation the samples were washed 3 times with PBS for 20 min each.

The specimens were post fixed with 1% osmium tetroxide for 1 h, subsequently washed with PBS and then dehydrated in the graded series of ethanol (30, 50, 70, 90 and 100% the 70 and 100% steps were done twice). The specimens in absolute ethanol were embedded in hard epoxy Spurr through serial changes of ethanol with the Spurr. In the first change the ethanol was removed by centrifugation and a mixture (1:2 Spurr:ethanol) was added and left for 2 h at room temperature. In the second change (1:1 Spurr:ethanol) was left overnight. The third change (2:1 Spurr:ethanol) for 4 h and for the last change pure Spurr for at least 4 h or overnight. The last change was repeated.

Polymerization of the Spurr was performed in a heating chamber at 70°C for at least 8 hours. Thin sections of 50-70 nm in thickness were prepared using a diamond knife in an ultramicrotome (Hoa *et al.*, 2000). The thin sections were double stained with lead citrate and uranyl acetate and then examined with the transmission 187 electron microscope H-500 (Hitachi Ltd., Tokyo, Japan) operated at 80 kV acceleration voltage.

Determination of Endospore Cortex Thickness

For each of the species both endospore cortex and core dimensions were measured using 6-20 representative electron micrographs of mature endospores thin sections. Cortex thickness was obtained by subtracting the radius of the core from the radius of endospore cortex plus core.

Selection of electron micrographs was based on good resolution of integument. Both elongated and round sections were chosen according to the similarity among micrographs.

RESULTS

Morphological Features of *Bacillus cereus* Endospores and their D-Values

Figure 1 and 2 show the General morphological features of *Bacillus cereus* native endospore. The endospores were oval; their length varies between 2 and 2.5 µm. All endospores studied possess exosporia and many of them possess pili that form net shapes in between (Fig. 3). D-values of some *Bacillus cereus* endospores are shown in (Table 1).

Heat treatment induced morphological changes in endospores of *Bacillus cereus*. The changes vary among the strains. Figure 4a and b show that *Bacillus cereus* CH52 endospores shrank significantly after heating (excluding exosporium). No significant changes in volume were observed in endospores of other strains of *Bacillus cereus* after heat treatment.

Heat Effect on Cores

TEM micrographs of native *Bacillus cereus* strains showed cores containing electron dense homogeneous materials that resemble ribosome-containing cytoplasm together with electron transparent materials that resemble DNA-SASP complex (Fig. 3). TEM micrographs of heated *Bacillus cereus* strains showed cores of less uniformity as more electron dense clump fragmented into small black spots distributed all over the core (Fig. 5).

Heat Effect on Cortices

TEM revealed that heating cause no visible damages in cortices. The integrity of cortex layers increased and a woven structure of the cortex became distinct (Fig. 6a, b and 7a, b). Enlargement of 70 to 75% in cortex thickness was observed in endospores of *Bacillus cereus* MK131. The increase in cortex thickness observed in strain CH72 reached 47%. Cortices thickness of *Bacillus cereus* strains MK52 and CH52 increased by 28%. *Bacillus cereus* MK81 cortices thickness decreased by 12% (Table 2). No significant changes appeared in cortices thickness of *Bacillus cereus* MS21.

Heat Effect on Inner and Outer Coats

The heat effect on outer endospore coat revealed by SEM showed a tortuous shape in some strains (Fig. 8). The tortuous shape of the outer coat did not appear in SEM micrographs of exosporium possessing strains unless the exosporium was destructed. The damage is manifested by changes in shape of ridges which became more tortuous than those

Table 1: D-values of some *Bacillus cereus* endospores

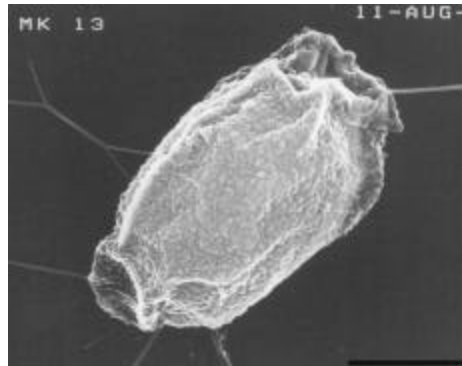| *Bacillus* strain	D-value (min)
Bacillus cereus MK81	1.28
Bacillus cereus MK51	1.66
Bacillus cereus MK131	2.52
Bacillus cereus MK54	5.55
Bacillus cereus CH52	5.90

Fig. 1: SEM micrograph recorded with secondary electrons shows the outside of a Bacillus cereus MK131 native endospore. The bar correspond to 500 nm
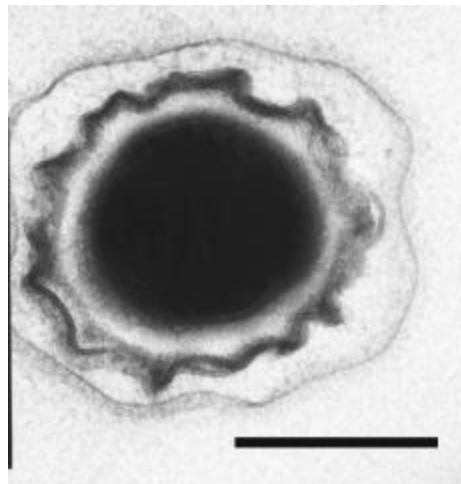

Fig. 2: TEM micrograph shows a cross-section through a *Bacillus cereus* native endosporeMK131. The bar corresponds to 455 nm

Fig. 3: SEM micrograph recorded with secondary electrons of *Bacillus cereus* endospores, which possess exosporia and pili forming net- like links between endospore
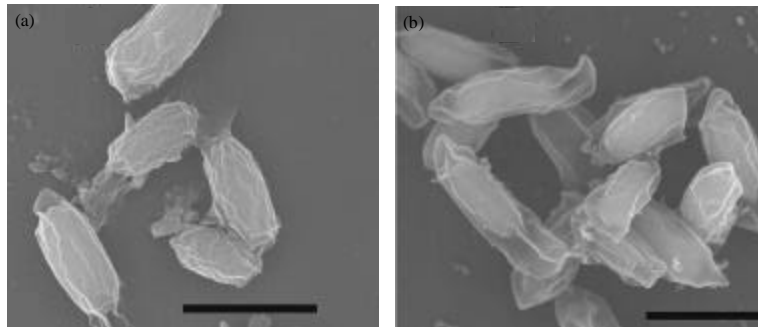
Fig. 4: SEM micrograph recorded with secondary electrons of *Bacillus cereus* CH52 spores (a) before and (b) after heating. The bars correspond to 2 μm
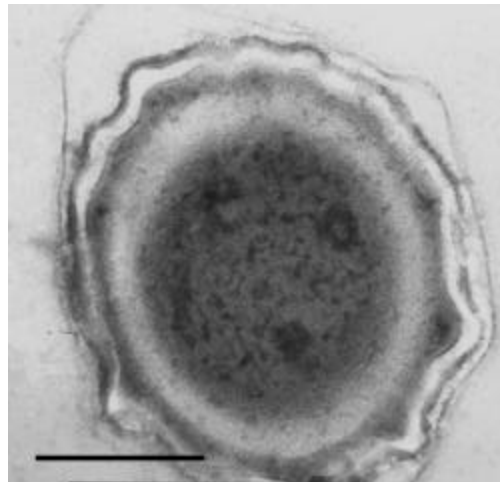

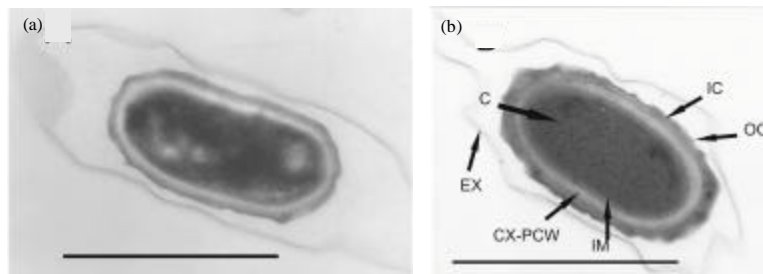Fig. 5: TEM micrograph of heated *Bacillus cereus* MK131 endospores. The bar corresponds to 295 nm

Fig. 6: TEM micrographs of *Bacillus cereus* MK81 spores (a) before and (b) after heating. Exosporium (EX), outer coat (OC), inner coat (IC), complex of cortex and primordial cell wall (CX-PCW), inner membrane (IM), core (C). The bars correspond to 1.3 μm
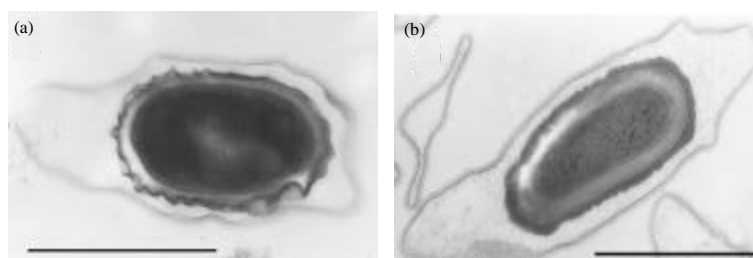
Fig. 7: TEM micrographs of *Bacillus cereus* MK131 spores (a) before and (b) after heating. The bars correspond to (a) 1.1 μm and (b) 1.23 μm, respectively
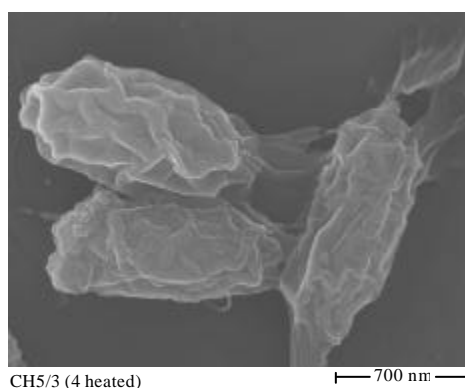

CH5/3 (4 heated)   ⊢——700 nm——⊣

Fig. 8: SEM micrograph recorded with secondary electrons of *Bacillus cereus* CH52 showing destructed exosporium and tortuous outer spore coat

Table 2: Approximate cortex thickness in *Bacillus cereus* strain measured in nanometres before and after heating

	Cortex thickness	
Bacillus cereus strain	Before heating	After heating
CH52	74	64
CH72	43	63
MK131	62	119
MK51	54	69
MK81	74	65
MS21	73	76

on native unheated endospores in which the ridges were more straight and running parallel to long axes of the endospores when the exosporium was removed by sonication and lysozyme treatment (data not shown).

TEM revealed no clear heat effect in the outer endospore coat or destruction of inner coat but endospore integuments became clearer in appearance and layers can be distinguished easily. *Bacillus cereus* MK52 is an exception; the inner layers of coat lost their integrity.

Heat Effect on Exosporia

The exosporia revealed by SEM were damaged by fast heat in *Bacillus cereus* strains MK131, MK52, MS21 and slightly destructed in *Bacillus cereus* CH72. No effects were
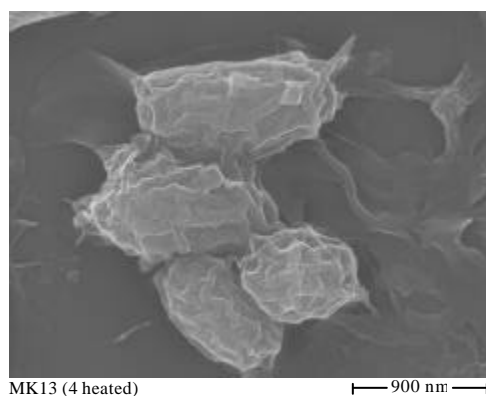
MK13 (4 heated)   ⊢——900 nm——⊣

Fig. 9: SEM micrograph recorded with secondary electrons of *Bacillus cereus* MK131 showing melting of exosporia after heat treatment

MK13 heated   ⊢——2 µm——⊣

Fig. 10: SEM micrograph recorded with secondary electrons showing aggregation and formation of a net shape after heating of *Bacillus cereus* MK131 endospores

observed in *Bacillus cereus* strains MK81 and CH52 exosporia. The electron non-dense exosporium of *Bacillus cereus* MK131 formed melting elastic parts after fast heat treatment (Fig. 9).

Bacillus cereus MK52 exosporium became fragmented and its residues were located at the endospore poles, this made the coat visible. Scanning electron microscopy of the fast heated endospores of strain MS21 also showed destructed exosporia.

Autoclaved endospores of *Bacillus cereus* MK131 showed no effect on exosporia even after four successive turns of autoclaving. The close-attached exosporium of *Bacillus cereus* MK31 endospore became very loose after heating indicating shrinkage of endospore volume.

Heat Effect on Pili

SEM micrographs showed that the straight pili of most *Bacillus cereus* endospores were bended or warped around the endospore. *Bacillus cereus* endospores MK131 pili tend to aggregate and form a net shape after heating (Fig. 10). Fast and slow heating processes had the same effect on pilli.

DISCUSSION

This is one of the few times that electron microscopy and imaging techniques are applied to investigate endospores from food isolates as most studies in this field were concentrated on limited number of type species strains. *Bacillus cereus* food stains MK31, MK51, MK81, MK131, CH72, CH52 and MS21 were all cultured in identical growth and sporulation media under the same conditions. However, they showed different D-values (Table 1). Variations in heat resistance among *Bacillus cereus* strains are not surprising; Gonzalez *et al.* (1999), Laurent *et al.* (1999) and Fernandez *et al.* (2001) studied endospores thermal inactivation of *Bacillus cereus* strains and found different D-values among the species.

Slow and fast heat showed the same effects on cores without major destruction in their integuments. The changes in the cores of *Bacillus cereus* strains were very clear after 20 min of heat treatment at 100°C. This is different from the observations made by Kulikovskii (1979), who stated that no effects or destructions were recognized on ultrathin sections of *Bacillus cereus* and *Bacillus anthracis* strains heated in water at 99°C even after 90 and 120 min of treatment. This is also different from his observation that autoclaving at 121°C for 15 min produced no effect although the *Bacillus cereus* endospores were not viable. The changes appeared in core support the suggestions that it is the site of damage in bacterial endospores exposed to moist heat, which damages the endospore membranes, cause protein denaturation and DNA strand breakage (Russell, 2003).

Setlow and Setlow (1996) stated that wet heat among other methods of treatment kill endospores that lack SASP by causing DNA damage and that the saturation of endospore DNA with $\acute{\alpha}/\beta$-type SASP provides protection against DNA base loss due to wet heat till 90°C . The logical assumption is that morphological destruction of endospores by heat should be more severe on the external layers (coat and cortex) but this is not the situation. It is clear from the electron micrographs that the effect observed in core is a systematic response to the heat. The micrographs of heated cores indicate the role of core content in wet heat resistance (Nicholson *et al.*, 2000). Depending on this observation we can suggest that there is a great possibility that SASP play more roles than expected in wet heat resistance (Novak *et al.*, 2003; Li and McClane, 2008). This view is more consistent when dipicolinic acid effect on resistance is excluded as it was been proved experimentally that its role in heat resistance may vary (Nicholson *et al.*, 2000; Orsburn *et al.*, 2008).

The spore cortices appeared striped after heating but we think these changes were destructive for the cortex mechanism in controlling water content of core. Hyun *et al.* (1983) suggest that higher degree of endospore heat resistance was associated with thicker cortex layer and that it is proportional to the ratio of endospore cortex volume to cytoplasmic volume but this cannot be approved for the strains studied. for example *Bacillus cereus* CH52 strain has similar cortex thickness to *Bacillus cereus* strain MK81 cortex although *Bacillus cereus* CH52 has higher D-value than *Bacillus cereus* strain MK81.

Slight changes observed in coats due to slow heating are to some extent agreed with Kulikovskii (1979) findings that the coat and layers were unaffected.

The micrographs showed exosporia in all the strains of *Bacillus cereus* investigated and the majority of them has pili flagellates. *Bacillus cereus* CH52 has the distinctive feature of multi-layered exosporium which may explain its relatively high D value. The study suggests that multi layer exosporium contribute in increasing heat resistance by working as a physical barrier. Thus, support the findings of Russell (2003) who stated that exosporium-less endospores of *Bacillus megaterium* are somewhat less heat-resistant than native

endospores. Exosporium and pili play some roles in endospore clumps formation, which may increase the resistance of the endospores by increasing endospores hydrophobicity, such development of resistance due to hydrophobicity has been mentioned before by Furukawa *et al.* (2005) but attributed to the denaturing of endospore coat surface proteins. *Bacillus cereus* exoporium has high protein content as stated by Matz *et al.* (1970), this enable *Bacillus cereus* exoporium to play the same role that is played by endospore coat surface proteins in heat resistance through protein hydrophobicity.

The fast heating destructed the endospores surfaces more than autoclaving, which preserved the original shape of endospore surfaces including exosporium. Fast heating cause fast boiling of water and denature protein within endospores causing destruction of the components while the slow heating process of conventional autoclaving give the proteins quite enough time for gradual coagulation before the water inside and outside the endospores reach the boiling point hence preserving the shape of the endospore.

Although, it is still far away to correlate endospore resistance to limited number of factors as stated before by Stecchini *et al.* (2006), the clear morphological changes of the cores and the relatively slight damage to the cortex and coats in dead endospore appeared in this study give core contents more role in endospore resistance than expected since it is the site of wet heat damage. Damage to the cortex, which supposed to control the water content of the endospore, never appeared clear in this study, but this may support the views of Lewis *et al.* (1960) and Stewart *et al.* (1980), who stated that heat damages the contractile mechanism of cortex. Loss of such mechanism cannot be seen, at least by the techniques used in this study. For better understanding of wet heat destruction mechanism more studies for numerous spore former bacteria is still highly needed. An important conclusion that 100°C destroy *Bacillus cereus* endospore structures more than autoclaving at 121°C, this may highlight the importance of using fast heating devices at lower temperature for liquid foods to preserve their sensory and nutritional properties.

ACKNOWLEDGMENTS

REFERENCES

Blackwood, K.S., C.Y. Turenne, D. Harmsen and A.M. Kabani, 2004. Reassessment of sequence-based targets for identification of *Bacillus* species. J. Clin. Microbiol., 42: 1626-1630.

Fernandez, A., M.J. Ocio, P.S. Fernandez and A. Martine, 2001. Effect of heat activation and inactivation conditions on germination and thermal resistance parameters of *Bacillus cereus* spores. Int. J. Food Microbiol., 63: 257-264.

Furukawa, S., N. Narisawa, T. Watanabe, T., Kawarai and K. Myozen *et al.*, 2005. Formation of the spore clumps during heat treatment increases the heat resistance of bacterial spores. Int. J. Food Microbiol., 102: 107-111.

Gonzalez, I., M. Lopez, S. Martinez, A. Bernardo and J. Gonzalez, 1999. Thermal inactivation of *Bacillus cereus* spores formed at different temperatures. Int. J. Food Microbiol., 51: 81-84.

Granum, P.E. and T. Lund, 1997. *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol. Lett., 157: 223-228.

Haas, J., D. Behsnilian and H. Schubert, 1996. Determination of the heat resistance of bacterial spores by the capillary tube method. I. Calculation of two borderline cases describing quasi-isothermal conditions. Lebensm.-Wiss. und-Technol., 29: 197-202.

Hansen, B.M. and N.B. Hendriksen, 2001. Detection of enterotoxic *Bacillus cereus* and *Bacillus thuringiensis* strains by PCR analysis. Applied Environ. Microbiol., 67: 185-189.

Harmsen, D., J. Rothganger, M. Frosch and J. Albert, 2002. RIDOM: Ribosomal differentiation of medical microorganisms database. Nulc. Acids Res., 30: 416-417.

Harrigan, W.F., 1998. Laboratory Methods in Food Microbiology. 3rd Edn., Academic Press, San Diego, Carlifornia, ISBN: 0-12-326043-4, pp: 36-84.

Hoa, N.T., L. Baccigalupi, A. Huxham, A. Smertenko and P.H. Van, 2000. Characterization of *Bacillus* species used for oral bacteriotherapy and bacterioprophylaxis of gastrointestinal disorders. Applied Environ. Microbiol., 66: 5241-5247.

Hyun, H.H., J.G. Zeikus, T.R. Longin, J. Millet and A. Ryter, 1983. Ultrastructure and extreme heat resistance of spores from *Thermophilic clostridium* species. J. Bacteriol., 156: 1332-1337.

Kulikovskii, A.V., 1979. Structural changes in spores under high temperature exposure. Zhurnal Mikrobiol. Epidemiol. Immunobiol., 6: 65-67.

Laurent, Y. S. Arino and L. Rosso, 1999. A quantitative approach for studying the effect of heat treatment conditions on resistance and recovery of *Bacillus cereus* spores. Int. J. Food Microbiol., 48: 149-157.

Leuschner, R.G. and P.J. Lillford, 2001. Investigation of bacterial spore structure by high resolution solid-state nuclear magnetic resonance spectroscopy and transmission electron microscopy. Int. J. Food Microbiol., 63: 35-50.

Lewis, J.C., N.S. Snell and H.K. Burr, 1960. Water permeability of bacterial spores and the concept of a contractile cortex. Science, 132: 544-545.

Li, J. and B.A. McClane, 2008. A novel small acid soluble protein variant is important for spore resistance of most *Clostridium perfringens* food poisoning isolates. PLoS Pathogens, 4: e1000056-e1000056.

Mallidis, C.G. and J. Scholefield, 1987. Relation of the heat resistance of bacterial spores to chemical composition and structure. II. Relation to cortex and structure. J. Appl. Bacteriol., 63: 207-215.

Matz, L.L., T.C. Beaman and P. Gerhardt, 1970. Chemical composition of exosporium from spores of *Bacillus cereus*. J. Bacteriol., 101: 196-201.

Molina-Garcia, A.D., S.E. Harding, L. De-Pieri, N. Jan and W.M. Waites, 1989. Dynamic light-scattering studies on the effect of heat and disinfectants on spores of *Bacillus cereus*. Biochem. J., 263: 883-888.

Nicholson, W.L., N. Munakata, G. Horneck, H.J. Melosh and P. Setlow, 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev., 64: 548-572.

Novak, J.S., V.K. Juneja and B.A. McClane, 2003. An ultrastructural comparison of spores from various strains of *Clostridium perfringens* and correlations with heat resistance parameters. Int. J. Food Microbiol., 86: 239-247.

Orsburn, B., S.B. Melville and D.L. Popham, 2008. Factors contributing to heat resistance of *Clostridium perfringens* endospores. Applied Environ. Microbiol., 74: 3328-3335.

Romeis, B., 1968. Mikroskopische Technik. R. Oldenbourg Verlag, München Wien.

Russell, A.D., 2003. Lethal effect of heat on bacterial physiology and structure. Sci. Prog., 86: 115-137.

Scheldeman, P. L. Herman, S. Foster and M. Heyndrickx, 2006. *Bacillus sporothermodurans* and other highly heat-resistant spore formers in milk. J. Applied Microbiol., 101: 542-555.

Setlow, B. and P. Setlow, 1996. Role of DNA repair in *Bacillus subtilis* spore resistance. J. Bacteriol., 178: 3486-3495.

Sneath, P.H.A., 1986. Endospore-Forming Gram-Positive Rods and Cocci. In: Bergey's Manual of Systematic Bacteriology, Holt, J.G., N.R. Krieg, P.H.A. Sneath, J.T. Staley and S.T. Williams (Eds.). Williams and Wilkins, Baltimore.

Stecchini, M.L. M.D. Torre, E. Venir, A. Morettin, P. Furlan and E. Maltini, 2006. Glassy state in *Bacillus subtilis* spores analyzed by differential scanning calorimetry. Int. J. Food Microbiol., 106: 286-290.

Stewart, M., A.P. Somlyo, A.V. Somlyo, H. Shuman, J.A. Lindsay and W.G. Murrell, 1980. Distribution of calcium and other elements in cryosectioned *Bacillus cereus* spores, determined by high-resolution scanning electron probe X-ray microanalysis. J. Bacteriol., 143: 481-491.