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Inactivation of *Bacillus cereus* spores in liquid food by combination treatments of heat and irradiation

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Abstract

Spores of *Bacillus cereus*, like other bacterial spores, are heat and radiation resistance causing problem in food processing because of the high temperature or irradiation dose needed to inactivate them. In this work, combination treatments of heat and irradiation were tested for their potential to reduce heat-resistance of *B. cereus* spores in raw milk, carrot juice and water. D_T and Z-values were used to characterize heat resistance of these spores, whereas D_{10}-values were used to characterize radiation resistance. The results obtained indicated that D_{85}-values ranged from 24.9 to 35.2 min, D_{90}-values ranged from 7.6 to 11.6 min and D_{95}-values ranged from 2.4 to 4.7 min. The Z-values of *B. cereus* spores in the used media ranged from 9.81 to 11.24^{o}C. The D_{10}-values ranged from 1.9 to 2.6 kGy. Pre-irradiation treatment at 4 kGy followed by heating reduced D_{90}-values 2.8 to 3.4 times. The obtained findings indicated the effectiveness of irradiation at 4 kGy followed by heating in a same process to ensure safety of raw milk or carrot juice contaminated with Bacillus cereus.

Key words: Bacillus cereus spores, heat resistance, radiation resistance.

1. Introduction

B. cereus is a sporeforming, Gram-positive, motile, rod-shaped and facultative anaerobic bacterium associated with food spoilage and food poisoning in humans. From the food safety point of view, *B. cereus* is considered the most important among other bacterial species contaminating food because of its ability to form two types of enterotoxins: thermostable emetic enterotoxin or a thermosensitive diarrheal enterotoxin (Goepfert *et al.* 1972 and Schneider et al. 2004). Farinaceous foods are the most common vehicles of the emetic type endotoxin whereas the diarrheal type is associated with meat, soups, sauces, vegetables and milk products (Kramer and Gilbert, 1989). *B. cereus* has been isolated from fresh vegetables and refrigerated-minimally processed foods (Valero *et al.* 2002), raw and cooked rice (Sarrias *et al*. 2003), spices (Choo *et al.* 2007), fresh and heat treatment milk (Bartoszewicz *et al.* 2008) and seafood (Rahmati and Labbé, 2008). *B. cereus* form endospores which are resistance to inactivation agents such as heating, desiccation, UV, γ-radiation, high pressure and oxidizing agents (Van German *et al.* 1999 and Seltow, 2006).

Heat treatment is commonly used to inactivate both spoilage and pathogenic microorganisms in liquid food in order to improve food safety and extend shelf-life. Spores produced by various *Bacillus* species including *B. cereus* (a causative agent of food poisoning) are heat resistance (Setlow, 2006 and Uemura *et al.* 2010). There are many factors affecting the heat resistance of bacteria to food preservation, among of which are the type of bacteria and its strains, sporulation conditions and the nature of suspending media (Farkas, 1990 and Mazas *et al.* 2007).

Irradiation of food using ionizing radiation (γ and x-rays or electron beam) is used to inactivate both spoilage and pathogenic microorganisms and to guarantee the hygienic quality of several foodstuffs (Diehl, 1990). This technology has been approved as a safe and alternative preservation technique by Codex Alimentarius Commission (1992), WHO (1994), and International Atomic Energy Agency (2006). Microorganisms differ greatly in their resistance to ionizing radiation. Vegetative cells of bacteria are sensitive to ionizing radiation; with D_{10}-value usually lower than 1 kGy (D_{10}-value is the radiation dose, usually expressed in kGy, necessary to reduce the bacterial counts one log cycle). Bacterial spores are found to be more resistant than yeasts, molds and vegetative cell of bacteria, their D_{10}-values usually in the range of 1-4 kGy (VanGermen *et al.,* 1999).

Microorganisms highly resistant to heat or ionizing radiation cause big problem in food processing because of the sever treatment required to eliminate them. Such severe treatment may adversely affect the quality of the processed food as a result of the induced changes in organoleptic characteristics and loss of nutrients (Valero *et al.,* 2006). Therefore, less severe heat or irradiation treatments, *i.e.* combination treatment of heat and irradiation

during food processing is preferable. A pre-irradiation treatment followed by heating has been found to have a synergistic effect on the destruction of bacterial spores (Farkas, 1990). Therefore, the heat resistance of microorganisms can be reduced by medium doses of ionizing radiation (Gombas and Gomez, 1978 and De-Lara *et al*., 2002). Since the degradation of food sensory quality by ionizing radiation and by thermal treatments are dose and temperature dependent, it suggests that reduction of the radiation dose and thermal temperature would result directly in improving sensory properties of the treated food.

The main objectives of the present work are to investigate the heat resistance of *B.cereus* spores in raw milk, fresh carrot juice and water. The radiation resistance of these spores in the above suspending media was also investigated. Sensitization of *B.cereus* spores to heat by gamma radiation and vice versa were also investigated in order to identify the optimum time, temperature and irradiation dose necessary to inactivate *B.cereus* spores in liquid food.

2. Material and Methods

2.1. Bacterial culture

B.cereus used in this experiment was isolated from fresh-cut carrots on polymyxin-manitol-egg yolk phenol red agar (PMYPA) medium at Food Microbiology Lab, Microbiology Department, National Centre for Radiation Research and Technology, Egyption Atomic Energy Authority. The isolated *B.cereus* was identified by conventional methods which include Gram-staining, shape and position of endospores and biochemical testing according to the description of Cowan and Steel (1974), Parry *et al.*, (1983) and Bergey's Manual of Systemic Bacteriology (Holt *et al.*, 1993) . The identified *B.cereus* was confirmed by the PCR amplification method of 16S rRNA method and the 16S rRNA was deposited in the Gene bank database under accessions (AB 599718.1).

2.2. Spore preparation

Stock culture of *B. cereus* on TSA slant was inoculated in 125-ml Erlenmeyer flask containing 50ml of nutrient broth (NB) using inoculated loopfull and incubated overnight at 30^{o}C to achieve the stationary phase. 0.2 ml of this culture were spread into the surface of Fortified nutrient Agar (5 plates were used) and incubated at 30^{o}C for 5 days. The produced spores were harvested by flooding the FNA plate with 10 ml sterile distilled water and scratching the surface with a glass spatula. After harvesting, spores were washed four times with saline solution (0.85% W/V) by centrifugation at 10 000 rpm for 20 min. The resultant pellet was re-suspended in sterile distilled water. Spore suspension was heat treated in a water bath at 80^{o}C for 10 min to kill remaining vegetative cells. The concentration of spore suspension was estimated by spread –plating 0.1 ml portions on plates of PCA which were incubated at 30^{o}C for 24 h. The spore suspension was adjusted to concentration 10^{7}–10^{8} cfu/ml and maintained 4^{o}C until use (De-Lara *et al.*, 2002).

2.3. Heat treatments

Experiments were conducted by adding 0.5 ml of spore suspension to 4.5 ml of radiation sterilization (20 kGy) raw milk, fresh carrot juice and water in 10 ml screw- capped test tubes. The tubes were completely submerged in a water bath at 85, 90 and 95 °C constant temperature for various times. The temperature of the water bath and inoculated tubes were monitored with a digital thermometer. After 5, 10, 20 and 30 min, a single tube was removed, cooled in ice-water bath. The spores survivor counts were enumerated by serial dilution and plating in duplicates on PCA. The inoculated plates were incubated at 30 °C for 24-48 h. All heat treatments were performed in triplicate.

2.4. Calcuation of D_T- and Z values

The Thermal-decimal reduction time/ (D_T- values) which is defined as the time in minutes at temperature needed to reduce the spores population by 10-fold, were calculating from the slope of the regression line obtained with the values of the heat survival curves. The survival curves were obtained by plotting the $\log_{10}$ numbers of surviving spores against heating time (min) at each temperature. Z-value (^{o}C) (the increase in temperature required to reduce the decimal reduction time by 10-fold or by 90%) were determined by plotting log D_T value means against the corresponding temperature.

2.5. Irradiation treatments

For irradiation treatment, 0.5 ml of the spore suspension was added to 4.5 ml of radiation sterilization (20 kGy) raw milk, fresh carrot juice and water in the 10 ml – screw capped test tubes. The tubes were exposed to gamma radiation doses of 0.0, 2.0, 4.0, 6.0, 8.0 and 10 kGy. The spores' survivor counts were enumerated by serially

dilution and plating in duplicates on PCA using pour plating technique. The inoculated plates were incubated at 30°C for 24-48 h. Three replicate were used in each dose.

2.6. Calculation of D_{10}-values

Radiation decimal reduction doses (D_{10}- values) were calculated from the regression line obtained with the values of the radiation survival curves which are obtained by plotting the log survivor curves vs. the corresponding dose of irradiation.

Irradiation processes were performed using Nordion International Gamma Cell 220, located at the National Centre for Radiation Research and Technology, Nasr City, Cairo, Egypt. The dose rate of this source was 3.08 kGy/h at the time of irradiation and irradiation treatments were achieved at room temperature. Irradiation dosimetry was carried out by putting reference alanine dosimeters traceable to National Physical Laboratory (NPL), UK.

2.7. Determination of heat resistance before and after irradiation

B. cereus spores were suspended in radiation (20 kGy) sterilized raw milk, fresh carrot juice and saline solution, in screw-capped test tubes, then heated at 90°C for 0, 5, 10, 20 and 30 minutes before and after irradiation at 4 kGy. Determination of viable counts and construction of thermal survival curves have been carried out as previously mentioned. All experiments were performed in triplicate and values were recorded as the mean of the three replicate.

3. Results

3.1. Heat resistance of B. cereus spores

Heat resistance of bacterial spores measured by the so-called thermal decimal reduction time (D_T-value). Figures (1, 2, 3) show thermal survival curves at 85, 90, and 95°C of *B. cereus* spores suspended in raw milk, fresh carrot juice and water.

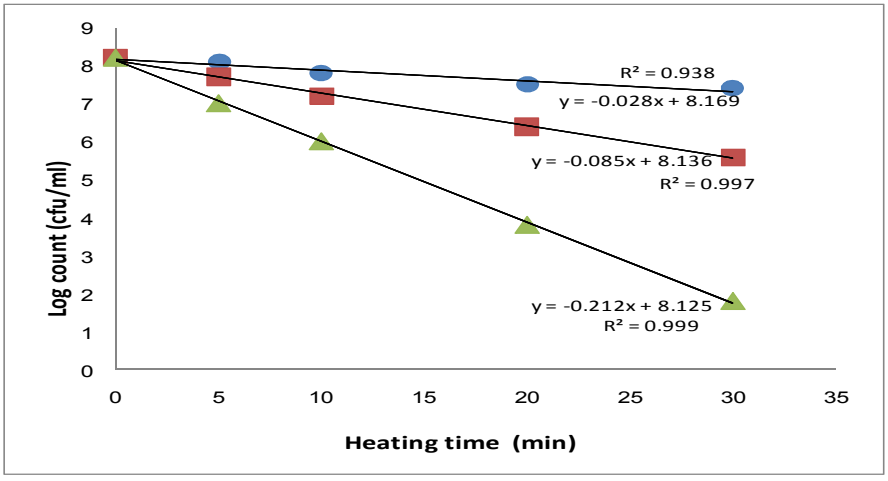

Fig (1): Thermal survival curves of *B. cereus* spores suspended in raw milk, heated at 85 (●), 90 (■) and 95 °C (▲).

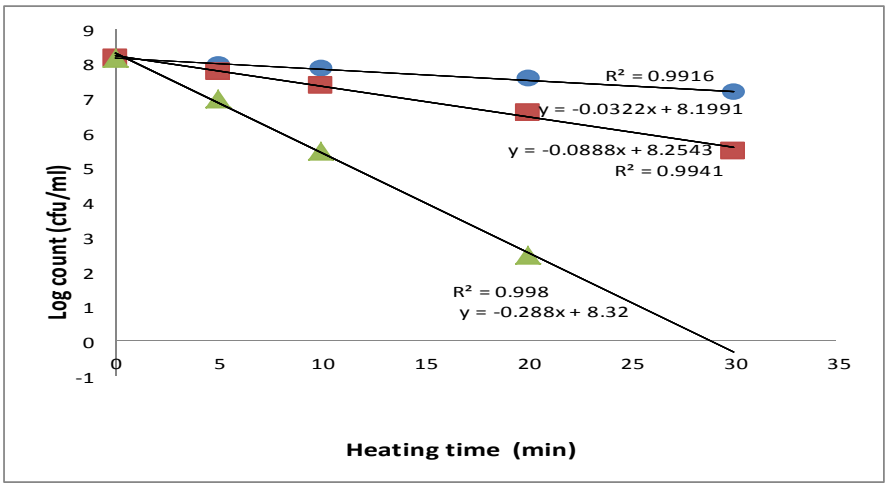

Fig (2): Thermal survival curves of *B. cereus* spores suspended in carrot juice, heated at 85 (●), 90 (■) and 95 ^{o}C (▲).

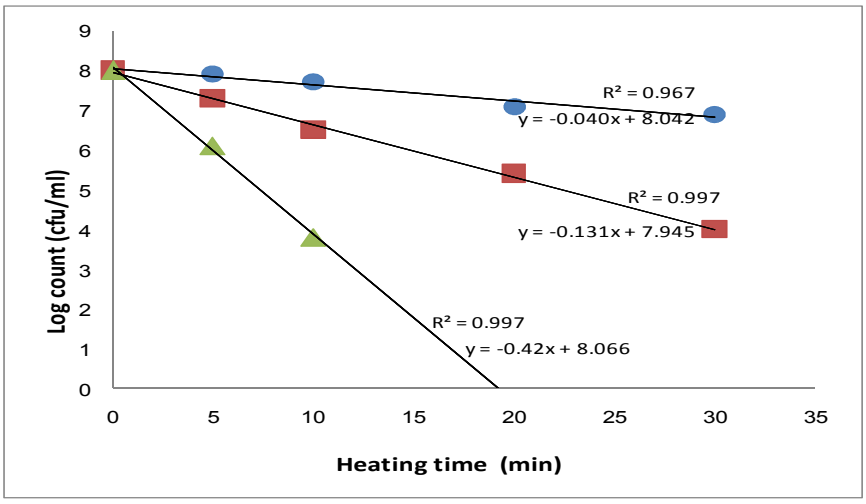

Fig (3): Thermal survival curves of *B. cereus* spores suspended in water, heated at 85 (●), 90 (■) and 95^{o}C (▲).

It is clear from these curves that D_{10}-values of *B. cereus* spores under investigation ranged from 24.9 to 35.2 min at 85 ^{o}C, calculated D-values at 90^{o}C ranged from 7.6 to 11.6 min and at 95^{o}C ranged from 2.4 to 4.7 min depending on the type of heating medium. It is obvious that *B. cereus* had low heat resistance in water than raw milk and fresh carrot juice.

Z-values which characterized and quantified spores heat resistance suspended in different media were determined and shown in Fig (4). Z-values ranged from 9.81 to 11.24^{o}C.

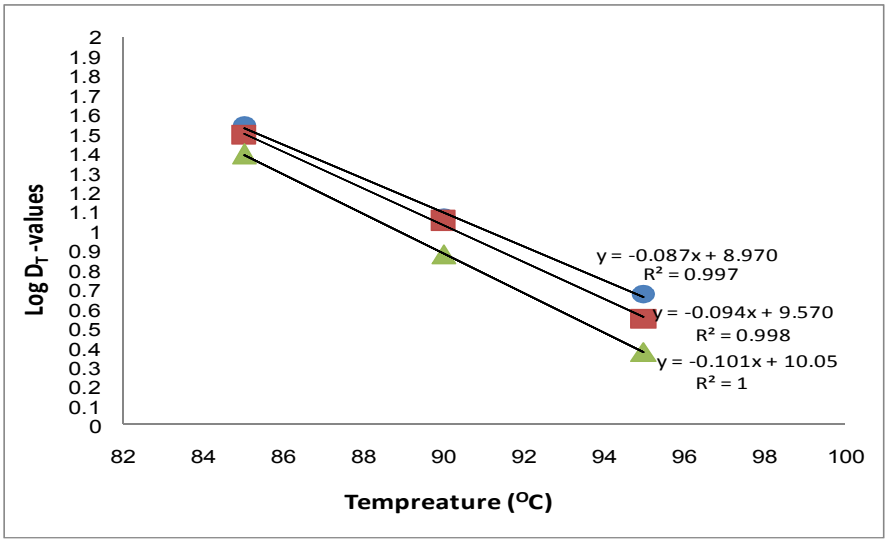

Fig (4): Z-values (°C) for *B. cereus* spores suspended in raw milk (●), fresh carrot juice (■) and water (▲).

3.2. Radiation resistance of B. cereus spores

Radiation resistance of bacterial spores measured by the so-called radiation decimal reduction dose (D_{10}-value). *B. cereus* spores suspended in raw milk, fresh carrot juice and water were exposed to incremental gamma radiation doses (0, 2, 4, 6, 8 and 10 kGy) to determine their radiation resistance. Fig (5) shows mean log survivors spores counts (cfu ml^{-1}) at different doses. Generally, there was a progressive decrease in survival counts as irradiation dose increased. The calculated D_{10}-values of *B. cereus* spores in raw milk, fresh carrots juice and water were 2.6, 2.4 and 1.9 kGy, respectively.

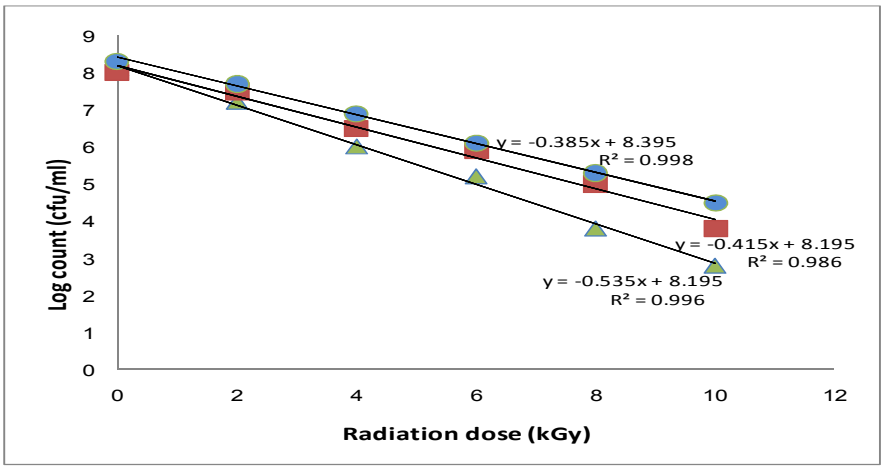

Fig (5): Radiation-dose response curves of *B. cereus* spores inoculated into raw milk (●), fresh carrot juice (■) and water (▲).

3.3. Heat resistance of B. cereus spores before and after irradiation

In this experiment, *B. cereus* spores suspended in raw milk, fresh carrot juice and water were tested for their heat resistance before and after irradiation to know what effect the reverse treatment. Figures (6, 7, and 8) illustrate the thermal survival curves of *B. cereus* spores suspended in the above media and heated at 90°C before and after irradiation at 4 kGy.

The calculated D_{90}-values of *B. cereus* suspended in raw milk decreased from 11.6 to 5.7 and 4.1 min when heat treatment achieved before and after irradiation, respectively. D_{90}-valules of *B. cereus* spores suspended in carrot

juice reduced from 11.3 to 5.4 and 3.8 min when heat treatment achieved before and after irradiation respectively. In water, the D_{90}-values reduced from 7.6 to 3.8 and 2.2 min.

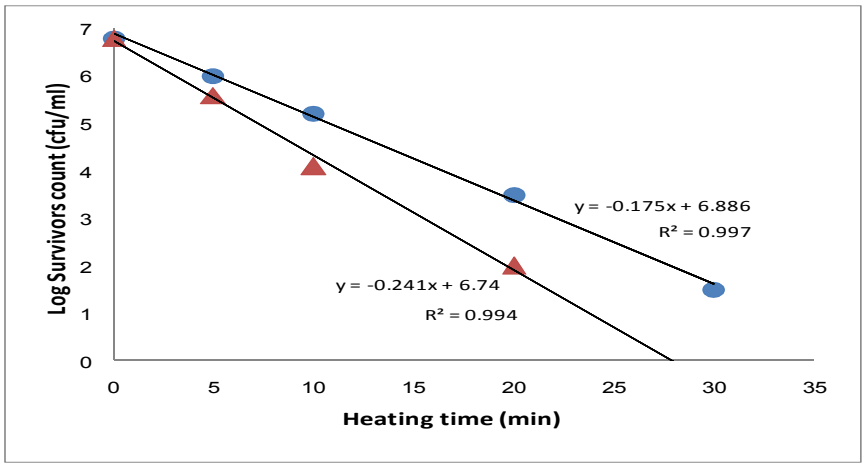

Fig. (6): Thermal survival curves of *Bacillus cereus* spores suspended in raw milk: heated at 90°C before (●), and after irradiation (▲) at 4 kGy.

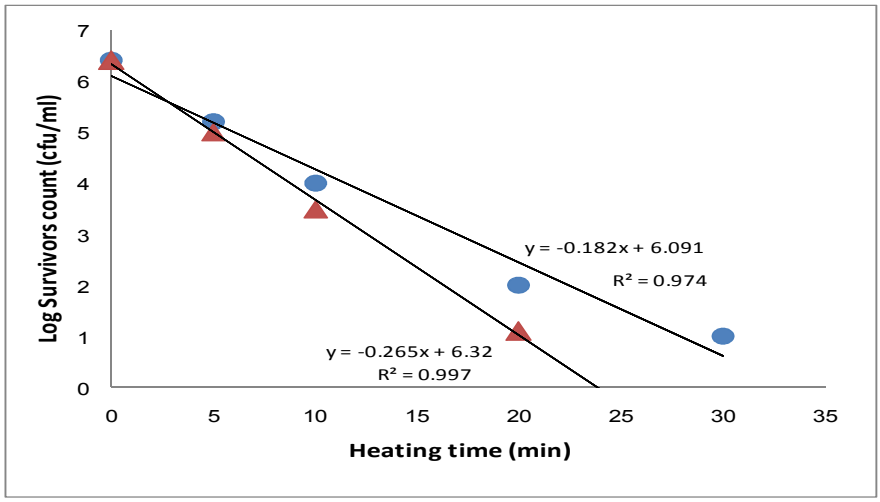

Fig. (7): Thermal survival curves of *Bacillus cereus* spores suspended in fresh carrot juice: heated at 90°C before (●), and after irradiation (▲) at 4 kGy.

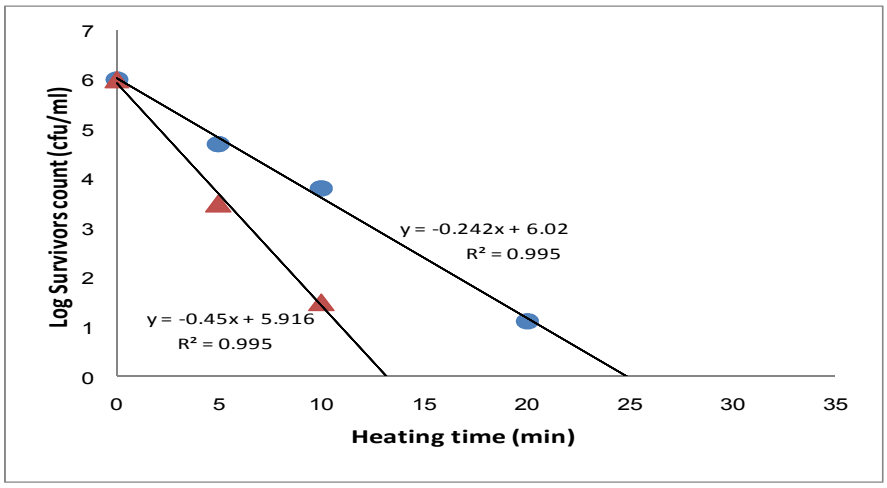

Fig. (8): Thermal survival curves of *Bacillus Cereus* spores suspended in water: heated at 90°C before (●), and after irradiation (▲) at 4 kGy.

4. Discussion

Spores of *B. cereus* are heat and radiation resistance, causing a big problem in food processing because of the high temperature or irradiation dose needed to inactivate them. In this work, the heat and radiation resistance of *B. cereus* spores in raw milk, carrot juice and water were investigated. Combination of heat and irradiation as reverse treatments was also studied to reduce the temperature or heating time required in liquid food processing for inactivation of these spores. D_{85}-values of *B. cereus* spores under investigation ranged from 24.9 to 35.2 min, D_{90}-values ranged from 7.6 to 11.6 min, whereas D_{95}-values ranged from 2.4 to 4.7 min depending on the type of suspending medium. The resistance of these spores was greater in raw milk than in the carrot juice or water. Heating at 95°C showed higher spore destruction than at 85°C or 90°C. The mechanism involved in the heat destruction of the bacterial spores towards to the most sensitive targets which likely seem to be core enzymes (Palop *et al.*, 1998) or spore membranes (Setlow, 1995), since spores DNA is very well protected by its combination with small acid soluble proteins (SASP).

The D_T-values of *B. cereus* spores obtained here in our investigation fell within those previous reported in the literature. Johnson *et al.*, (1982) examined spores of eight strains of *B. cereus* representing diarrheal, emetic and toxigenic strains. They found that D_{85}-values in sodium phosphate buffer (pH 7) ranged from 32.1 to 106 min, D_{95}-values ranged from 1.2 to 20.2 min. El-Fouly *et al.*, (1989) found that D_{90}-value for *B. cereus* spores in phosphate buffer (pH 8) was 17.8 min. Dufrenne *et al.*, (1994) published D_{90}-values from 4.6 to 14 min for *B. cereus* strains isolated from different sources. Valero *et al.*, (2002) examined thirty-two strains of *B. cereus* isolated from different kinds of fresh vegetables and refrigerated minimally processed foods for their spores heat resistance. They found that D_{90}-values in sterilized water ranged from 1.4 to 21.2 min. Sarrias *et al.*, (2002) tested spores of eight *B. cereus* strains isolated from Spanish rice and found D-values at 90°C in the range of 2.23 to 23.26 min, the calculated D_{95}-values ranged from 0.69 to 5.17 min. and D_{100}-values were in the range of 0.43 to 1.09 min. Montville *et al.*, (2005) examined spores of three *B. cereus* strains (ATCC 9818, ATCC 4342 AND ATCC 7004) for their heat resistance. They found D_{90}-values in buffer (pH 4 and 7), milk and orange juice in the range of 0.74 to 22 min according to the strains and type of suspending medium. Aguirre *et al.*, (2012) found D_{94}-values of *B. cereus* spores suspended in whole milk, saline solution and tryptone soya broth (TSB) to be 9.26, 8.64 and 8.49 min, respectively.

It is obvious from the D_T-values obtained in this work and from the published data in the literature that there was a wide range in spores heat resistance among *B. cereus* strains and our data fell within the published range. In this concern, Mazas *et al.*, (2007) reported that the properties of *B. cereus* spores thermal resistance are influenced by strain, sporulation conditions and heating medium.

Z-values on the basis of the D_T-values data obtained in our investigation, varied from 9.18 to 11.24°C. These data are similar to that obtained by Montville *et al.*, (2005), who found Z-values for spores of eight strains of *B. cereus*, *B. thuringinesis*, *B. mycoid* and *B. subtilis* in pH 4.5 and milk averaged 10.5°C, whereas in pH 7.0 and orange juice were significantly higher and averaged 12.9 and 13.9°C, respectively. Johnson *et al.*, (1982) found

Z-values in the range of 6.8 to 13.9 for spores of eight strain of *B. cereus*. Meanwhile Z-values obtained here in our investigation were higher than those reported by Sarrias *et al.,* (2002) who found Z-values for spores of eight *B. cereus* strains in the range of 7.4^{o}C to 8.2^{o}C, with an average 7.7^{o}C.

Radiation-decimal reduction doses (D_{10}-values) are used to characterize radiation resistance of *B. cereus* spores suspended in raw milk, carrot juice and water. It was found that *B. cereus* spores were higher resistance to gamma radiation in raw milk than in carrot juice or water indicating protective effect of the raw milk on the spores. This is true and could be attributed to that raw milk may contain certain compounds such as protein and fat which act as quencher or scavengers of the free radicals formed upon radiolysis of water in milk, leaving fewer ions to react with the spores thereby reducing the net effect of radiation damage (Urbain, 1986).

The mechanisms of inactivation viable microbial cells or spores by ionizing radiation include both direct and indirect action. Direct action involves absorption of photon energy by a target molecule in the cells (e.g. DNA) leading to damaged to that target. Indirect action resulted from absorption of photon energy by a nearby molecule (e.g. water) leading to the formation of highly reactive free radicals (H^{o}, OH^{o}, é) which in turn react with target molecule in the cells causing its damage (Thompson and Blachely, 2000).

The obtained D_{10}-values (1.9 - 2.6) in this work fell within the range previously reported by various investigators. Thayer and Boyde (1994) found D_{10}-values of approximately 2 kGy for spores of different strains of *B. cereus* gamma irradiated in several food products. Monk *et al.,* (1995) revised D_{10}-values of several foodborne microorganisms, including *B. cereus* spores and vegetative cells, and found D_{10}-values between 1.25 and 4.0 kGy when *B. cereus* spores were irradiated in different media. Also, Van German *et al.,* (1999) reported that the typical D_{10}-values for bacterial spores exposed to ionizing radiation coming from radionuclides were in the range of 1 ~ 4 kGy. De-Lara *et al.,* (2002) found higher D_{10}-values of spores of two strains of *B. cereus* being 3.0 and 3.8 kGy. Sarrias *et al.,* (2003) calculated D_{10}-values for spores of eight *B. cereus* strains suspended in distilled water. They observed diversity in radiation resistance among strains with average D_{10}-values ranged from 2.07 to 2.68 kGy. Valero *et al.,* (2006) found that the D_{10}-values for spores of two *B. cereus* strains suspended in sterilized water ranged between 2.48 and 2.75 kGy with an average of 2.62 kGy which is higher than the obtained D_{10}-value in our investigation. Ayari *et al.,* (2012) studied radiation resistance of *B. cereus* spores in sterile saline solution and in minced meat. They found that the D_{10}-values in saline was 1.75 kGy whereas in minced meat was about 3.7 kGy. *B. cereus* spores in our investigation showed radiation resistance values corroborate the previous findings.

Figures (6,7,8) show that heating before irradiation reduced D_{90}-values of *B. cereus* spores suspended in raw milk, fresh carrot juice and water by two times. Whereas, pre-irradiation treatment at 4 kGy followed by heating reduced D_{90}-values by 2.8, 3.0 and 3.4 times, respectively. This indicates that combination treatment of heat and irradiation greatly reduced the heat resistance of *B. cereus* spores. A pre-irradiation treatment had more synergistic effect on the destruction of *B. cereus* spores than pre-heating treatment. The synergistic effect of heating and irradiation would be easily explained, since their targets are different, and these exclude the possibility of cross-resistance (De-Lara *et al.,* 2002). Stegeman (1977) suggested that the sensitizing mechanism of pre-irradiation induced chain breaks in the peptidoglycan polymer and reduced the ability of spores to maintain the hydrated state of the cortex.

The pre-irradiation induced heat-sensitivity of *B. cereus* spores has been reported by other investigators. El-Fouly *et al.,* (1989) found that heating (90^{o}C for 30 min) before irradiation (6 kGy) reduced spore counts of *B. cereus* by 4.25 log cycles, whereas heating after irradiation reduced the counts by 7.82 log cycles. De-Lara *et al.,* (2002) found that radiation had an important heat-sensitizing effect on bacterial spores. After 3.3 kGy treatment D_{T}-values were reduced more than three times for both *B. cereus* and *B. subtilis* spores suspended in double distilled water. Valero *et al.,* (2006) investigated the effect of electron beam irradiation on spore heat resistance of two *B. cereus* strains. They found that after irradiation doses of 1.3, 3.1 or 5.7 kGy followed by heating at 90^{o}C calculated D_{90}-values were reduced more than 1.3, 2.4 and 4.6 times.

It could be concluded that pre-irradiation treatment followed by heating was more effective in reducing heat resistance of *B. cereus* spores suspended in different media. These combination treatments can be used to ensure safety of raw milk and carrot juice contaminating with heat resistant *B. cereus* spores without impairing product quality. 4 kGy irradiation dose used here in our investigations will not enough for inactivating bacterial spores in foodstuffs, but it sensitize spores to heat treatment applied in food processing.

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