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HOW DOES MOIST HEAT INACTIVATE MICROORGANISMS?

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Microorganisms are living organisms, and the environment in which they live can positively and negatively affect them. The environmental stress exerted on the microorganism leads to specific responses, dependent upon the organism species, the strain, the methods and media used to culture the organisms, the environmental conditions to which the organism was exposed during the stress condition, and the source or type of stress (Pflug 1999). When one subjects a microorganism to a stress condition or agent, attempting to inactivate the microorganism, the results may be variable depending upon the stress agent (Russell 1993). Furthermore, each stress condition or agent used to inactivate microorganisms has a specific mechanism via which the cell is inactivated (Pflug 1999). This chapter is devoted exclusively to using moist heat sterilization as a method of microbial inactivation. These principles may not be applicable to other types of sterilization agents, e.g., dry heat, ethylene oxide, chemical agents, and radiation.

WHAT DO WE MEAN BY MOIST HEAT STERILIZATION?

Steam sterilization or moist heat sterilization processes are accomplished by heating in the presence of moisture (water). There is no one temperature that must be achieved to attain sterilization conditions, although 121.1°C or 250°F are commonly used. The length of time required to achieve microbial inactivation depends on the length of time that the microorganism is subjected to

a specific temperature and the inherent resistance of the microorganism to inactivation. Accordingly, use of a lower temperature, e.g., 115°C, requires a longer exposure dwell period (time) to achieve the equivalent microbial inactivation as a higher temperature, e.g., 121.1°C. In summary, higher exposure temperatures require shorter exposure times and lower exposure temperatures require longer exposure times. Both, however, may be used and validated to achieve microbiological inactivation (Russell 1993).

Vegetative cells (i.e., bacteria that do not produce spores), fungi, and protozoa have minimal resistance to heating. They are typically destroyed by exposure to temperatures of 50°C to 60°C (Russell 1993). Most viruses are killed by exposure to 60°C for approximately 20 minutes, with DNA denaturation initiating at approximately 45°C. Bacterial spores have the potential to be more resistant to moist heat conditions. The organism species and strain may have an effect on the resistance. The most commonly reported moist heat resistant microorganisms are *B. stearothermophilus* (reclassified by ATCC to *Geobacillus stearothermophilus*), *B. subtilis* variety 5230, *C. sporogenes,* and *B. coagulans* ATCC 51232 (Owens 1993). The level of resistance to moist heat sterilization is generally represented by the D-value or z-value, discussed below. It is also believed that prions are excessively heat resistant (Russell 1993).

The thermal death time (TDT) is defined as the time in minutes that is required to kill all of the spores in a given suspension at a given temperature. Since the number depends on the total number of spores present in the solution or suspension, it has limited value. More frequently, the D-value is used to represent the heat resistance (Russell 1982).

The D-value is defined as the time in minutes, at a specified temperature, to kill one logarithm or 90% of the bacterial population. Some documents refer to D-value as the decimal reduction time (DRT) (Russell 1982). All D-values are specific to a temperature. Most frequently, they are expressed/evaluated at 121.1°C or 250°F, but they can be determined at any temperature. The heating characteristics at one specified temperature may not be true at another temperature. This value is represented by the following equation (Russell 1982):

$$\text{D-value} = \frac{\text{Duration of heat treatment (minutes)}}{\text{Log}_{10}\text{ Initial Population} - \text{Log}_{10}\text{ Final Population}}$$

$$= U / (\text{Log}_{10}\, N_o - \text{Log}_{10}\, N_u)$$

where:

U = time in minutes.
N_o = initial number of spores present.
N_u = final number of spores present.

It is expected that for any given spore, the D-value would decrease as the sterilization temperature increases (Russell 1982).

The z-value is another way to measure microbial moist heat sterilization resistance. It is defined as the number of degrees (usually expressed as degrees centigrade) necessary to change the D-value 10 fold (e.g., from 10 to 100) (Russell 1982). This number is obtained from the exponential curve obtained when the temperature (in degrees centigrade) is plotted on an arithmetic scale against the D-value on a logarithmic scale (i.e., use of semi-log graphs).

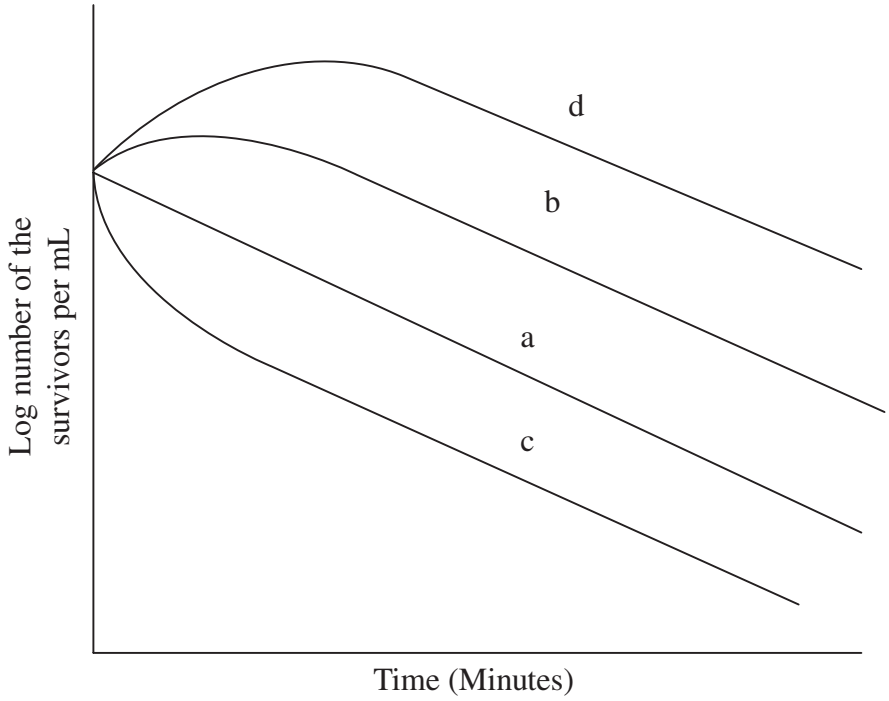
D-values and z-values allow the user to determine the relative sensitivity of different bacterial spores to moist heat sterilization processes. Biological indicators are organisms that have a high resistance to a specified sterilization process. These organisms are typically used as the bacterial challenge organisms in validation studies, representing the "worst-case" biological challenge. D, z, and F values and their relationships to effective sterilization are discussed in more detail in Chapters 3, 11, and 16 of this book.

COMPARISON OF MICROBIAL SENSITIVITY

There are many factors that affect the sensitivity or resistance of a microorganism to moist heat sterilization. Figure 1 graphically represents the various types of sensitivity/resistance to moist heat sterilization (survivor curves). The line designated "a" shows an exponential death rate of kill. The line designated "b" has a shoulder followed by an exponential death rate, and "c" shows an exponential death rate initially, followed by a tailing off. Line "d" shows when some spores initiate an apparent increase in viability and then inactivation. It is believed that the initial increase in viability is the result of heat activation breaking the bonds of dormancy in the spore. The shoulder effect shown in line "b" is postulated to be a result of clumping, or cases when more than one target site must be attacked to inactivate a cell. The tailing effect is postulated to be due to the presence of a very small population of highly resistant cells (Pflug 1999; Russell 1982).

Additionally, the microorganism resistance may be affected by the carrier solution or substrate (the product or component with which the microorganism comes into contact), and the recovery methods used. Pflug, Berger, Moldenhauer and others have published data on the effects of solution carrier on the biological indicator resistance. Table 1 is an abbreviated summary of some of the available data. Rubio and Moldenhauer (1995) published data on the effects of stopper composition on the heat resistance of *B. stearothermophilus* (reclassified by ATCC to *Geobacillus stearothermophilus*). Table 2 provides an abbreviated summary of this data.

Figure 1. Graphical Representation of Time-Temperature Survivor Curves



MECHANISMS OF INACTIVATION

There are several reported types of injuries caused to microorganisms by moist-heat.

Vegetative Cells

Mackey (1983) indicated that mild heat treatments (45°–50°C) cause damage to the outer membranes of Gram-negative bacteria, e.g., *E. coli.* This causes the cells to have increased sensitivity to hydrophobic inhibitors. There is also significant damage to the cytoplasmic membrane yielding RNA degradation, protein coagulation, and chromosomal injury. Heat can damage almost all cell structures and functions. However, cellular repair (including DNA) can take place only if the DNA remains functional. DNA injury occurs with this type of exposure, but it is postulated that the injury occurs as a result of enzymatic activity after the initial thermal injury (Russell 1993).

Table 1. Effects of Carrier Solution on Bacterial Moist Heat Resistance

Carrier Solution	Biological Indicator	D-Value, °C, Min.				z-Value	Table Reference
		110°	115°	120°	121.1°		
Adenosine Inj. USP (3 mg/mL)	*B. coagulans* ATCC 51232	–	–	1.61	–	–	9
Aminophylline Inj. USP (25 mg/mL)	*B. coagulans* ATCC 51232	–	–	1.36	–	–	9
Antifungal in 0.9% sodium chloride	*C. sporogenes*	–	–	–	0.5	10.5	3
Atrophine sulfate Inj. USP (0.3–0.4 mg/mL;	*B. coagulans* ATCC 51232	–	–	~0.8	–	–	9
1.0 mg/mL)		–	–	1.03	–	–	9
Butorphenol with benzethonium chloride (0.2 mg/mL)	*B. coagulans* ATCC 51232	–	0.54	–	–	–	9
Calcium chloride (100 m/mL; 10%)	*B. coagulans* ATTC 51232	–	–	1.29	–	–	9
	C. sporogenes ATCC 7955	–	–	–	0.2	10.7	3
	*B. stearothermophilus** ATCC 12980	–	–	–	4.9	6.3	3
Calcium gluconate Inj. USP (100 mg/mL)	*B. coagulans* ATCC 51232	–	–	1.92	–	–	9
Dextrose (5% in water)	*B. coagulans* ATCC 51232	–	4.9	1.20	–	8.2	7
	*B. stearothermophilus** ATCC 7953	87.8	32.0	3.27	2.42	10.3	11
	B. subtilis 5230	–	–	0.47	0.34	8.0	4
	C. sporogenes ATCC 7935	–	–	–	0.3	14.7	3
	C. sporogenes PA 3679	1.34	–	0.05	–	10.7	11

*Note: *B. stearothermophilus* was subsequently reclassified as *Geobacillus stearothermophilus.*

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Table 1 continued from the previous page

Carrier Solution	Biological Indicator	D-Value, °C, Min. 110°	115°	120°	121.1°	z-Value	Table Reference
Gentamicin sulfate Inj. USP (40 mg/mL)	*B. coagulans* ATCC 51232	–	–	0	–	–	9
Glycopyrolate Inj. USP (0.2 mg/mL)	*B. coagulans* ATCC 51232	–	0.52	–	–	–	9
Hyudroxyzine HCl Inj. USP (50 mg/mL; 120 mg/mL)	*B. coagulans* ATCC 51232	–	–	0.4	–	–	9
	B. coagulans ATCC 51232	–	–	1.13	–	–	9
Lidocaine HCl Inj. USP (20 mg/mL)	*B. coagulans* ATCC 51232	–	–	0.50	–	–	9
Magnesium sulfate Inj. USP (500 mg/mL)	*B. coagulans* ATCC 51232	–	–	1.13	–	–	9
Multilyte (20)	*B. coagulans* ATCC 51232	–	–	1.38	–	–	9
(40)	*B. coagulans* ATCC 51232	–	–	1.51	–	–	9
Neostigmine	*B. coagulans* ATCC 51232	–	–	0	–	–	9
0.025 M Phosphate (M/40)	*B. coagulans*	2.4	–	0.19	–	9.0	12
pH = 6.8	*B. coagulans*	6.1	–	0.42	–	8.6	6
0.067 M Phosphate (M/15) pH =7.0	*B. stearothermophilus** ATCC 7953	–	88.4	4.40	3.36	7.6	11
	B. subtilis 5230	23.9	6.70	0.39	–	8.3	10
	B. subtilis 5230	–	–	0.42	0.3	7.6	1
	C. sporogenes PA36779	42.6	9.17	0.84	–	9.0	11
	C. sporogenes PA3679	–	11.80	1.36	–	10.8	2

*Note: *B. stearothermophilus* was subsequently reclassified as *Geobacillus stearothermophilus.*

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Table 1 continued from the previous page

Carrier Solution	Biological Indicator	D-Value, °C, Min.				z-Value	Table Reference
		110°	115°	120°	121.1°		
0.1 M Phosphate (M/10) pH = 7.0	*B. stearothermophilus**	–	–	2.60	2.10	8.5	8
0.00031 M (M13200) Phosphate pH = 7.2	*B. stearothermophilus** ATCC 7953	–	72.5	6.17	4.70	9.1	11
	C. sporogenes PA 3679	21.2	7.15	0.70	–	10.2	11
0.0153 M Phosphate (M/65) pH = 7.8	*B. coagulans* ATCC 51232	–	9.7	2.50	–	8.2	7
Potassium acetate Inj. USP (2 mEq/mL)	*B. stearothermophilus** ATCC 7953	–	–	–	3.2	–	3
	*B. stearothermophilus** ATCC 12980	–	–	–	6.6	6.5	3
	B. coagulans ATCC 51232	–	–	1.62	–	–	9
	C. sporogenes ATCC 7955	–	–	–	1.4	8.6	3
4 mEq/mL	*B. coagulans* ATCC 51232	–	–	1.84	–	–	9
Potassium chloride Inj. USP (2 mEq/mL)	*B. coagulans* ATCC 51232	–	–	1.22	–	–	9
	*B. stearothermophilus** ATCC 7953	–	–	–	2.9	–	3
	*B. stearothermophilus** ATC12980	–	–	–	5.9	–	3
	C. sporogenes ATCC 7955	–	–	–	0.8	11.4	3
Potassium phosphate Inj. USP (3 mM/mL)	*B. coagulans* ATCC 51232	–	–	1.36	–	–	9

*Note: *B. stearothermophilus* was subsequently reclassified as *Geobacillus stearothermophilus.*

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Carrier Solution	Biological Indicator	D-Value, °C, Min. 110°	115°	120°	121.1°	z-Value	Table Reference
Sodium acetate Inj. USP (4 mEq/mL)	*B. coagulans* ATCC 51232	–	–	1.45	–	–	9
Sodium bicarbonate Inj. USP (84 mg/mL)	*B. coagulans* ATC 51232	–	–	0	–	–	9
Sodium chloride Inj. USP (0.9%)	*B. coagulans* ATCC 51232	–	9.1	2.20	–	8.2	7
	*B. stearothermophilus**	–	–	2.90	2.20	7.9	4
	*B. stearothermophilus** ATCC 7953	–	–	–	1.9	–	3
	B. subtilus 5230	–	8.46	0.48	–	8.0	10
Sodium chloride Inj. USP (0.9%)	*C. sporogenes* ATCC 7955	–	–	–	1.5	11.8	3
Sodium chloride Inj. USP (234 mg/mL)	*B. coagulans* ATCC 51232	–	–	0	–	–	9
Sodium phosphate Inj. USP (3 mM/mL)	*B. coagulans* ATCC 51232	–	–	0.85	–	–	9
SMZ/TMP for Inj. USP (80 mg/mL; 16 mg/mL)	*B. coagulans* ATCC 51232	–	–	0	–	–	9
Water for Inj. USP	*B. coagulans* ATCC 51232	–	6.8	1.60	–	8.2	7
	*B. stearothermophilus** ATCC 7953	–	61.1	4.16	2.98	9.4	11
	*B. stearothermophilus** ATCC unknown	–	–	2.50	1.80	8.5	8
	B. subtilis 5230	–	11.8	0.67	–	8.0	10
		–	–	0.61	0.44	7.7	5

*Note: *B. stearothermophilus* was subsequently reclassified as *Geobacillus stearothermophilus.*

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Table 1 Continued from the previous page

Carrier Solution	Biological Indicator	D-Value, °C, Min.				z-Value	Table Reference
		110°	115°	120°	121.1°		
	C. sporogenes PA 3679	13.7	4.08	0.78	–	12.4	11
	C. sporogenes ATCC 7955	–	–	–	0.7	10.1	3

*Note: *B. stearothermophilus* was subsequently reclassified as *Geobacillus stearothermophilus.*

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Bacterial Spores

Bacterial Spores Defined

The bacterial spore is the result of bacteria that have been subjected to an adverse environment. When a stress condition occurs, the organism stops replicating, causing the organism to remain in a static state. As the stress is extended or increased, the organism begins to die. Some bacteria have the ability to develop structures capable of surviving the stress, so that instead of dying, they form a bacterial spore (as a survival mechanism). A bacterial spore is different from a fungal (mold) spore in that fungal spores are part of the normal reproductive cycle, not the result of an adverse condition, and therefore

Table 2. Effect of Rubber Stopper Composition and Treatment on the Moist Heat Resistance of *B. stearothermophilus* (Rubio and Moldenhauer 1995)

Rubber Stopper Composition	Detergent/Preservative(s) Treatment	D-value (min) Range*
1888 gray/red	None	5.84–6.66
	2.0% EDTA	5.51–5.84
	2.5% EDTA	5.67–6.48
	(0.02% Propylparaben, 0.l8% methylparaben)	5.69–5.97
	(0.04% Propylparaben, 0.36% methylparaben)	4.90–5.81
	(0.01% Propylparaben, 0.10% methylparaben)	5.09–5.37
	0.2% Methylparaben	6.16–6.30
	0.5% Chlorobutanol	6.29–6.64
1704 gray	2.0% EDTA	6.44–6.95
4416/50 gray	None	6.91–6.96
6104 red	None	5.85–6.29
	(0.10% Propylparaben, 0.10% methylparaben)	5.09–5.71
1888 gray Teflon	AFCO	6.44
890 gray	None	6.11

*Across various rinse-water temperatures

not particularly resistant. Spores are only formed by Gram-positive, rod shaped bacteria. *Bacillus* (*Geobacillus*) and *Clostridium* genera are the most commonly known genera that produce moist-heat resistant spores. Bacterial spores are the organisms most likely to survive moist heat sterilization (Pflug 1999).

Spore Characteristics

Certain characteristics are common to bacterial spores. For example, the amount of water present in the spore is decreased from the amount of water bound within the vegetative cell, spores are refractile and more difficult to stain than their associated vegetative cells, they have high resistance to adverse environmental conditions, and they have the ability to germinate and reproduce, even after long dormant periods (Pflug 1999). Other characteristics are similar within a specific strain of bacterial species, e.g., the spore size, spore shape, spore position (Pflug 1999).

Spores may be described in several ways, e.g.,

- projecting, spherical, and terminal
- nonprojecting, ovoid, and central,
- nonprojecting, ovoid, and subterminal.

The terminal, central, and subterminal descriptions refer to the position of the spore in relationship to the parent cell. Most spores have an oval shape, approximately 0.5–1.5 μm by 1.0–2.5 μm. The estimated volume of a spore is approximately 10^{-12} mL (Pflug 1999).

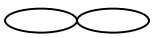
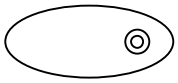
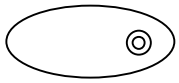
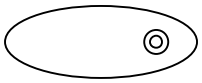
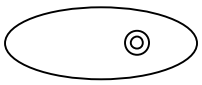
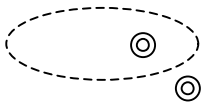
Spore Formation

There are seven basic stages in the development of a spore. In the pre-Stage I phase, the cell exists in its vegetative state, in a friendly environment. When stress is encountered in the environment (Stage I), the cell becomes unsuccessful at dividing. The cell generates one small cell and one normal size cell, instead of two equally sized daughter cells (Stage II). By Stage III, the smaller structure has been engulfed by the larger structure and this becomes the bacterial spore. There are two membranes, one facing outward and an internal membrane. In Stage IV, the peptidoglycan is present between the two membranes. Mineralization , particularly Ca^{++}, occurs. Concentrated Ca^{++} moves to the prespore. Dipocolinic acid (DPA) is synthesized and concentrated in the prespore. In Stage V, the water is highly bound (dehydrated). The prespore is highly calcified, is surrounded by tough layers (refractile), is hard to stain, and has no detectable metabolic activity and little respiratory activity. It can live for long periods of time in this condition. The cell proceeds to Stage VI, where heat resistance occurs after full mineralization occurs. This may take days or weeks (Czechowitz 1992). Stage VII is when spore lysis and release of the spore takes place. The spore is dormant and highly resistant to its environment. Data from the food industry show that spores have been dormant and revived after hundreds of years (Pflug 1999). The development of spores is depicted in Figure 2.

Mineralization

Compositionally, one of the biggest differences between spores and vegetative cells is the content of minerals. It has been shown that removal of divalent cations from the spore reduces its ability to resist heat (Pflug 1999; Russell 1982). Vegetative cells primarily have potassium present, which balances the negative charges of RNA, DNA, and proteins. It also is useful for metabolism, growth, and cell division. Conversely, spores are highly calcified. Manganese and calcium both provide heat resistance to the spore. It has been shown, however, that all of the minerals present in the spore can contribute to its heat resistance. Few data are available for the heat resistance related to the presence of sodium, however (Pflug 1999).

Figure 2. Spore Formation and Development

Stage of Development	Characteristics
Pre-Stage I 	Vegetative cell, reproducing at will, in a friendly environment.
Stage I 	Stress is encountered in the environment.
Stage II 	Cell is unsuccessful at dividing. The cell generates one small cell and one normal size cell rather than two equally sized cells.
Stage III 	The smaller structure is engulfed by the larger structure, and the smaller structure becomes the bacterial spore. Two membranes exist (facing outward and inward)
Stage IV 	Peptidoglycan is present between the two membranes. Mineralization, particularly calcium occurs. Concentrated Ca^{++} moves to the prespore. Dipocolinic acid (DPA) is synthesized and concentrated.
Stage V 	Prespore becomes refractile (highly dehydrated). Highly calcified, surrounded by tough layers, hard to stain, no metabolic activity, and little respiration. Long lived at this point.
Stage VI 	Heat resistance occurs in late Stage VI, following full mineralization.
Stage VII 	Spore lysis and release of the spore in a dormant, highly resistant form.

The Transition from Spore to Vegetative Cell

The spore moves to a vegetative state in three stages. In the first stage, triggering or activation occurs, conveying to the spore that the adverse environment no longer exists. Following activation, the spore proceeds to germinate. During germination the spore swells, peptidoglycan breaks down, water is taken in, and the cell becomes stainable and sensitive to heat. The spore structure is depolymerized rapidly, releasing the DPA, calcium, and manganese, and half the peptidoglycan. The cell takes in potassium and heat resistance is lost. As heat resistance is lost, the minerals are lost. This, in turn, results in the first vegetative cell, which has the ability to reproduce normally (Pflug 1999).

Thermal Injury to Spores

Thermal injury to bacterial spores is expressed in several cellular reactions: denaturation of vital spore enzymes, membrane damage resulting in leakage of calcium dipocolinate (CaDPA), impairment of germination/outgrowth, cellular structural damage, damage to the spore chromosome (e.g., mutations, DNA strand breaks), and increased sensitivity to inhibitory agents. DNA single strand breaks (SSB) have been reported in *B. subtilis* spores heated at 90°C. They resulted in loss of viability (Russell 1993).

The DNA and the CaDPA are reported to be in close proximity within the spore coat. The CaDPA has an important influence on the condition of the DNA within the spore. It is also reported to provide a heat stabilizing capacity on the enzymes and the DNA (Russell 1982, 1993).

Bacterial DNA is different in that it occurs in the A-form. The A-form is tightly coiled and the base pairs are tilted nonperpendicularly to the helical axis, with 11 base pairs for each turn. The A-form indicates a low water activity in the environment. More often, DNA in other cells is in the B-form, where the base pairs are perpendicular to the helical axis, with 10 base pairs per helical turn (Russell 1982, 1993).

Dehydration

There is a great deal of published literature on the effects of water content in spores and the location of the water within the spore (Gould 1985; Lindsay, Murell, and Warth 1985; Russell 1993) Spores inherently have a low water content, and the dehydration of the core (protoplast) is an important, if not the most important, factor in thermal resistance (Russell 1993).

Demineralization

Demineralization is also reported to change the heat resistance of microorganisms. One way this can happen is to induce H-form spores, for example, when chelating agents are used in a product formulation and the heat of the process activates the agent (adds energy) to demineralize the spore and affect its heat resistance. Calcium from the spores may also be remineralized and the spores will regain their original thermoresistance (Pflug 1999; Russell 1993).

Adaptation

The spores of certain organisms are more heat resistant than others. For example, thermophilic organisms are more resistant to heat than mesophilic or psychrophilic bacteria. Certain bacteria have adapted to provide optimum growth at higher temperatures, e.g., 52°C, 55°C (Russell 1982).

CONCLUSION

Bacterial cells can be affected by their environment. Moist heat sterilization can be used to inactivate the microorganisms by dehydration, mineralization/demineralization, or adaptation. Within the cells, proteins are the most important heat sensitive compounds. Several mechanisms have been postulated to account for their stability. Some believe that they are intrinsically heat stable. Others theorize that there may be stabilizing substances present in the spore that allow for heat resistance. Lastly, some believe that the loss of water content is the key factor to protein stability (Pflug 1999; Russell 1993; Warth 1978).

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