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The Effect of Bioindicator Preparation and Storage on Thermal Resistance of *Bacillus stearothermophilus* Spores

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Abstract

Paper strips inoculated with spores of *Bacillus stearothermophilus* ATCC 7953 were conventionally dried (lot 1) and lyophilized (lot 2); stored in defined environments of 32 and 86% relative humidity at 10, 25 and 33°C for 210 d; and submitted to moist heat treatments at 121°C. A significant decrease in thermal resistance from initial starting levels was found for lyophilized bioindicators stored at 86% relative humidity. The respective average $D_{121°C}$ values were 1.55 ± 0.05 and 1.37 ± 0.10 min for lyophilized bioindicators stored at 32 and 86% relative humidity; and 1.65 ± 0.15min and 1.57 ± 0.11 min for dried bioindicators stored in the same environments.

Index Entries: *Bacillus stearothermophilus*; decimal reduction time; biological indicators; thermal resistance; sterilization.

Introduction

A bioindicador represents a standard preparation with specific microorganisms of known concentration and defined resistance to a physical or chemical agent *(1–3)*.Bioindicator is recommended to be used routinely to monitor a set sterilization or disinfection cycle, and periodically to revalidate previously documented cycles *(4)*.

Bacillus stearothermophilus spores are used as bioindicators to monitor moist heat processes, which at low temperatures may be associated with radiation, chemical sterilants, or disinfectant agents. Bioindicators should be placed in those portions of the load that are the most difficult to access,

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and also into the load unit, for an established process run, so as to evaluate the effectiveness of the environmental conditions on the bioindicator population, which presumably should be greater in number and resistance than the presterilization bioburden of the item.

Therefore, bioindicators are an important tool for assisting in the practices required to ensure reproducibility of the processes in the manufacture of sterile products. Bioindicators are commercially available in suspension form, inoculated on paper carriers (individually packed in a container) or in a self-contained ampoule in which spores are suspended in a recovery medium.

In general, strips of paper with a defined number of thermal-resistant *B. stearothermophilus* spores are used and are easily placed in strategic positions in order to verify the homogeneity of distribution of the moist heat in an autoclave chamber and in a load unit. The main advantages of the paper carriers are low cost, small size, and ease in handling.

Because of the logarithmic nature of the kinetics of microbial death, the bioindicator is the biologic parameter chosen to verify that the minimum safe sterility assurance level (SAL) established for a product is attained (a safety margin of just one contaminated unit in every 10^4–10^6 units of thermal labile and thermal stable product, respectively, depending on the bioburden under consideration) *(2,4)*. Therefore, it is of the utmost importance that the user make a complete evaluation of the biologic indicator before placing it in use, including viable spore count per carrier (bioindiator population) and required resistance characteristics (decimal reduction time $-D_{Tr}$ value, and survival/kill times) according to the sterilization conditions, in order to confirm the information on the label *(2–5)*.

Control of the preparation and storage of the bioindicator such as conditions of spore growth *(6)*, harvest and suspension *(6,7)*, filter paper characteristics and previous treatment before being inoculated with spores *(7)*, the final drying of the inoculated strips, the storage conditions of the shipped bioindicator during shelf life *(5)*, and the recovery conditions of the processed bioindicator *(8)*, is of fundamental importance in order to keep its desired characteristics for a particular sterilization cycle being monitored.

When used appropriately, the bioindicator quantifies satisfactorily the efficacy of the sterilization process through the integration of the lethal factors of exposition time, vapor temperature, and chamber pressure on the bioindicator population *(5)*. Therefore, the function of the bioindicator is to establish, evaluate, and monitor the physical parameters of the sterilization cycle for the defined equipment; to qualify the level of sterility reached; and to document the efficiency of the process.

The purpose of the present work was to study a preparation of a bioindicator, that would keep the viability and heat resistance characteristics and that would easily attain equilibrium with the saturated steam during the process, ensuring a real and similar behavior of the considered product, without causing over- or understerilization. The effects of drying

methods (conventional and lyophilization) of inoculated strips (bioindicators) and of long-term storage conditions in two different conditions of relative humidity and at three different temperatures were investigated on the spore viability and thermal resistance of *B. stearothermophilus* ATCC 7953.

Materials and Methods

Preparation of B. stearothermophilus

B.stearothermophilus ATCC 7953 spores were developed at 65°C for 6 d on plate count agar (Difco,Detroit, MI) supplemented with 0.10% soluble starch, 0.05% magnesium sulfate, 0.05% manganese sulfate, 0.05% calcium chloride, at pH 7.0 *(7)*. The growth was harvested in 0.02 *M* calcium acetate solution, adjusted to a final pH of 9.7 with 0.14% (w/v) calcium hydroxide ($Ca[OH]_2$) solution, and centrifuged four times (2000 *g* for 30 min). The pelleted spores were suspended in chilling 0.02 *M* calcium acetate solution, the suspension was adjusted to pH 9.7 with 0.14% $Ca(OH)_2$ solution, and stored (4°C) and used as required. The viability of 10^6–10^7 CFU/mL of heat-shocked (98.7°C for 30 min) spores in suspension was estimated through pour plate on trypticase soy agar (65°C for 48 h).

Preparation of Bioindicator

Bioindicator, formed by the spores impregnated on strips, was prepared as in previous studies *(6)*. Sterile paper strips (250 g/m^2, 7 mm wide, 0.2 mm deep, 20 mm long) were immersed in sterile 0.02 *M* calcium acetate (pH 11.0/0.14% $Ca[OH]_2$) for 12 h to a final pH of 9.0, dried at 45°C for 24 h and stored at –18°C. The monolayer disposed strips were individually inoculated with 0.1 mL of *B. stearothermophilus* suspension in 0.02 *M* calcium acetate (pH 9.7). The initial decimal reduction time of the bioindicator at 121°C in saturated steam ($D_{121°C}$) was 1.9 min. The impregnated spore strips (bioindicator) were divided into two lots; one lot was submitted to conventional drying (convection) at 45°C for 24 h and the other was lyophilized at room temperature. The initial spore populations were 5.30 and 6.30 $\log_{10}$ for the conventionally dried and the lyophilized bioindicator lots, respectively.

Storage of Dried Bioindicator

After drying, every 20 dried strips were placed in 20-mL bottles, which were kept open inside glass desiccators, and showed a relative humidity of approx 32 (saturated solution of $MgCl_2$) and 86% (saturated solution of potassium chromate) *(9–12)*. The desiccators were stored at 10, 25 and 33°C and remained in the reference temperature until the strips in the bottles presented constant weight equilibrium with the environment. The bottles were then removed from the desiccators and sealed and stored at 10, 25 and 33°C for a period ranging from 30 to 210 d.

Thermal Resistance Parameters

Thermal resistance parameters of each bioindicator lot to steam sterilization at 121°C were determined after storage under different conditions through the serum bottle technique apparatus *(7)*. The bioindicator spore activation, which ranged from 5 to 7 min, was equivalent to the come-up-time required by the system to achieve the process temperature of 121°C. Ten *B. stearothermophilus* spore strips, previously humidified by instantaneous contact with sterile distilled water, were transferred to 20-mL glass (400 brand borosilicate) serum bottles (Wheaton SB205A) and placed between 10 Durham tubes (30 mm high × 3 mm diameter), which were glued upright on the internal wall of the bottle in a vertical line and on the internal base center of the bottle. The distance between neighboring parallel tubes was 4.0 ± 0.5 mm, and each Durham tube was filled with water to two-thirds of its total capacity. The bottle (60 mm high × 25 mm bottom diameter) was closed with a sterile flange rubber stopper and then sealed with an aluminum seal. The air was removed (5 min) by suction (vacuum pump; 1725 rpm, 1/6 hp; General Electric), using a 22-gauge stainless steel needle (30 × 7 mm) through the rubber stopper of the sealed bottle. A thermocouple sensor end, inside a 316 stainless steel needle (1.5 mm diameter × 100 mm length), was placed in the geometric center of the steam surrounding the spore strip inside the bottle, using the hole through the rubber stopper left by the pump needle The serum bottle containing the spore strips was laid in a stainless steel wire basket (50 mm diameter × 90 mm depth × 100 mm holder length) suspended in a thermostatically controlled oil bath and continuously agitated to keep the oil (Dow Corning® silicone 200/220 CS, $\rho = 0.948$ g/cm^3) circulating at the set temperature. Triplicate sets of bottle-counting spore strips were heated at 121°C at different time intervals.

For every test, each bottle was fitted with a thermocouple sensor end to measure temperatures in the geometric center of the steam that surrounds the spore strip or suspension. The temperature of the oil bath was measured simultaneously with a PT 100 thermocouple (IOPE therm 48, calibrated to 1.0 ± 0.1°C) and monitored visually with reference calibrated thermometers (nitrogen filled, ASM 67C, external Hg scale, +95 to +155°C, subdivided and calibrated to 1.0 ± 0.2°C; Incoterm). Eleven units of thermocouples, type J (2x32AWG), overwrapped with Teflon/Teflon, were attached to a reading, and the data registered simultaneously using multipoint recording equipment (IOPE therm 400-CE 12, recording rate: 30 s). The equipment for collecting the temperature data was an indicator of multichannel temperature (12 points) with a resolution of 0.1°C for the temperature range from 0°C to 199.9°C. The thermocouples were found to equilibrate at 121.0 ± 0.1°C, and holding times were determined with an electronic timer. The experimentally determined lag correction of 7 min was used. Triplicate heated sets of the 10 spore strip bottles used for each assay were cooled and held in a 40-L ice-water bath until spore recovery.

Survivors on the strips were quantified for spore viability and expressed by decimal logarithms of the average colony-forming units per strip from at least 10 plates for each time heating condition and system used.

Decimal Reduction Times

Decimal reduction times (D_{Tr} values) at 121°C were determined from the negative reciprocal of the slopes (b) of the regression lines, using the linear portions of the survivor curves ($\log_{10}$ population vs time of exhibition at a constant temperature). The slope SE (b) and the multiple determination coefficient (R^2) were also calculated. The estimated value of the D_{Tr} value SE (D) was obtained from the root of the variance $(V[D])^{1/2}$, which was calculated as follows: $V(D) = ([1/b^2]^2 \cdot [\mathrm{SE}(b)]^2)$. The upper ($D$–mean + SE[$D$]) and lower ($D$–mean–SE[$D$]) D-values were also expressed.

Statistical Analysis

Regression variance analysis (analysis of variance) *(13)*, estimated parameters, and respective confidence intervals at significance levels ($p < 0.05$) were calculated using the SGWIN program (Statgraphics Plus for Windows version 1.4; Statistical Graphics, 1995).

Results and Discussion

Wet-heat sterilization by saturated steam in an autoclave is the simplest and most efficient, efficacious, and low-cost process for producing sterile products. Because of their extreme heat resistance, *B. stearothermophilus* spores on strips are widely recommended as bacteriologic controls for autoclaving processes and are utilized as biologic monitors of the effectiveness of saturated steam sterilization processes, at a reference temperature of 121°C. The influence of storage environmental conditions over thermal resistance of bioindicator (spores in strips previously treated with calcium acetate) was expressed by decimal reduction time ($D_{121°C}$ value, the time in minutes to kill 90% of a given microbial population at a given temperature) at a reference temperature (121°C), as shown in Tables 1 and 2.

The immersion of paper strips in 0.02 *M* calcium acetate (pH 11.0/ 0.14% $Ca[OH]_2$) solution for 12 h replaced ions H^+ by Ca^{++} in the structure and provided homogeneity to the directions of the fibers, releasing the starch presented and favoring distribution of the spore suspension throughout the strip. The structure of the strip paper was altered by convection drying, which provoked irregular porosity of the material both inside and in distribution, making free exchange of water unfavorable to the environment. The inner heterogeneity of the convection-dried paper structure protected the microorganism, hindering access of steam to the spore. In lyophilization, the structure of the paper strip became more homogeneous with better distributed porosity, facilitating moist heat steam diffusion throughout the paper structure. The spores in the lyophilized paper structure remained homogeneously exposed to the sterilizing agent.

Table 1
D and *F* values at 121°C of *B. stearothermophilus* Bioindicator Dried by Convection, Equilibrated at 32 and 86% Relative Humidity, and Stored in Different Climatic Conditions[a]

Storage		Relative humidity 32%					Relative humidity 86%				
d	°C	Log N_0	$D \pm$ SD	Log N_f	SLR (log N_o/N_f)	F value (min)	Log N_0	$D \pm$ SD	Log N_f	SLR (log N_o/N_f)	F value (min)
90	10	4.65	1.61 ± 0.12	−4	8.65	13.96	4.70	1.61 ± 0.08	−4	8.70	14.04
120	10	4.70	1.93 ± 0.09	−4	8.70	16.79	4.90	1.68 ± 0.06	−4	8.90	17.18
30	25	5.40	1.59 ± 0.07	−4	9.40	14.95	4.89	1.69 ± 0.04	−4	8.89	14.14
130	25	4.90	1.68 ± 0.05	−4	8.90	14.95	4.30	1.48 ± 0.19	−4	8.30	13.90
55	33	3.36	1.51 ± 0.07	−4	7.36	11.11	4.48	1.50 ± 0.08	−4	8.48	12.81
130	33	3.70	1.52 ± 0.04	−4	7.70	11.68	4.48	1.45 ± 0.05	−4	8.48	12.89
		Mean = 1.65 ± 0.15					Mean = 1.57 ± 0.11				
		SD = 0.15					SD = 0.11				

[a]Total convection (32 + 86%); mean = 1.61; SD = 0.13. $D_{121°C}$ = decimal reduction time at 121°C in minutes; SD = standard deviation ($p < 0.05$); Log N_f = SAL 10^{-4} spores/strip; SLR = log N_o − Log N_f ; $F_{121°C}$- values = $D_{121°C} \times$ SLR = equivalent time in minutes.

For conventionally dried lots (Table 1), the average $D_{121°C}$ value of 1.57 (SD = 0.11) min for bioindicator at 86% was lower than the average *D* value of 1.65 (SD = 0.15) min for bioindicator at 32%, although they were independent of storage (p <0.05) environments, considering 32 and 86% relative humidity, respectively. The variation between average *D* values was not significant (p <0.05) and was independent of storage conditions for temperatures of 10°C and shorter than 30 d at 25°C. There was a tendency for *D* values to decrease at temperatures of 25°C (>30 d) and 33°C, notably for strips equilibrated at 86%.

For lyophilized strips (Table 2) kept in 32% relative humidity environments, there was an insignificant variation between the average *D* values (p <0.05), although the average *D* value of 1.55 (SD = 0.048) min for the lot was lower than the average *D* value (1.65 min) obtained for the conventionally dried strip lot kept under the same climatic conditions. The regularity of thermal resistance maintenance reached for lyophilized 32% relative humidity strips was observed to be the best for the exposition environments under consideration. This phenomenon was not verified for lyophilized strips at 86% relative humidity, which was shown to be the most sensitive system for the storage conditions selected. It was confirmed that the variations between average *D* values were significant for each storage environment, confirming a nonstable system. The total average value of 1.37 (SD = 0.100) min was the lowest in relation to the other drying and warehousing conditions. It was equal to the minimum limit recommended by USP XXI *(14)*, considering a kill time of 8D and an initial spore population of 4 $\log_{10}$, and was also acceptable for heat-sensitive solutions, including aqueous preparations, as suggested by the European Pharmacopoeia *(15)*. The average *D* value for the freeze-dried bioindicator lots appears to be dependent on relative humidity and storage conditions at the 95% confidence level.

The drying method showed remarkable influence on the moist heat resistance of the freeze-dried bioindicators lot equilibrated to 86% relative humidity in comparison with the same lot equilibrated to 32% relative humidity, the thermal resistance of which was kept stable for the storage temperatures, under consideration. A significant decrease in moist heat resistance from initial starting levels was found for bioindicators stored at 86% relative humidity and 33°C, as shown in Table 2.

It has been demonstrated that spores are highly permeable, with free exchange of water occurring between a spore and its environment. Therefore, the water activity of a spore changes with the relative humidity of the environment *(13)*. The thermal resistance for *Bacillus megaterium* spores *(16)* has been shown to increase in water activity ranging from 0 to 0.3 A_w and to decrease for values greater than that *(13)*. Water activity of the bioindicator lot after drying ranged from 29 to 34% for conventionally dried strips and remained below 10% (between 7 and 8% A_w) for lyophilized strips. Therefore, freeze-dried bioindicator on strips should be equilibrated

Table 2
D F values at 121°C of *B. stearothermophilus* Bioindicator Dried by Lyophilization, Equilibrated at 32 and 86% Relative Humidity, and Stored in Different Climatic Conditions[a]

Storage		Relative humidity 32%					Relative humidity 86%				
d	°C	Log N_0	$D \pm$ SD	Log N_f	SLR (log N_o/N_f)	F value (min)	Log N_0	$D \pm$ SD	Log N_f	SLR (log N_o/N_f)	F value (min)
90	10	5.73	1.61 ± 0.06	−4	9.73	15.57	5.78	1.39 ± 0.10	−4	9.78	13.70
120	10	5.96	1.63 ± 0.06	−4	9.96	16.23	5.76	1.52 ± 0.08	−4	9.76	14.84
30	25	5.70	1.53 ± 0.07	−4	8.70	13.31	5.62	1.41 ± 0.07	−4	8.62	12.15
92	33	5.22	1.50 ± 0.06	−4	9.50	14.58	6.17	1.18 ± 0.04	−4	10.17	11.35
130	33	5.87	1.52 ± 0.08	−4	9.87	14.96	6.74	1.38 ± 0.10	−4	10.74	14.82
170	33	5.94	1.53 ± 0.10	−4	9.94	15.20	6.20	1.42 ± 0.12	−4	10.20	11.62
210	33	5.85	1.52 ± 0.11	−4	9.85	14.96	6.88	1.33 ± 0.10	−4	10.88	14.47
		Mean = 1.55 ± 0.05 SD = 0.05					Mean = 1.37 ± 0.10 SD = 0.10				

Average $D_{121°C}$ for 32 and 86% relative humidity =1.46 ± 0.12. $D_{121°C}$ = decimal reduction time at 121°C in minutes; SD = standard deviation (p <0.05); Log N_f = SAL 10^{-4} spores/strip; SLR = log N_o – Log N_f ; $F_{121°C}$- values = $D_{121°C}$ × SLR = equivalent time in minutes.

at an environment of 32% relative humidity before being used, since thermal resistance falls when the relative humidity is below 32%.

There was a tendency for a 1 to 2 $\log_{10}$ cycle reduction in the bioindicators spore viability from the initial population in function of set environmental storage conditions. This decrease was notably observed for the conventionally dried lot at 32% relative humidity stored at 33°C. For freeze-dried bioindicator lots, the initial spore population was kept constant and independent of either the relative humidity or the temperature conditions. Reich and Morien *(17)* did not observe any significant difference between the initial *B. stearothermophilus* or *B. subtilis* spore populations and the spore populations of bioindicators stored at 20°C and 0, 20, 44, or 55% after 12 mo.

The influence of moisture on spore viability and resistance to the sterilant agent has been investigated extensively. Spores stocked at 20°C and in the interval of 20 and 44% relative humidity have presented greater moist heat resistance than those stored over phosphorous pentoxide (0% relative humidity) or in the interval of relative humidity between 80 and 90% *(1,3,17,18)*.

Young and colleagues *(15,19)* showed that in environments with a low level of moisture, spores remain viable for a lengthy period, becoming more resistant when the relative humidity approaches 32%. Moist heat resistance was also observed to increase in the range of 0–32% relative humidity and to decrease when the relative humidity increases above 44% *(17)*. According to Young *(15)*, these effects can result in a variation in *D* value of up to 100 times.

Because freeze drying helped to maintain a certain regular structure related to paper porosity distribution, facilitating the exchange of water (vapor phase) with the environment, and because the presence of moist heat allows peptide linkages to rupture easily *(20)*, the process requires higher energy in the absence of the free water required, consequently providing the spores with greater thermal resistance. The presence of free water in the system permits the peptide bond to rupture through heat, a process that requires more energy when there is no water, and consequently confers greater resistance of the spores to moist heat. The destruction of spores, under oscillatory pressurization, was efficient with the rise in temperature, promoting water permeability into the cell wall and spore protoplasm *(21)*.

Only calibrated spores can be used to determine the capacity of moist heat treatments and the level of destruction of the product bioburden. An appropriate bioindicator must have a thermal resistance to the sterilization process greater than the thermal resistance of the microorganisms originally present in the product to be sterilized. The number of reduced log cycles in the initial population of bioindicator spores to evaluate the sterilization cycle depends on the safety level established for the system in bioindicator thermal resistance.

The 10^{-4} SAL suggested by USP XXI *(14)* for *B. stearothermophilus* spores defined the final number (N_f) as one surviving spore for every 10^4 treated

strips. The sterility level of 10^{-4} may be considered for aqueous solutions and for heat-sensitive products manufactured under good manufacturing practices, presuming an initial 10^2 spores in the product and an SAL (N_f) equal to 10^{-6}, as recommended by USP 24 *(4)*, British *(22)* and European pharmacopoeias *(15)*.

A spore log reduction (SLR) corresponds to the reduced decimal log cycles between the number of spores initially present in the bioindicator and the SAL, which establishes the final population (N_f) for the system. For an SLR of 8, considering the product's bioburden (N_o) of approx 10^2 and a final SAL with a probability of 10^{-6}, the SAL for the bioindicator, with an initial population of 10^4 less than 5×10^4 spore count per strip, depends on its labeled *D* value for the same moist heat treatment. The level of lethality established for the sterilization process is confirmed by the equivalent time (*F* value), which also depends on the *D* value of the bioindicator spores and on the initial count (N_o).

The *F* value is the interval of time required at the constant reference temperature in order to attain the preestablished SAL. The *F* value is the time, in minutes, equivalent to the reference temperature (T_r = 121°C), at which the product should be maintained, taking into consideration instantaneous heating and cooling, as calculated by multiplying the number of log reductions by the specific *D* value at the T_r = 121°C ($F_{121°C} = D_{121°C} \times$ SLR). For an SLR of 8, the *F* value will be on the order of 8D, considering bioindicator presenting an initial spore population per strip between 5×10^4 and 5×10^5. A $D_{121°C}$ value of 1.3 min, with an SLR of 8 means that the sterilization process at 121°C ($F_{121°C} = D_{121°C} \times$ SLR) will not be <10.4 min *(2,4,14,15,22)*.

Despite a lower initial spore population, $<10^4$ spores per conventionally dried strip, for bioindicator at 32% and 33°C (Table 1), the $D_{121°C}$ of 1.5 min guaranteed an $F_{121°C}$ higher than 10.4 min, even though the SLR ranged from 7.3 to 7.70 lower than the SLR of 8, established for the process. For a bioindicator under 86% relative humidity storage (Table 1), the *D* value remained stable, averaging from 1.7 min at 10°C to 1.5 min at 33°C, independent of the storage time, satisfying a minimum SLR of 8 and an *F* >12 min. Following USP XXIII *(2)* recommendations, bioindicator of *B. stearothermophilus* with a $D_{121°C}$ of 1.5 min, when treated at 121°C for 12 min, has a lethality input of 8D. The *D* value for bioindicator of *B. stearothermophilus* should be verified and compared to the labeled value by determining survival curves or by fraction-negative (method) achievement, and every change in a specific program of validation should be recorded in order to reestablish appropriate sterilization cycles related to a certain product. A suitable bioindicator preparation should be employed in routine sterilization cycles and periodically checked for viable spore population and resistance, in addition to other attributes, to detect any changes in the bioindicator article. Since the level of destruction (*n*) was higher than 8 log cycles, the *D* value was 1.3 min higher, the minimum limit recommended *(14,22)*.

The lyophilized bioindicator showed $F_{121°C}$ values >10.4 min for all climatic conditions (Table 2). The spore population remained stable, between 5×10^5 to 5×10^6 spores per strip, greater than for the conventionally dried bioindicator (Table 1). For the system referring to lyophilized bioindicator at 32% relative humidity, the average *D* value of 1.55 min guaranteed an SLR of about 9 and an $F_{121°C} > 13$ min (Table 2). For bioindicator at 86% relative humidity, despite the instability of the system related to low *D* values, varying between 1.33 (at 33°C) and 1.4 min (all temperatures) and an average of 1.5 min at 10°C, the stable spore viability with high counts of 6 $\log_{10}$ guaranteed an SLR greater than 8 log cycles, providing a minimum $F_{121°C}$ value of 11.6 min (Table 2) corresponding to the lower *D* value for bioindicator at 33°C for 210 d. According to USP *(2,13)*, for *D* values outside the specified range between 1.3 and 1.9 min, the survival time (>3.9 min) and kill time (<19 min) should relate to the *D* values and SLR range defined by the spore count per strip. A population with less than 5×10^4 to 5×10^5 viable spores per bioindicator does not invalidate its use when such bioindicators ensure the minimum *F* value established by the process. According to the British Pharmacopoeia *(22)*, a moist heat sterilization process at 121°C provided with a minimum *F* value of 8 min may be considered satisfactory in the treatment of especially thermal-sensitive products, for which strict control must be exercised during the stages of manufacture in order to ensure that a consistent SAL is achieved.

D values can vary with the pH of the suspended medium. For *B. stearothermophilus* ATCC 7953 spores, suspended in Mcllvaine buffer at different pH values of 7.0, 6.0, 5.0, and 4.0, López et al. *(23)* obtained $D_{120°C}$ values of 2.10, 1.25, 0.59 and 0.43 min, respectively. Fernandez et al. *(24)* confirmed the influence of acidity on thermal resistance when *B. stearothermophilus* spores were suspended in bidistilled water (pH 7.0) and in natural mushroom (pH 6.7), giving $D_{121°C}$ values of 3.08 and 1.77 min, respectively. Spores suspended in acidified mushroom extract at pH values of 6.7, 5.34, and 4.65 resulted in *D* values of 1.70, 1.12, and 0.62 min, respectively.

Suspension of *B. stearothermophilus* spores gave a $D_{120°C}$ value of 2.0 min inoculated onto a paper carrier, and onto rubber tops, which were submitted to the moist heat sterilization process. Rubio and Moldenhauer *(25)* verified an increase in the thermal resistance of the spores, $D_{120°C}$ values of which remained between 4.9 and 6.9 min. Because of their high thermal resistance ($D_{121°C}$ 3.0 – 6.0 min), *B. stearothermophilus* spores are widely used in the food and pharmaceutical industries and are employed as bioindicators in several sterilization processes. In accordance with Young *(15)* and Hayakawa et al. *(26)*, for bioindicator used as controls in the sterilization process, the $D_{121°C}$ value typically varies between 1and 2 min and when inoculated into the product, $D_{121°C}$ values may change within 0.1 and 14.0 min, typically between 4.0 and 5.0 min *(20)*.

The system referring to spores inoculated on strips, dried by freeze drying, equilibrated at 32% relative humidity and stored at 10, 25, and 33°C

for 210 d presented the best stable spore population of 5 to 6 $\log_{10}$ and the best performance of thermal resistance with invariable $D_{121°C}$ values averaging 1.55 min.

The preparation of the bioindicator determines the effectiveness of its performance as monitor of moist heat sterilization processes. The saturated steam temperature is directly related to the vapor pressure of the surrounding environment in the autoclave, which directly affects the rate of spore inactivation. The quality and distribution of the saturated vapor determine the homogeneity of the activation of the starting spore population in the paper carrier, during the come-up-time. The come-up-time is the exposure phase at which the chamber is maintained under saturated steam conditions, long enough to ensure that the product and bioindicator have achieved the desired thermal exposure, before the inactivation of the spores in the bioindicator.

The bioburden of the commercial bioindicators is usually determined before they are employed as sterilant monitors, although the thermal characteristics labeled by the manufacturer are not usually confirmed by the user. Our studies have demonstrated that the carrier preparation *(7)* as well as the final dehydration of the paper inoculated with the spore suspension affect bioindicator performance and interfere in the calculation of the *F* value and the SAL for the specific product.

The effect of moisture on heat sterilization is evidenced by the difference in temperature required for dry heat processes (160–180°C) vs saturated steam processes (115–135°C). The effect of moisture reduces the temperature required to denature or coagulate proteins. In dry heat sterilization, microorganisms are dehydrated before the temperature rises enough to cause denaturing of the proteins. Consequently, dry heat is an oxidative process with different kinetics from moist heat sterilization.

It has been demonstrated that the spores are highly permeable, and that free exchange of water occurs between a spore and its environment. If the drying of spores by conventional oven procedure causes an oxidative dehydration of the spores resulting in the denaturing of the proteins, the permeability of the spores is also affected. Thus, the free exchange of water will be poor between the spore and the surrounding environment, making the spore vulnerable to variations in temperature. The greater loss of starting spore population in conventional dried carriers over freeze-dried ones could also be explained by the oxidative damage of the spores during oven drying, parallel to the difficult access of moisture throughout entangled spots when moisture is not easily accessible. The rigorous, hard desiccation conditions of the bioindicator must damage a proportion of the starting spore population so that they become nonviable. The conditions of freeze drying mostly preserved the starting population compared to conventional drying, which provoked irregularity of the paper as well as desiccation of heterogeneous portions of the starting

spore population. The final A_w after lyophilization did not cause any stress to the starting spore population, which was equilibrated at 32 and 86% relative humidity immediately before storage.

Manufacturers should explain the preparation conditions of the bioindicator, specifically for each carrier. When conventionally dried, the bioindicator in paper carrier should be tested in bioburden; the necessary come-up-time should be determined to enable regular steam heat distribution throughout the carrier, permitting free moisture access to the spores. The steam exposure times should be selected so that a surviving population range of approx four decimal logarithmic cycles might be inactivated.

The carrier should be equilibrated before being used as a sterilant monitor. If this is not possible, the come-up-time of the process should provide the necessary equilibrium between the spores and their surrounding ambient. Dehydrated bioindicator may cause superheating within the carrier itself. When exposed to steam, these carriers readily absorb both heat and moisture, seeking thermal and moisture equilibrium with the surrounding steam *(13)*. Heat and moisture are simultaneously transferred to the material as steam condenses. If the material is dehydrated (A_w<0.86), more steam will condense to satisfy the equilibrium of the moisture, and, consequently, additional thermal energy will be transferred to the material. This can result in superheating of the inner carrier because the temperature is higher than that of the saturated steam surrounding it. This phenomenon is the cause of super heated steam, which inhibits the transfer of moisture and, consequently, causes an increase intemperature without attaining the necessary activation energy and entropy for the inactivation of spores and denaturing of the proteins.

In the freeze-dried carriers, steam penetration into the fibers was easier and the equilibrium with the environment was promptly attained. As a result, the come-up-time was reduced in contrast to the conventional dried carriers, which demand a longer come-up-time to attain equilibrium of the carriers with the environment.

In summary, we recommend using a freeze-dried bioindicator equilibrated at 32% relative humidity before storage and avoiding extreme conditions of freeze drying or oven drying and irreversible damage to the starting spores, which causes loss of the easy rehydration property during steam heat treatments. In addition, the conventional dried bioindicator should be equilibrated to $A_w > 0.86$ before being used to monitor a moist heat sterilization process, by quick immersion in water to help the steam heat distribution along the strip *(7)*, guaranteeing the rapid access of vapor in every part of the paper and permitting regular activation of the starting spore population during the come-up-time of the process. Finally, bioindicator in lyophilized or conventionally dried papers should be stored at 32% relative humidity at ambient temperature to ensure that the viability and thermal resistance of the spores are maintained.

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