RESEARCH ARTICLE

Effect of Carrier Materials on the Resistance of Spores of *Bacillus stearothermophilus* to Gaseous Hydrogen Peroxide

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ABSTRACT: The testing of the H_2O_2 decontamination process using spores of *Bacillus stearothermophilus* has gained widespread acceptance. Usually, commercially available Biological Indicators (BIs) with a specified resistance to H_2O_2 are challenged to qualify the process. The question arises whether the resistance of test spores is dependant on the type of carrier material and whether the resistance is representative for the system under test.

The objective of the study is to quantify the effect of different carrier materials on the resistance of spores of *Bacillus stearothermophilus* to H_2O_2. Materials from which isolators were built, as well as those used in disposables during daily work were investigated. These materials were inoculated with 10^6 spores of *Bacillus stearothermophilus* (ATCC 7953). The spore resistance was tested to a well defined H_2O_2 decontamination cycle by determining the D-value using the "Fractional Negative" method.

This paper reports on the effect of different carrier materials to the resistance of the test organism against H_2O_2. Various materials have significantly increased resistance of the spores and should be avoided in isolator systems. If commercially available BIs are used for process qualification, the resistance of the BI used, the fluctuation in resistance caused by isolator materials, the required log reduction, and at least the bioload of isolator surfaces need to be known.

Introduction

The testing of the H_2O_2 decontamination process using spores of *Bacillus stearothermophilus* has gained widespread acceptance (1, 2, 3). In practice, commercially available Biological Indicators (BIs) with a specified resistance to H_2O_2 are applied for the process qualification (3). BIs with CrNi steel carriers are generally used, whereas various materials like other metals, glass, and plastics can be found in decontamination chambers. The use of such BIs for process qualification has prompted the authorities (4, 5, 6, 7, 8, 9) to demand more detailed information on the extent to which the use of such BIs is associated with a successful decontamination effect on different surfaces in the chamber.

Studies showing the influence of different materials on successful inactivation pose a considerable challenge even for thermal sterilization methods. A surface decontamination study with gaseous H_2O_2 requires a thorough understanding of the mechanisms of H_2O_2 decontamination, a defined and reproducible decontamination system, and extreme care in the individual steps to produce inoculated material samples. The time and effort involved make such studies unsuitable for routine qualification.

As part of the initial qualification of several filling isolators, material pass throughs, and sterility test isolators it was possible to conduct a single study to address this question. The goal of the study was to detect any variation in the resistance of spores of *B. stearothermophilus* to various materials and to incorporate the results into the design, cycle development, and final qualification of decontamination chambers. In conclusion, it should be possible to use these data as a basis for carrying out cycle qualification and requalification using commercially available BIs.

Material and Methods

Biological Indicators

BIs consist of an inoculated carrier with a defined number of a specified test organism, which is sealed into a primary packaging that is permeable to the decontamination medium. A BI generally indicates a defined resistance to a specified inactivation method (1, 2, 10,

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11, 12). In order to compare the resistances between various spore-carrier combinations and the resistance of commercially available BIs, the following test organism and carrier materials were used.

Test Organism

According to international standards (1, 2, 3), spores of *B. stearothermophilus* are specified to qualify the H_2O_2 decontamination process. In the present study, spores of *B. stearothermophilus* ATCC 7953 from a 40% ethanol solution with a concentration of $\geq 10^8$ spores per 1.0 mL were used as the test organism.

Carrier Materials

All construction materials and consumable materials used in routine isolator operation were evaluated. The selection process was based on two criteria: 1) the material quantity of the entire surface to be decontaminated, and 2) the risk of a contamination from a particular surface. The materials selected for the study and their use in the different production systems are shown in Table 1.

Primary Packaging

The inoculated carrier materials were sealed in a Tyvek bag permeable to H_2O_2.

Commercially Available BI

The commercially available BI used as reference shows the specifications described in Table 2.

Resistance Determination

The decimal reduction rate, D-value, was determined as a direct measure for the resistance comparison between the various spore carrier combinations and the commercially available BI against H_2O_2 decontamination. The Fractional Negative Method (in particular, the Limited Spearman Karber Method, LSKM) (10, 11, 12, 13) was used to determine the D-values of BIs.

Limited Spearman Karber Method (LSKM)

With the LSKM, the "Mean Time to Sterility" is calculated from the final stages of microbiological inactivation

Table 1. Selected materials and their use in production systems.

Materials	Use
CrNi steel, different qualities: 1.4301 (304) unpolished 1.4301 (304) polished (Ra < 0.8 μm) 1.4435 (316L) unpolished 1.4435 (316L) polished (Ra < 0.8 μm)	Main chamber material Parts of filling line Steritest pump
Glass	Window and door material Media bottles, product units
Polycarbonate, PC	Window material
Hypalon	Glove material
Polyvinylchloride, PVC, soft	Material of glove gauntlets
Polyvinylchloride, PVC, hard	Glove ports
Polyvinylchloride, PVC	Package of steritest units
Polyoxymethylene, POM	Conveyer system of filling line, shelfs of steritest isolator
Polypropylene, PP	RTP-ports
Polyethylene, PE-UHMW	Conveyer system of filling line, RTP-ports
Polytetrafluorethylene PTFE / (Teflon)	Tubings, parts of filling line
Aluminium anodized, different qualities	Material sample commercially available Material sample filling line Material sample air sampler
Butyl caoutchouc	Stoppers of media bottles and product units
Laminated foil 1; from inside to outside: Polyethylene, Aluminium, Polyester	Package of media plates for cleanroom monitoring
Laminated foil 2; from inside to outside: Polyethylene, Polypropylene	Package of media plates for cleanroom monitoring
Tyvek	Package of Steritest units
HEPA-filter pad	HEPA-filter

Table 2. Specification of reference BI.

Test organism	*Bacillus stearothermophilus*, ATCC 7953
Carrier material	CrNi steel
Initial population [CFU]	$\geq 1.0 \times 10^6$ spores per carrier
Primary packaging	Tyvek
Specified inactivation method	Gaseous H_2O_2

and the D-value is derived from this value. In practice, several groups of BIs are exposed simultaneously to a decontamination cycle. The groups are sequentially removed from the inactivation atmosphere at a constant time interval and then evaluated using the growth test (10, 11, 12, 13). An iterative test procedure was used to determine the D-value. During the first test, the D-value was estimated with a minimized LSKM (14). Next, the estimated D-value was solidified using a modified test method. Furthermore, the result of the LSKM allows one to determine the model behaviour of the BI in relation to the survival time model (14). As an example, the result of the minimized LSKM is shown and interpreted in Table 3.

The D-value for the spore-carrier combination in Figure 1 can be estimated to be 1.2 [mins]. The BI shows acceptable model behaviour for germ reduction with the transition from overall positive groups in a fractional field to overall negative groups. In particular, there are no late-positive results at higher exposure times. Such late-positives would raise doubts about the inactivation kinetic of this BI. A minimized LSKM is a very good tool for the D-value estimate and for the assessment of the model behaviour (14). For a more detailed D-value determination, a larger number of BIs were used in this study (10, 11, 12, 13).

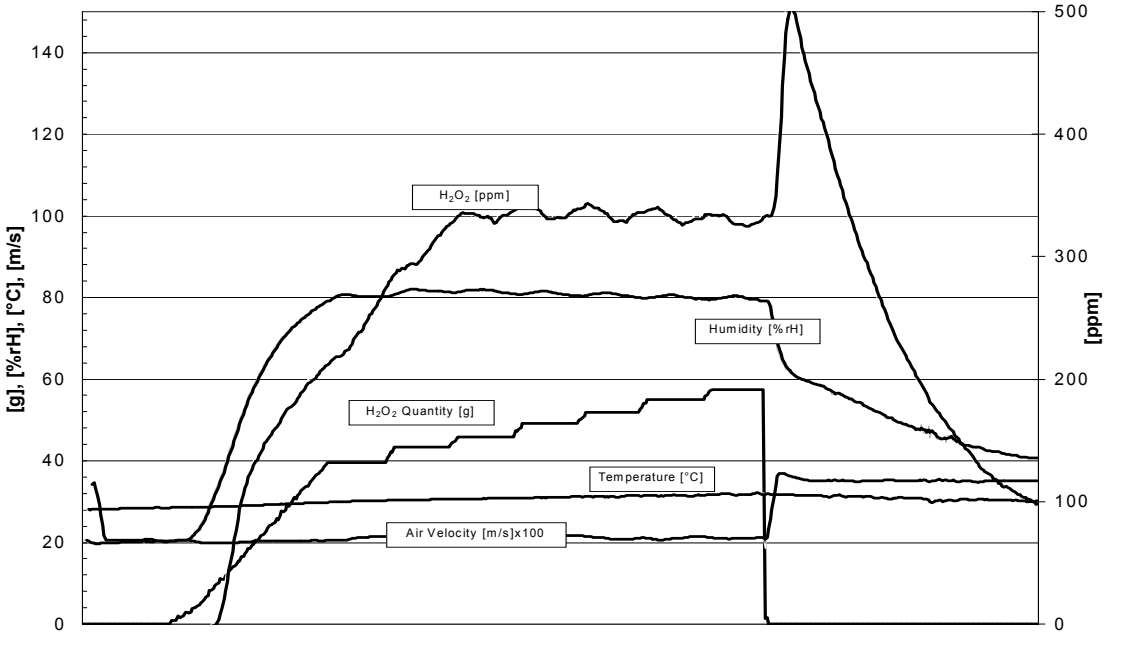
Table 3. An example of the results of minimized LSKM.

Test organism	*Bacillus stearothermophilus* ATCC 7953
Carrier material	CrNi steel 1.4301 (304) unpolished
Initial Population [CFU]	1.3 x10^6 spores per carrier

+ = growth
-- = no growth

Group	**01**	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**	**pos**	**neg**
Exposure time [mins]	2	4	6	8	10	12	14	16	18	20		
Result 1	+	+	+	+	+	--	--	--	--	--	+	--
2	+	+	+	--	--	--	--	--	--	--		
3	+	+	--	--	--	--	--	--	--	--		

Figure 1. The H_20_2 cycle of the reference isolator.



Decontamination Method

All inactivation tests were carried out in a reference isolator with a defined and reproducible H_2O_2 decontamination cycle. This reference isolator represents state of the art isolator technology and is comparable to modern systems available on the market. The construction is mainly CrNi steel and glass; the isolator volume is 1.4 m^3. It features unidirectional air supply via terminal HEPA filters. The H_2O_2 is directly vaporized by an integrated decontamination system into the recirculated air flow. The H_2O_2 cycle was microbiologically qualified in regards to the achieved decontamination effect and the stability of this decontamination effect over time (14). During the study, the initial chamber conditions and decontamination parameters were kept stable for each decontamination cycle. Figure 1 illustrates the cycle parameters of the reference isolator.

Since 1998, this reference isolator has been in use as a test isolator with constant cycle parameters and reproducible decontamination effect over this period. This isolator is currently being used as a "Biological Indicator Evaluator Resistometer" (BIER) vessel (10, 12, 13) for the characterization of BIs and for decontamination studies.

Production Systems Examined

The present study served as the basis for the design qualification, the H_2O_2 cycle development and the process qualification of the production systems mentioned in Table 4. The integrated decontamination units, the design and the air supply of the reference isolator and the examined productions systems are comparable.

Table 4. A breakdown of the production systems examined.

Type of System	Numbers of Systems	Volume [m^3]
Reference Isolator	1	1.4
Sterility Test Isolator	2	2.2
Filling Isolator	5	9.0
Material Pass Through	2	15.0

Execution

Material Testing in the Reference Isolator

The selected carrier materials were cleaned, sterilized, and subsequently inoculated with at least 1.0 x 10^6 spores of *B. stearothermophilus* and sealed into the Tyvek bag. D-value estimates were carried out in the reference isolator for each lot of BIs. At the same time, the recovery rate [spores/carrier] of each batch was determined. The iterative procedure produced two D-value estimates for each spore-carrier combination. Over the full period of the study, the commercially available BI was included in each D-value estimation as a standard to verify the reproducibility of the decontamination effect in the reference isolator. All results of the material tests from the reference isolator were evaluated in terms of reproducibility of the D-value estimates and compliance with the model behaviour. This evaluation described how suitable the different materials were for use with H_2O_2 decontamination (14). Furthermore, these results gave the basis for a risk assessment study in regards to materials used in current production systems.

Transferability of the Resistance Tests

A transferability study was carried out, based on selected spore-carrier combinations. The target of this part of the study was to show that the results obtained in the reference isolator are also applicable for the current production systems with a specified decontamination cycle. The spore-carrier combination with the highest established resistance and the one with the lowest resistance were used for the evaluation, as well as the commercially available BI. Then a complete D-value determination was carried out with these three different BIs in each type of production system to verify the range and the constellation of the resistances established in the reference isolator. If the resistance behaviour of these three selected BIs found in each type of production system is comparable to the results of the reference isolator, it would be possible to transfer all results established in the reference isolator to all production systems.

Resistance Factor

The material tests would have a specific influence on the evaluation of the H_2O_2 decontamination cycles if the resistance of the commercially available BI is lower than the highest resistance established in the material tests. As a consequence, a resistance factor is to be calculated for each type of system as a measure of this influence. The resistance factor reflects the relationship between the resistances established from the commercially available BI and the most resistant spore-carrier combination; see Formula 1. The resulting

resistance factor shall then be used for the qualification of the H_2O_2 decontamination cycles of the production systems.

$$\text{Resistance Factor} = \frac{\text{D-value: most resistant spore-carrier combination}}{\text{D-value: commercially available BI}} \quad \text{(Formula 1)}$$

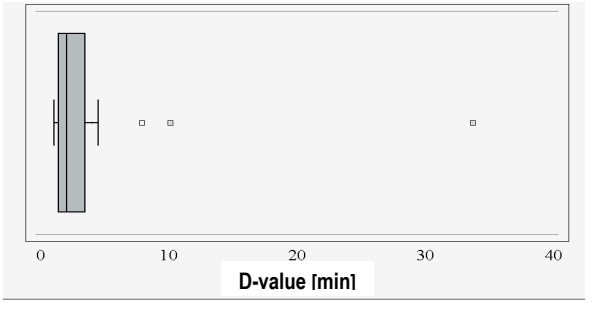
Results

Results of Material Tests in the Reference Isolator

Table 5 shows the D-value estimates and the qualitative evaluation of the model behaviour of material tests and the commercially available BI from the reference isolator.

The D-value estimates for the various spore-carrier combinations are in a range from 1.0 to > 33.7 [mins]. 22 out of the 23 combinations show a D-value estimate between 1.0 and 4.6 [mins] with a mean resistance of 2.3 [mins]. The three various aluminium samples have D-value estimates between 7.9 and > 33.7 [mins], significantly higher than the mean value for the other 20 systems. This relationship can be seen in the Box and Whisker Plot for the D-value (Figure 2).

Figure 2. The D-value relationships of three various aluminium samples.



As an example, the result for the minimized LSKM of anodized aluminium (sample commercially available) is shown in Table 6.

The BI out of commercially available anodized aluminium initially produced negative results at an exposure time of 9 [mins]. However, no shift to all-negative results was observed up to the maximum exposure time of 57 [mins]. While the D-value can be mathematically estimated with a verifiable limit of > 3.1 [mins], the model behaviour observed does not show any inactivation kinetics matching this theoretical D-value. In a further test, this stochastic pattern continues up to exposure times of 70 [mins]. The model behaviour with the occurrence of

Table 5. The results of the material tests in the reference isolator.

Carrier Material of BI	D-value Estimations [mins]	Model Behaviour
Glass	1.0 / 1.1	given
CrNi steel 1.4435, polished	1.3 / 0.9	given
CrNi steel 1.4301, unpolished	1.0 / 1.2	given
CrNi steel 1.4435, unpolished	1.0 / 1.4	given
CrNi steel 1.4301, polished	1.3 / 1.4	given
PVC, hard	1.0 / 1.8	given
PTFE	1.3 / 1.6	given
PE, UHMW	1.6 / 1.6	given
PP	1.3 / 2.0	given
PVC	2.0 / 1.6	given
Laminated foil 1, outside	1.6 / 2.5	given
PC	2.2 / 2.3	given
BI, commercially available	2.6 / 2.3	given
Tyvek	2.0 / 3.1	given
Laminated foil 2, outside	2.5 / 3.2	given
Butyl chaouchouc	2.9 / 3.1	given
Hypalon	3.0 / 4.1	given
HEPA-filter pad	3.6 / 3.6	given
PVC, soft	4.3 / 4.6	given
POM	4.6 / 4.4	given
Aluminium, anodized, commercially available	> 3.1 / 7.9	**not given**
Aluminium, anodized, air sampler	>8.3 / 10.1	given
Aluminium, anodized, filling line	>17.1 / > 33.7	**not applicable**

Table 6. The results for the minimized LSKM of the commercially available anodized aluminium sample.

Test organism	*Bacillus stearothermophilus* ATCC 7953
Carrier material	Aluminium, anodized, commercially available
Initial population [CFU]	1.3 x10^6 spores per carrier

+ = growth
-- = no growth

Group		**01**	**02**	**03**	**04**	**05**	**06**	**07**	**08**	**09**	**10**	**pos**	**neg**
Exposure time [mins]		3	9	15	21	27	33	39	45	51	57		
Result	**1**	+	+	+	+	--	+	+	--	--	+	+	--
	2	+	--	+	--	--	--	--	--	--	+		
	3	+	--	--	--	--	--	--	--	--	--		

late-positive results makes the decontamination effect on this material sample uncertain.

The BI out of anodised aluminium (sample filling line) shows only all-positive results up to the selected maximum exposure time of 100 [mins]. The model behaviour could not be evaluated, while the D-value was estimated at >33.7 [mins].

The H_2O_2 decontamination does not guarantee any reliable germ reduction, or significantly delayed reduction, on the qualities of anodized aluminium tested. Questioning surface quality, it should be mentioned that the aluminium samples had an extremely porous structure, into which the inoculated spore suspension was absorbed. Tests carried out later on with other material samples of anodized aluminium with better surface quality produced much lower D-values. However, the three qualities of aluminium studied could be easily replaced by more suitable materials. As a consequence these three D-value estimates were ignored for the further study.

The remaining results for the following steps of the study are summarized in Figure 3.

Result of Transferability and Resistance Factor

Based on these results, the spore-carrier combinations for the transferability study were selected. The combination with glass has shown the lowest resistance and the combination with POM the highest resistance. For both of these combinations and for the commercially

Figure 3. A summary of the results of the material tests in the reference isolator.

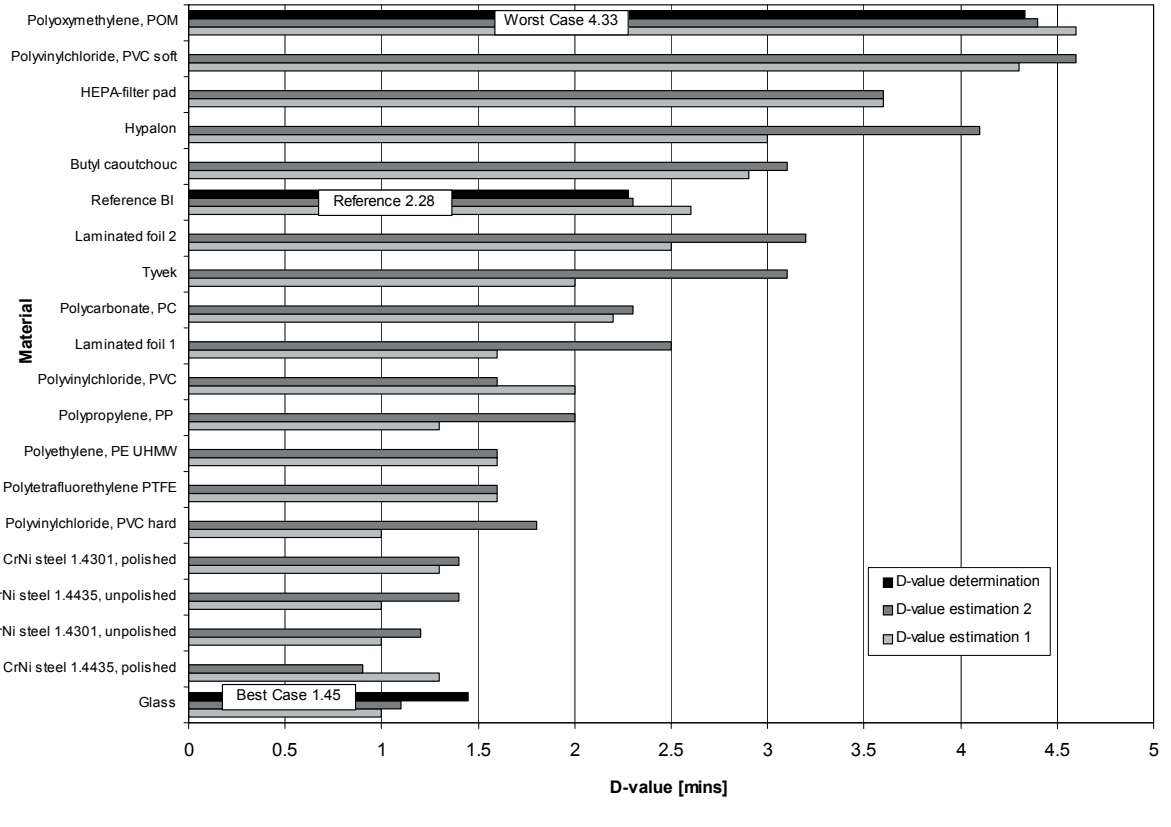


Table 7. The results of the transferability study and resistance factors.

	D-value [mins] Reference Isolator	D-value [mins] Sterility Test Isolator	D-value [mins] H_2O_2 Material Pass Through	D-value [mins] Filling Isolator
B. stearothermophilus **on Glass**	1.45 ± 0.07	1.41 ± 0.04	0.96 ± 0.05	1.18 ± 0.20
Biological Indicator Reference	2.28 ± 0.06	2.22 ± 0.06	1.62 ± 0.08	1.72 ±0.07
B. stearothermophilus **on POM**	4.33 ± 0.20	4.00 ± 0.30	3.38 ± 0.16	3.41 ± 0.23
Resistance Factor	1.89	1.80	2.09	1.98

available BI, a complete D-value determination was conducted in the reference isolator and subsequently in all the production systems. The resistance factor for each production system was determined. At this point of study, a decontamination cycle comparable to the one in the reference isolator had been developed for every production system, each containing a description of the achieved decontamination effect and its stability (14). The result of the transferability study and the resistance factors are shown in Table 7.

The resistance range established in the reference isolator and the relationship of the individual resistances to each other can be qualitatively reproduced in all production systems. Quantitatively slight variations in the decontamination effect can be observed in the D-values. In any case, the once established range of resistances and the relationship between them was maintained in each production system.

In consequence, all results obtained from the reference isolator could be transferred to the different production systems. The transferability is further supported by the minor deviation of the resistance factors from 1.80 to 2.09 among the different production systems. To summarize, all the tested production systems, irrespective of size and design, display a comparable decontamination effect.

Interpretation

The resistance range observed between 1.0 and 33.7 [mins] for the spore-carrier combinations was compared to physical and chemical properties of the carrier materials in order to determine the cause of the shift in resistance. Therefore, the surface structures of the different materials were examined under a microscope and characterized. In addition, various compositions of the spore suspension were tested to compensate for the effect of different wettabilities given by the carrier materials.

The results revealed no clear correlation between different material properties and the shift in resistance. Apparently, other influencing factors appear to be responsible for the results obtained. For example, the very high resistance of samples from anodized aluminium seems clearly to derive from the rough, porous surface structure. The inoculated spores are absorbed into this surface structure, and their exposure to gaseous H_2O_2 is only minimal and not guaranteed. However, Hypalon also shows a rough surface but still gives acceptable D-values. POM, on the other hand, despite its very smooth surface structure, shows increased resistance. Furthermore, material with poor wettability, such as PTFE, gives comparable resistance to materials with good wettability, such as glass or CrNi steel. With these materials, however, a difference was observed in the form of the inoculum with an identical composition of the spore suspension. In this case, the difference is not reflected in the resistances obtained. For other samples, the form of the inoculum could be interpreted as one reason for the increased resistance. It is not possible to generally explain the observed range of resistances based on one factor. The cause of the shifts in resistance is clearly the result of combinations of many influencing factors which were not examined in more detail in this study. The results showed in Figure 3 have now been incorporated into the cycle qualification of the different production systems. Two methods were considered to adjust the decontamination cycle developed based on commercially available BI.

Cycle Adjustment 1: Extension of Decontamination Phase

The duration of the decontamination phase, determined by the D-value for the commercially available BI, is extended in order to achieve the required log reduction on the material with the highest D-value (POM, worst case). In practice, this extends the decontamination period by the resistance factor (Figure 4).

Cycle Adjustment 2: Bioload Consideration

This approach requires the bioload determination on the material with the highest resistance (POM, worst case).

The duration of the decontamination phase based on the D-value for the commercially available BI can be retained if the bioload for the material with the highest resistance does not exceed a specific population. The log reduction achieved on the material with the highest resistance is obtained by dividing the log reduction achieved on the commercially available BI by the resistance factor. Based on the required decontamination target, it is thus possible to calculate the maximum permissible bioload on the material with the highest resistance. If the bioload obtained exceeds this maximum permissible level, the duration of the decontamination phase has to be extensioned accordingly (Figure 5).

Depending on the resistance factor, the extension results in an unacceptable long and uneconomical decontamination cycle. Furthermore, a higher quantity of H_2O_2 is vaporized which increases the length of the aeration phase. But the determination of the bioload is required for neither initial qualification nor for requalification.

The requalification of the decontamination cycle can be carried out based on commercially available BIs using the unadjusted cycle length. The other method of cycle adjustment requires the bioload determination on worst case materials, but results in a shorter decontamination cycle. The requalification is based on commercially available BIs and the result of the bioload determination on worst case materials. Both options ensure the achievement of the required decontamination target. Considering the level of productivity of each production system examined, the decontamination cycles were adjusted using both methods.

The required basis to apply such cycle adjustments is the quantification of the inactivation factors initial population, bioload, D-value and decontamination target. In particular, the suitability and the resistance of the commercially available BI have to be assessed in advance (14). During all requalifications carried out up to now, the bioload determination on all materials has shown very low values. In addition,

Figure 4. Depiction of cycle adjustment 1, extension of cycle time.

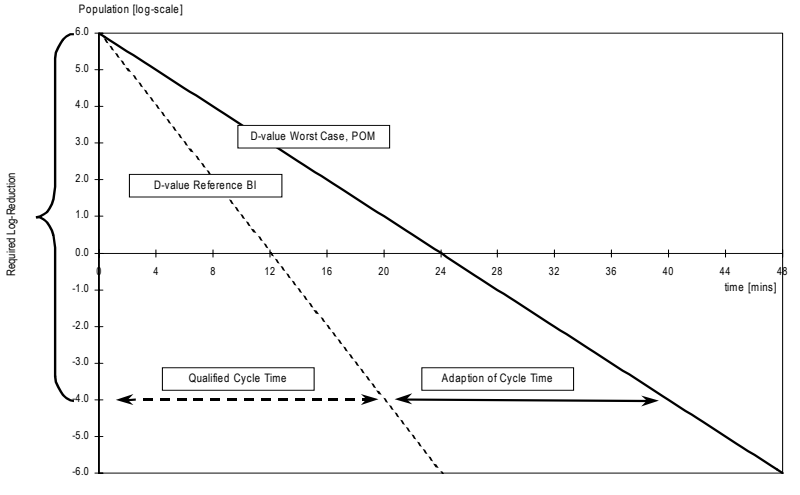
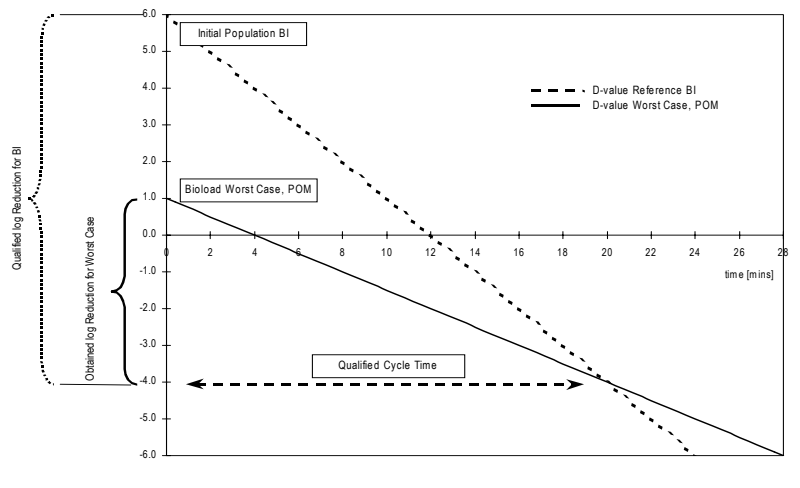


Figure 5. Depiction of cycle adjustment 2, consideration of bioload.



almost only vegetative germs were identified as bioload. In further tests, these germs have shown much lower resistances compared to the resistances of bacteria spores. These observations lead to the conclusion that initial qualifications and requalifications of H_2O_2 decontamination cycles based on commercially available BIs ensure the required safety level of decontamination on all chamber surfaces. Requirements, therefore, are the use of BIs with the highly resistant spores of *B. stearothermophilus* and the knowledge and control of the bioload on various materials.

These results represent only a small part of a more comprehensive study of H_2O_2 decontamination. Over a period of approximately 20 months, tests were carried out on commercially available BIs, various types of germs, and other issues relating to the qualification of the H_2O_2 decontamination process. There was unlimited access to the described reference isolator. A great deal of laboratory work was required in preparing and evaluating the samples. It was only possible to carry out the present study as a basic investigation for the simultaneous initial qualification of the mentioned production systems. Finally, the results of the study indicate that such an investigation should not be required for routine qualifications.

Summary

The study shows reproducible different resistances of *B. stearothermophilus* to a H_2O_2 decontamination on different carrier materials. Because of their surface structure and properties, certain materials seem to be not suitable for the H_2O_2 decontamination. The resistances found were transferable to each tested production system in terms of range and relationships. It was possible to adjust the decontamination cycle developed on the basis of commercially available BIs using quantified inactivation factors. The results of this study and the experience gained in routine operation indicate that such an investigation should *not* be required for routine qualifications. Cycle qualification using commercially available BIs with a highly resistant organism and the knowledge of the bioload on the various materials ensure the required safety level of decontamination in the whole chamber.

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