Test method to determine the microbiological resistance and characterization of the reaction kinetics of hydrogen peroxide sterilization processes

Characterization of biological indicators for validation and routine monitoring

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A method to determine the resistance of biological indicators in hydrogen peroxide disinfection und sterilization processes is described. D- and z-values in aqueous hydrogen peroxide solutions are established and the influence of the hydrogen peroxide concentration on the reaction kinetics is determined. The results enable the use of biological indicators for process validation and routine monitoring.

KEY WORDS
- hydrogen peroxide sterilization processes
- biological indicator resistance determination
- reaction kinetics
- hydrogen peroxide solution

Introduction

Hydrogen peroxide is used as a disinfecting and sterilizing agent in various processes. In industry and healthcare hydrogen peroxide/plasma processes in the gas phase are used as low temperature sterilization processes worldwide [1].

Disinfection processes use vaporized hydrogen peroxide/water mixtures [2, 3]. Amongst others this principle is used for room disinfection and decontamination in isolators. Depending on the manufacturer these decontamination systems claim to use gaseous [2] or condensing liquid hydrogen peroxide [3, 4] as active agent.

Finally aqueous hydrogen peroxide solutions are used by the food industry to extend the shelf life of perishable liquids by spraying bottles with hydrogen peroxide before filling them.

Whether hydrogen peroxide is used as a gas, as an aerosol or in an aqueous solution, the advantage of this sterilizing agent is that only oxygen and water are formed as non-toxic end products.

On the market hydrogen peroxide is available as hydrogen peroxide/water mixtures with up to 60% hydrogen peroxide. Higher concentrations are not in use since pure or higher concentrated hydrogen peroxide is instable and may decompose explosively to oxygen and water, if traces of catalysts are present. Water stabilizes hydrogen peroxide as a hydrate complex and prevents its sudden decomposition.

In connection with the sterilization/disinfection reaction kinetics of hydrogen peroxide processes different processes are discussed:

1. low pressure gas phase processes with different steam and hydrogen peroxide partial pressures
2. processes at atmospheric pressure, either with gaseous or condensing hydrogen peroxide on surfaces
3. liquid phase processes using aqueous hydrogen peroxide solutions.

Problem description

The knowledge of the reaction kinetics is a general requirement for validation and routine monitoring of disinfection and sterilization processes [5]. Also biological indicators of known resistance shall be used for each process as monitoring devices. Until today, there is no standardized test method available for biological indicators to test H_2O_2 processes. Therefore biological indicators for hydrogen peroxide processes can presently not be compared with respect to their resistance properties. Thus their use for validation and routine monitoring of disinfection and sterilization processes is not without difficulty.

To characterize the reaction kinetics of the process and the resistance characteristics of indicators, test sterilizers (resistometers) are necessary which keep all critical process variables constant over time [6]. Only under such conditions reproducible resistance values can be determined. H_2O_2 test sterilizers currently used can keep the critical variables temperature and pressure constant over time but not the critical variables steam and the hydrogen peroxide partial pressure in the gas phase. Especially if isolators supplied with vaporized hydrogen peroxide/water mixtures are operated as resistometers, local variations in the hydrogen peroxide concentration are to be expected as a result of condensation and decomposition [2, 3]. Besides the obvious process relevance of the hydrogen peroxide partial pressure it is up for discussion to which extent the steam partial pressure influences the inactivation of germs [4, 7].

Various studies draw different conclusions about the kill kinetics of bacterial endospores exposed to vaporized hydrogen peroxide/water mixtures [7, 8, 9, 10]. They relate linear [9] or nonlinear [10, 8]

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graphs in a semi-logarithmic plot of the number of viable spores against the time (survivor curve). Concluding knowledge about the kill kinetics in processes with vaporized hydrogen peroxide/water mixtures as sterilizing agent is still pending.

Kill kinetics of bacterial endospores in aqueous solutions of hydrogen peroxide have already been reported [11, 12, 13, 14, 15]. Survivor curves determined by these publications are mainly nonlinear [11, 12, 13, 14]. However, Toledo et. al. [15] observed linear survivor curves after an initial lag phase. Although the influence of the hydrogen peroxide concentration on the kill rate of spores has already been analyzed [11, 13, 15], the authors did not establish a kill kinetic reaction-equation which links the D-value (decimal reduction value required time to reduce the population by 90%) to the hydrogen peroxide concentration.

This also applies to the temperature. Among the referenced authors only Toledo et. al. [15] determined z-values for *Bacillus atrophaeus* in aqueous hydrogen peroxide solutions. The z-values state the temperature difference in K by which the reaction temperature must be increased to reduce the D-value to one tenth of the original value.

Differences in the reaction kinetics of hydrogen peroxide in gas phase and liquid phase are to be expected and have been reported [16]. In the absence of a working resistometer for hydrogen peroxide sterilization in the gas phase there is currently no reproducible test method available to determine the reaction kinetics of aforementioned processes or to characterize biological indicators with respect to their resistance properties. Until this situation is changed it seems sensible to use an already successfully employed technique in the liquid phase [15] in spite of possible differences between mechanisms in gaseous and liquid hydrogen peroxide. Using this method all the critical variables can be kept constant over time.

Test description

Resistance determinations of spore suspensions in aqueous hydrogen peroxide solutions have already been carried out successfully [15]. To determine the reaction kinetics the procedure has been optimized.

Geobacillus Stearothermophilus was used as test germ. The D-value of this germ was initially determined in a steam resistometer in compliance with the standard EN ISO 18472 [17] under saturated steam conditions according to EN ISO 11138-1 [18] and -3 [19]. Naturally other spores like those of *Bacillus atrophaeus* can be used as well. To determine the initial population of the spore suspension a heat shock according to US pharmacopoeia (15 minutes at 95 – 100 °C) was carried out.

D-values were determined for a spore suspension of *G. stearothermophilus* with about 10^7 CFU/ml (gke GmbH) using the survivor curve method.

100 µl of the spore suspension was mixed with 9.9 ml tempered aqueous hydrogen peroxide solution (Applichem, stabilized with 190 – 220 mg/l phosphate) and homogenized for approximately 10 seconds with a vortex shaker. For the required exposure time the solution is left in a tempered water bath. The inactivation is stopped abruptly by transferring 1 ml of the solution to 9 ml iced catalase solution (lyophilized, from bovine liver, Roth, 2000 U/ml in phosphate buffer, pH 7).

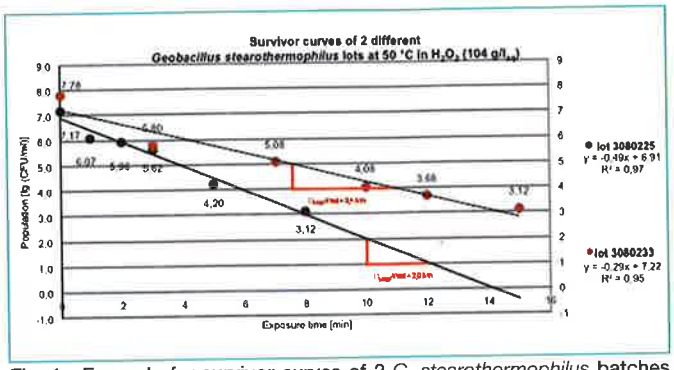

Fig. 1: Example for survivor curves of 2 *G. stearothermophilus* batches (lot 3080233 und lot 3080225) with different D-values in steam sterilization processes, at 50 °C in H_2O_2 (104 g/l_{aq}) (see table 1).

The number of surviving germs was determined using TSA (tryptic soy agar, Becton, Dickenson and Company) and standard plate count techniques. Culture plates were incubated at 57 °C. Applying this procedure the critical variables temperature and hydrogen peroxide concentration could be kept constant over time and the remaining spore population could be determined.

To determine the reaction kinetics of the microorganisms inactivation in aqueous hydrogen peroxide solutions, the effect of the variables temperature and hydrogen peroxide concentrations on the D-value were tested.

To evaluate the effect of the hydrogen peroxide concentration on the D-value of *G. stearothermophilus*, D-values were determined at constant temperature in different hydrogen peroxide concentrations. Test series at 40 and 50 °C were carried out. To further ensure the findings an additional test series at 50 °C with a different spore suspension batch was carried out.

Furthermore, the effect of the temperature on the reaction kinetics has been analyzed by D-value determinations at constant hydrogen peroxide concentration and different temperatures. With the obtained data z-values could be calculated.

To test for a possible influence of the pH value on the D-value, aqueous solutions of hydrogen peroxide with a pH values of 2 (H_2O_2 in water, acidified with H_3PO_4), pH 3.8 (H_2O_2 in water) and pH 6 (H_2O_2 in 0,1 M phosphate buffer) were prepared and used for resistance determinations as described above.

Results

1. Resistance of G. stearothermophilus in hydrogen peroxide solution at constant hydrogen peroxide concentration an constant temperature

For these measurements spore suspensions were used which differed strongly in their resistance in steam sterilization processes. A semi-logarithmic plot of the population against the time yields a linear relationship (see figure 1). The coefficients of determination (R^2) of the survivor curves evaluated for this study were greater than 0.85 and are approx. 0.95.

As already known for other sterilization processes different batches of *G. stearothermophilus* spore suspension show substantial differences in their resistance (see figure 1 and table 1).

Table 1: D-values of 2 different *G. stearothermophilus* spore suspensions in steam and H_2O_2

lot spore suspension	D-values [min]					
	In steam	In H_2O_2 (104 g/l_{aq}) at 50 °C				
		Single values of 3 tests			Mean value	Standard deviation
3080225	1.5 min	2.05	2.16	1.75	1.98	0.17
3080233	2.8 min	3.48	3.40	3.43	3.44	0.03

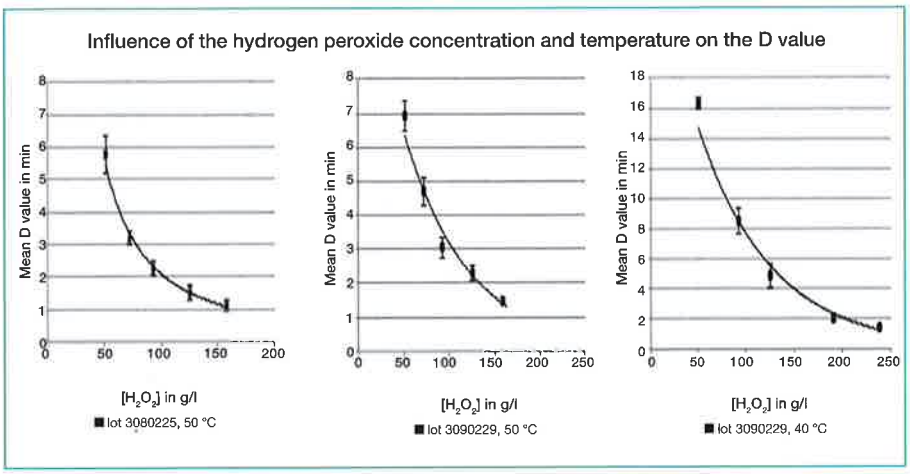

Fig. 2: Plot of the D-values of *G. stearothermophilus* (lot 3080225, lot 3090229) at 40 °C and at 50 °C against the concentrations of the used aqueous hydrogen peroxide solutions. Error bars state the standard deviation.

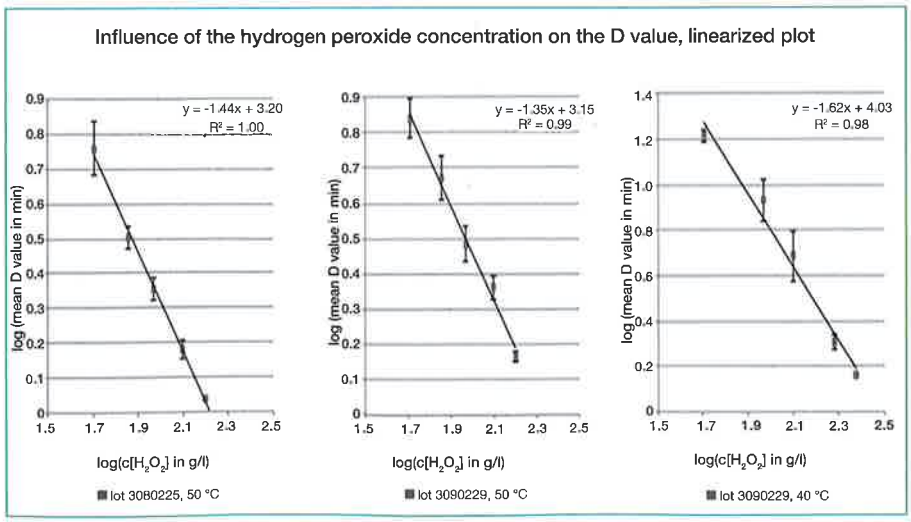

Fig. 3: Logarithmic plot of the D-values of *G. stearothermophilus* (lot 3080225, lot 3090229) at 50 °C and at 40 °C against the concentration of the aqueous hydrogen peroxide solutions. Linear equation of the linear regression and the coefficient of determination are given. Error bars indicate the standard deviation.

2. Influence of the hydrogen peroxide concentration on the D-value at constant temperature

The influence of the hydrogen peroxide concentration on the D-value of *G. stearothermophilus* has been analyzed by determining D-values at constant temperature and at different hydrogen peroxide concentrations (tables 2 – 4). The determined D-values decreased with increasing hydrogen peroxide concentration. A plot of the D-values against the hydrogen peroxide concentration shows a non-linear correlation of both variables (see figure 2).

3. Description of the reaction kinetics

When the D-value and the hydrogen peroxide concentration are plotted with a logarithmic scale they show a straight line (figure 3). From the linear equation of this plot the exponent of the hydrogen peroxide concentration in the reaction kinetics can be calculated.

The inactivating rate $R=-dN/dt$ is defined as the reduction of microorganisms over time. Since the survivor curves in a semi-logarithmic plot are linear at constant hydrogen peroxide concentrations, the reaction is first-order with respect to the population. The inactivation rate is described by equation (1).

$$R = -\frac{dN}{dt} = k \times N^n \qquad (1)$$

Abbreviation	Unit	Description
R		reaction rate of inactivating microorganisms
N	CFU	Population
t	Min	Time
k	min^{-1}	Temperature dependent reaction rate constant (for the logarithm to the base 10) at constant hydrogen peroxide concentration
n		Exponent of N = 1

If D-values of *G. stearothermophilus* are plotted against the hydrogen peroxide concentration (see figure 2) the influence of the hydrogen peroxide concentration on the inactivation rate is visible.

Consequently, the reaction rate constant k is a function of the hydrogen peroxide concentration:

$$k = k' \times [H_2O_2]^m \qquad (2)$$

Abbreviation	Unit	Description
k'	$g^{1-(m+n)\times l/(m+n)-1} \times min^{-1}$	Temperature dependent reaction rate constant (for the logarithm to the base 10)
$[H_2O_2]$	g/l	Hydrogen peroxide concentration
m		Exponent of $[H_2O_2]$

Substituting the D-value (equation (3)) in equation (2) results in equation (4). Re-writing and a logarithmic transformation result in equation (5).

$$D = -\frac{1}{k} \quad (3)$$

$$\frac{1}{D} = k' [H_2O_2]^m \quad (4)$$

$$D = -m \log [H_2O_2] + \log \left(\frac{1}{k'}\right) \quad (5)$$

Hence the exponent m can be calculated from a logarithmic plot of D-values against the hydrogen peroxide concentration (figure 3).

From the test results at 50 °C with spore suspension lot 3080225 an exponent m of 1.4 is determined. The evaluation of the test result at 50 °C and 40 °C with spore suspension lot 3090229 results in an exponent m = 1.4 – 1.6 (table 5). By approximation 1.5 can be accepted for m (equation (6)).

$$R = k' \times N \times [H_2O_2]^{1,5} \quad (6)$$

4. Influence of the temperature on the D-value at constant hydrogen peroxide concentration

To determine the effect of the temperature on the D-value of *G. stearothermophilus*, D-values have been measured at constant hydrogen peroxide concentration and different temperatures (table 6). From this data z-values could be calculated (figure 4, table 7).

Since the D-value and the reaction rate constant k are connected via equation (3), k can be calculated from the D-values (table 8).

According to the Arrhenius equation (7), the frequency factor A and the activation energy E_A can be calculated from the regression line in the Arrhenius graph (see figure 5). This yields a frequency factor A of approximately $3.75 \times 10^8\ s^{-1}$ and an activation energy E_A of approx. 65846 J·mol^{-1}.

$$k = A \times e^{-\frac{E_A}{R \times T}} \quad (7)$$

Abbreviation	Unit	Description
k'	s^{-1}	Temperature dependent reaction rate constant (for the logarithm to the base 10)
A	s^{-1}	Frequency factor
E_A	$J \times mol^{-1}$	Activation energy
R	$J \times mol^{-1} \times K^{-1}$	Universal gas constant
T	K	Absolute temperature

Table 2: D-values of *G. stearothermophilus* (lot 3080225) in aqueous hydrogen peroxide solutions

$[H_2O_2]$ in g/l	D-values [min] at 50 °C					
	Single values of 4 tests				Mean value	Standard deviation
50.88	6.57	5.53	5.22	5.67	5.75	0.58
71.73	3.08	3.40	2.94	3.27	3.17	0.20
92.88	2.27	2.45	1.95	2.38	2.26	0.22
125.17	1.53	1.35	1.33	1.84	1.51	0.24
158.16	0.98	0.95	1.17	1.28	1.09	0.16

Table 3: D-values of *G. stearothermophilus* (lot 3090229) in aqueous hydrogen peroxide solutions

$[H_2O_2]$ in g/l	D-values [min] at 50 °C				
	Single values of 3 tests			Mean value	Standard deviation
50.88	6.60	6.74	7.44	6.93	0.45
71.73	4.58	4.34	5.17	4.69	0.43
92.88	2.71	3.13	3.30	3.05	0.30
125.17	2.55	2.23	2.13	2.30	0.22
158.16	1.60	1.42	1.36	1.46	0.13

Table 4: D-values of *G. stearothermophilus* (lot 3090229) in aqueous hydrogen peroxide solutions

$[H_2O_2]$ in g/l	D-values [min] at 40 °C				
	Single values of 3 tests			Mean value	Standard deviation
50.88	15.89	16.39	16.58	16.29	0.35
92.88	7.90	9.43	8.12	8.48	0.83
125.17	5.75	4.36	4.40	4.84	0.80
191.89	2.26	2.03	1.81	2.03	0.22
238.02	1.45	1.54	1.38	1.45	0.08

Table 5: $[H_2O_2]$ exponent m, determined in two independent test series with different spore suspensions and at different temperatures

	lot 3080225, 50 °C	lot 3090229, 50 °C	lot 3090229, 40 °C
$[H_2O_2]$ exponent m	1.4	1.4	1.6

Table 6: D-values of *G. stearothermophilus* (lot 3080225) in aqueous hydrogen peroxide solution (104 g/l_{aq}) at different temperatures

Temperature [°C]	D-value [min] at 104 g H_2O_2/l_{aq}				
	Single values of 3 tests			Mean value	Standard deviation
35	6.54	7.65	6.39	6.98	0.61
40	4.19	3.65	4.09	3.99	0.23
45	3.11	3.12	2.56	2.91	0.26
50	2.05	2.16	1.75	1.98	0.21
55	1.10	1.42	1.10	1.20	0.15
60	1.13	1.02	0.92	0.97	0.13

Table 7: z-values of *G. stearothermophilus* (lot 3080225) in aqueous hydrogen peroxide solution (104 g/l_{aq}) and indication of the coefficient of determination R^2 of the regression rate.

z-value in K at 104 g H_2O_2/l	R^2
30.51	0.966
30.35	0.974
28.76	0.989

Table 8: Reaction temperatures and corresponding reaction rate constants of inactivation processes of *G. stearothermophilus* (lot 3080225) in H_2O_2 (104 g/l_{aq}).

Temperature in K	k in s^{-1}
308.15	2.43×10^{-3}
313.15	4.19×10^{-3}
318.15	5.68×10^{-3}
323.15	8.40×10^{-3}
328.15	1.38×10^{-2}
333.15	1.63×10^{-2}

Table 9: D-values of *G. stearothermophilus* (lot 3090229) in aqueous hydrogen peroxide (104 g/l_{aq}) of different pH at 50 °C. Average values and standard deviations were calculated from at least 3 separate experiments.

	D-value in min at 104 g H_2O_2/l and 50 °C	
pH value	Mean value	Standard deviation
2.0	0.92	0.06
3.8	2.24	0.16
6.0	1.87	0.13

5. Influence of the pH value on the D-value

The influence of the pH value on the D-value was examined (table 9). Among the tested pH values the resistance of *G. stearothermophilus* was highest at pH 3.8. A low pH value of 2 significantly decreased the D-value.

Discussion

With the described method the resistance of biological indicators in aqueous hydrogen peroxide solutions could be determined reproducibly. This method allows a resistance determination of spore suspensions but can also be used for biological indicators on carriers after slight modifications of the testing procedure.

At constant hydrogen peroxide concentration and temperature the reaction rate is only depending on the population; it is a first-order reaction with respect to the population. Therefore the semi-logarithmic plot of the CFU against the time is linear. Neither a tailing of the survivor curves, as described by Cerf and Metro [14], nor an initial lag phase, as reported by Toledo et al. [15], could be observed. Coefficients of determination of the survivor curves were higher than 0.85 and were mostly at approximately 0.95.

As it is already known from other sterilization processes, the D_{H2O2}-values of different batches of *G. stearothermophilus* can differ widely. This emphasizes the urgency to establish a reproducible method for resistance determinations. The identity of the test germ alone does not provide reliable information about its resistance.

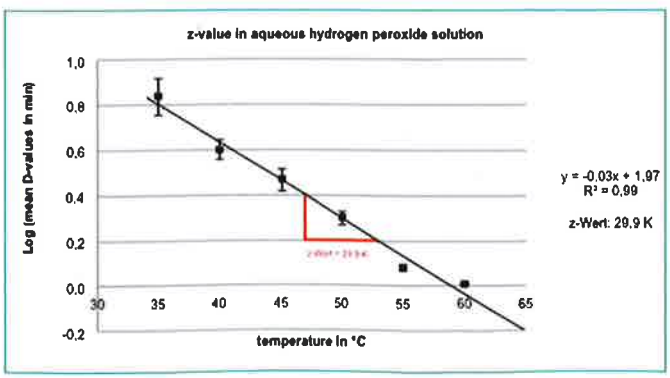

Fig. 4: Semi-logarithmic plot of average D-values of *G. stearothermophilus* (lot 3080225) in H_2O_2 (104 g/l_{aq}) against the temperature. Error bars state the standard deviation. The z-value results from the negative reciprocal slope of the stated regression line.

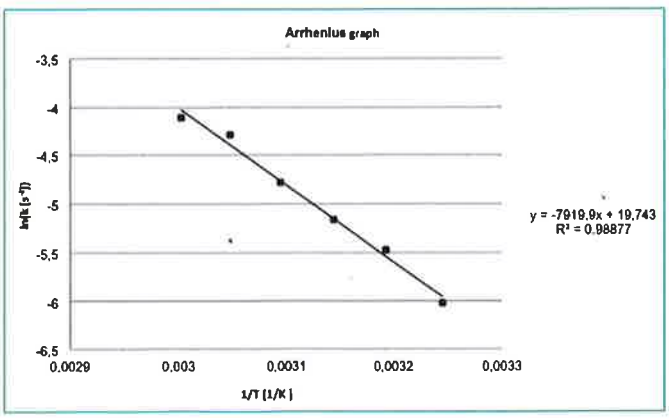

Fig. 5: Arrhenius graph of the inactivation reaction of *G. stearothermophilus* (lot 3080225) in H_2O_2 (104 g/l_{aq}).

By independent analysis of the effect of the reaction temperature and the hydrogen peroxide concentration on the D-value, their influence on the inactivation rate could be determined. z-values were at approximately 30 K.

In the reaction rate the hydrogen peroxide concentrations enter with an exponent m. Independent experiments at 40 and 50 °C determined values of 1.4 and 1.6 for m. The reaction kinetics of the inactivation of *Geobacillus stearothermophilus* in aqueous hydrogen peroxide solutions is therefore approximately of 1.5 order in this reactant. A fractional order of reaction of the inactivation of spores in liquid hydrogen peroxide appears not astonishing when taking the background of complex oxidation processes at DNA, proteins, plasma membranes, lipids and the spore coat into consideration [20, 21].

Experiments analyzing the influence of the pH value on the D-value indicate a decrease in resistance at a strongly acidic pH. Other authors already reported an influence of the pH value on the hydrogen peroxide resistance of bacterial spores [13]. The quality of hydrogen peroxide must not be neglected as a relevant factor for the inactivation of spores, as frequently used stabilizing agents like phosphoric acid and others modify the pH of hydrogen peroxide. ■

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