Keywords
- *Bacillus subtilis*
- ethylene oxide
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- kill kinetics

Kill Kinetics Study of *Bacillus subtilis* Spores in Ethylene Oxide Sterilisation Processes

D. Heider[1], J. Gömann[2], U. Junghannß[1], U. Kaiser[2]

The kill kinetics of *Bacillus subtilis* spores in ethylene oxide sterilisation processes were determined, while varying the process parameters ethylene oxide concentration, relative humidity (RH), temperature and inert gas type. The experiments were carried out in an ethylene oxide resistometer in accordance with EN 866 Part 2. The D values inferred from the kill kinetics were used to determine the reaction velocity changes of the various processes conducted.

The study showed, on changing the ethylene oxide concentration from 50 to 1200 mg/l, that the reaction velocity of the process is greatly influenced by the ethylene oxide concentration and that it corresponds somewhat to a reaction of the first order kinetics across the entire concentration range. On varying the relative humidity from 10 to 100%, we found that there was a correlation between the reaction velocity in the range between 10 and 60% RH, whereas no further changes were observed at higher levels. On varying the temperature between 30 and 64 °C, it was observed that a 10 K increase in temperature doubled the reaction velocity. On using an ethylene oxide concentration between 50 and 150 mg/l for sterilisation, no changes were observed on adding air, whereas on adding carbon dioxide the reaction velocity declined by up to 32%.

Introduction and Task Definition

Ethylene oxide sterilisation processes are being used worldwide on a broad scale in the medical devices industry when thermal or irradiation sterilisation processes cannot be used. To minimise the consumption, release of ethylene oxide into the environment as well as the residual amount of the gas in the devices, attempts are being made to operate with minimal ethylene oxide concentrations and short sterilisation times. Whereas formerly concentrations between 600 mg/l up to 1000 mg/l were used, concentrations of even less than 300 mg/l are being used at present. Moreover, inert gases are added to the pure sterilisation gas in order to curtail the risk of explosions.

To guarantee the safety of the sterilisation process, the influence exerted by all relevant process parameters must be quantitatively elucidated, as required by the standard EN-ISO 14937. In our study we investigated the influence generated by the parameters ethylene oxide concentration, relative humidity and inert gas type (air and carbon dioxide) on the reaction velocity in sterilisation processes with ethylene oxide. In order to be able to evaluate the influence generated by these parameters, one parameter was varied in each case while keeping the others constant in accordance with the requirements specified in the standards EN 866-2 and ISO 11138-2. The reaction kinetics was experimentally determined by plotting survival curves in accordance with the European standard EN 866-1 and the international standard ISO 11138-1 in an ethylene oxide resistometer which conformed to the standards EN 866-2 and ISO 11138-2. A mathematical model of the reaction kinetics was inferred from the results.

Material and Methods

The investigations were conducted in an ethylene oxide resistometer designed by the firm gke-mbH. The principle component of this resistometer was a two-litre glass reaction vessel. An external water thermostat via a water jacket with a precision of ± 0.1 K of the set point was used to provide for temperature control of this vessel. A vacuum-tight agitator device in the vessel made provision for homogenous conditions within the reaction chamber. The active ingredients were fed via supply pipes, controlled by magnet valves, from supply containers into the chamber. A vacuum pump was connected for evacuation of the reaction vessel. The sterilisation conditions were controlled via a Pt 100 temperature sensor of precision class B (90.2221, manufactured by Juchheim GmbH & Co., Fulda) and a pressure sensor with a characteristic deviation of 0.5% (4753, manufactured by Juchheim GmbH & Co., Fulda); a recorder (L 250, manufactured by Linseis, Selb) was used for data registration. A sample holder was used to secure the samples in the reaction chamber.

The test organisms employed were biological indicator strips from gke-mbH containing the test organism *Bacillus subtilis* (ATCC 9372). The mean population was 2.0×10^6 cfu/strip. A D_{EtO} value of 2.9 minutes was obtained at 54 °C under the sterilisation conditions specified in EN 866-2.

The test procedure set out in the standards EN 866-2 and ISO 11138-2 was used as a reference test condition to ascertain the D value at 54 °C (see table 1). Then one process parameter was changed in each case and the change in the reaction velocity was obtained by measuring the D value.

1 Hochschule Anhalt, Bernburger Str. 52–57, D-06366 Köthen

2 gke-mbH, Auf der Lind 10, D-65529 Waldems-Esch

Parameter	Reference conditions as per EN 866-2	Variation range of the parameters used in this study
EtO Conc.	600 mg/l ± 30 mg/l	50 – 1200 mg/l ± 10 mg/l
Relative atmospheric humidity	60% RH ± 10%	10 – 100% RH ± 3%
Temperature	54 °C ± 1 °C	30 – 64 °C ± 0.1 °C
Inert gas-added	none*	none*, air, CO_2

Table 1 The standard process conditions set out in EN 866-2 and the variation range of the parameters investigated in this study.

* due to the nature of the process, minimum quantities of residual air remain in the reaction chamber

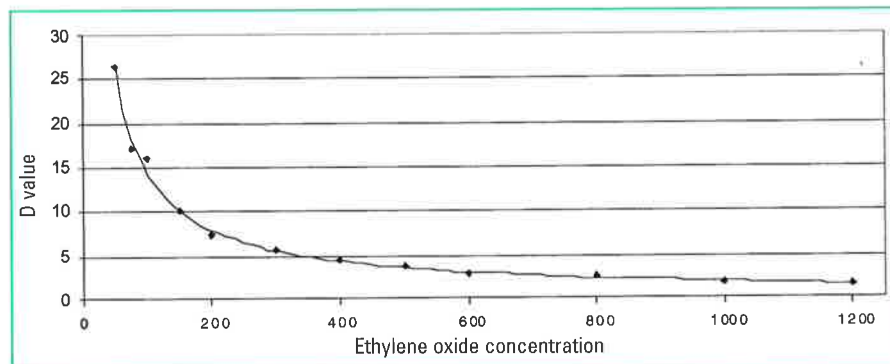

Figure 1 Correlation between the D value and the ethylene oxide concentration (at 54°C, 60% RH)

As a preparation for measurements, and having used a thermostat to maintain the temperature of the resistometer at the operating temperature, the biological indicators to be measured were fixed on the sample holder in the upper section of the reaction vessel. The agitator was switched on to ensure homogeneous conditions throughout the entire reaction vessel. The reaction vessel was evacuated to 60 mbar to remove air. Since it was not possible to fully remove the air, a residual air concentration of 2.2 mmol/l was present for each measurement. Having evacuated the reaction vessel and reached the set point temperature, the requisite active ingredients were supplied. For preconditioning, the amount of steam required for setting the relative atmospheric humidity was supplied from a thermostat-controlled water reservoir and kept constant for 30 min. Then the other gases were added. The concentration of the gases was set by measuring their partial pressure values. For sterilisation with the inert gases "air" (as ambient air) or "carbon dioxide", the gases were added until the ambient pressure of 1000 mB had been reached, in order to achieve as high as possible a concentration of the inert gases. The equipment settings remained unchanged until the end of the gassing duration. The pressure and temperature values were continually monitored for conformance with the set point. At the end of the experiments, the gases were withdrawn from the reaction chamber and the chamber was ventilated. Air removal was repeated on four occasions so as to completely remove the EO. The biological indicators were removed from the reaction vessel and their population assessed immediately. To determine the population, the biological indicators were homogenised in a blender filled with 100 ml water. The suspension obtained was subjected to a defined dilution process and plated onto a trypticase solid medium in accordance with the requirements of the USP 24 and the German Pharmacopoeia (DAB) 10. Colonies were counted after a two-day incubation period at 35 °C.The population of surviving organisms was calculated by multiplying with the dilution factors.

Based on the requirements of EN 866-2, it is not necessary to neutralise the ethylene oxide. The ethylene oxide is completely removed by the ensuing and repeated evacuation of the reaction chamber and any residual amounts of ethylene oxide are greatly diluted by the subsequent treatment of the biological indicators.

The experiments were evaluated by determining the D values of the biological indicators employed. The D value is defined as the time in minutes needed to reduce the baseline microbial count of a particular microorganism by one power of ten in accordance with the conditions stipulated in the standard, or to reduce the microbial count by 90%. The reaction velocity of the sterilisation process can be inferred by measuring the D value. The D value is inversely proportional to the reaction velocity r of the process ($D \sim 1/r$).

Having plotted the experimental D values against the experimental variables, the influence exerted by the measured parameters is illustrated.

For the kinetics study, the influence generated by the parameters ethylene oxide concentration, relative atmospheric humidity, temperature and addition of air and carbon dioxide, as inert gases, was investigated.

T [°C]	30	44	54	64
D value [min]	13.6	5.7	2.9	1.5

Table 2 Correlation between D values and temperature (600 mg/l EtO, 60% RH)

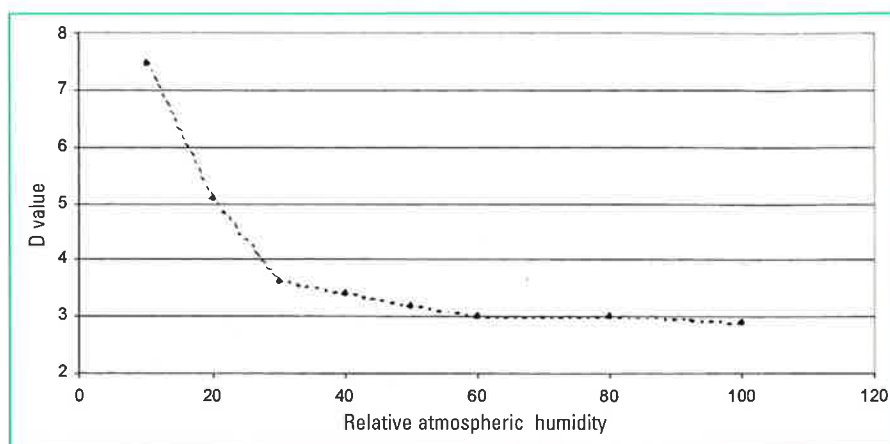

Figure 2 Correlation between the D value and the relative atmospheric humidity (at 54°C, 600 mg/l EtO)

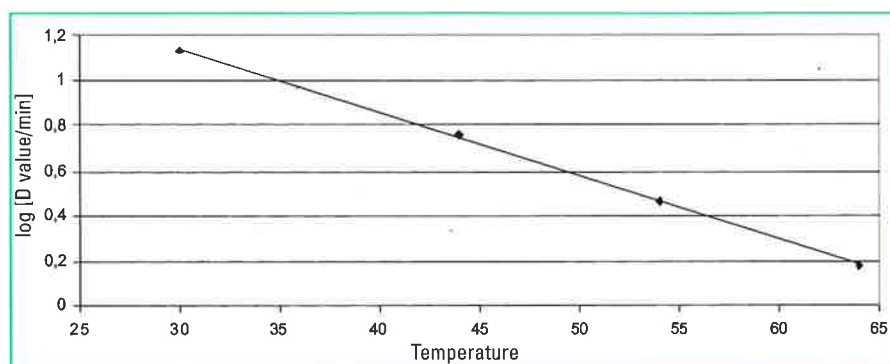

Figure 3 Ascertainment of the z value (at 60% RH, 600 mg/l EtO)

Experim. series EtO [mg/l]	Total pressure in reaction vessel [mbar]	Concentrations of Components [mmol/l]				D values [min]
		EtO	H_2O	air	CO_2	
	181	1.12	3.31	2.21	–	26.5
50	1000	1.12	3.31	32.36	–	26.6
	1000	1.12	3.31	2.21	30.15	28.1
	212	2.24	3.31	2.21	–	16.0
100	1000	2.24	3.31	31.24	–	15.8
	1000	2.24	3.31	2.21	29.03	19.4
	243	3.37	3.31	2.21	–	10.0
150	1000	3.37	3.31	30.11	–	10.1
	1000	3.37	3.31	2.21	27.91	13.2

Table 3 Correlation between the D values and the addition of air or carbon dioxide. Three series of experiments were conducted with an ethylene oxide concentration of 50, 100 and 150 mg/l and different concentrations of the gases air and carbon dioxide at a constant temperature of 54 °C and RH of 60%.

Results

Influence Exerted by the Ethylene Oxide Concentration

The correlation between the D value and the ethylene oxide concentration was measured over a concentration range between 50 mg/l and 1200 mg/l (see Figure 1). The D value was somewhat inversely proportional to the ethylene oxide concentration across the entire measuring range.

Influence Exerted by the Relative Humidity

The correlation between the D value and the relative humidity in the range 10 – 100% RH was measured (Figure 2). For a relative humidity between 10 and 30 %, it was demonstrated that there was a strong correlation between the D value and the relative humidity. In the range between 30 and 50% relative humidity it was possible to observe a sort of transition range, while the D value approached a saturation value when the relative humidity increased to above 60% (see Figure 2).

Influence Exerted by the Temperature

In addition, the correlation between the D value and the temperature in the range 30 to 64 °C was studied. An increase in temperature led to a reduction in the D value and thus to an increase in the reaction velocity.

The z value gives the temperature in °C or K required to change the D value by one power of ten. The Q_{10} value is given as the quotient reaction velocity constant k for each 10 K change in temperature. A z value of 35.4 °C (Figure 3) and a Q_{10} value of 2.0 were calculated from the results. The latter means that the reaction velocity doubles if the temperature is changed by 10 K.

Influence Exerted by Air or Carbon Dioxide

The correlation between the reaction velocity and the addition of air or carbon dioxide was measured. To this effect, the D values obtained when conducting sterilisation with a mixture of ethylene oxide, steam and with a small quantity of residual air as a standard were compared with those obtained on using, on the one hand, a mixture ethylene oxide, steam and air and, on the other hand, a mixture of ethylene oxide, steam and carbon dioxide. The results (see Table 3) demonstrate that

compared with the standard, the D value or the reaction velocity is hardly changed by adding air, whereas the D value is increased by up to 32 % by adding carbon dioxide.

When conducting tests to ascertain the D value after addition of carbon dioxide, a pH paper was exposed together with the spore strips. The pH paper showed a pH value = 4.5 during sterilisation.

Discussion

The results show that the D value depends greatly on the ethylene oxide concentration. An increase in the ethylene oxide concentration reduces the D value and thus expedites the entire sterilisation process. The results from this series of measurements were used to record the reaction kinetics, which are described in the next chapter. The concentration ranges used in this present study were on a broader scale than those used in the measurements conducted by Ernst and Shull (4). In our study it has been proven that even with an increase of the ethylene oxide concentration to 1200 mg/l the reaction velocity is still accelerated and that a reaction of the first order ensues.

The investigations of the relative atmospheric humidity demonstrate that with a relative atmospheric humidity of less than 60% RH, the D value is significantly prolonged and that it actually increases precipitously if the relative atmospheric humidity is very low. It is recommended that a relative atmospheric humidity of more than 60% RH be ensured at all inner and outer surfaces to be sterilised so that the effectiveness of the process is not compromised at any time.

The Q_{10} and z values obtained in this study, which concord with those cited in the literature (2, 4) attest to the good reproducibility of the measurements. Increasing the temperature can be viewed as an easy means of reducing the reaction time when carrying out ethylene oxide sterilisation.

In industrial processes ethylene oxide concentrations between 3 and 15% are used in carbon dioxide with an increased pressure between 2 and 5 bar. These conditions correspond to EtO concentrations of approx. 100 – 300 mg/l. The reaction velocity of chemical reactions in the range below 10 bar shows only a negligible correlation with the pressure. The increased pressure levels serve only to provide for an absolute increase in the EtO concentration with a concomitantly lower mixture concentration of the inert gas. The results obtained show that slower ethylene oxide sterilisation is achieved on adding carbon dioxide and that the process must be prolonged on adding CO_2 compared with pure EtO as an inert gas. One possible reason for this could be the reduction in the pH value in the reaction chamber caused by the addition of carbon dioxide. Addition of air did not change the reaction velocity.

Reaction Kinetics

It was established that – when conducing sterilisation in accordance with EN 866-2 – the reaction velocity in ethylene oxide sterilisation processes is a function of the microbial count of the microbes to be killed, of the concentration of the ethylene oxide and steam as well as of the temperature. The reaction velocity showing the reduction in the microbial count over time is generally illustrated by the following equation:

$$r = -dN/dt = k \times N \times [EtO]^m \times [H_2O]^n \quad (1)$$

reaction velocity

Symbol	Unit	Description
N	[cfu/strip]	Microbial count/strip
r	[– CFU/min]	Reaction velocity
T	[min]	Time
k		Temperature-dependent reaction velocity constant
[EtO]	[mg/l] or. [mmol/l]	Ethylene oxide concentration
[H2O]	[mg/l] or [mmol/l]	Steam concentration
m, n	Count	Exponents for the [EtO] or $[H_2O]$

For the exponent m of the ethylene oxide concentration, a value of 1.2 at 30 °C and 0.9 at 54°C was calculated in the experiments (see Figure 1.), hence a value of m = 1 can be assumed as initial approximation. The exponent n was null if the relative humidity values were greater than 60% (see Figure 2). Accordingly, the following equation is obtained for atmospheric humidity values greater than 60% after integration of the equation from N_0 to N_t and conversion from k to the decadic logarithm

$$\lg N_0 - \lg N_t = k' \times [EtO]^1 \times t = \frac{[EtO]^1}{D_{Ref\,EtO}} \times t = IF \quad (2)$$

The inactivation factor IF, also called the decadic reduction factor, describes the reduction of the microbial count and is the exponential difference between the decadic logarithm of the baseline concentration and the surviving residual population after time t.

Symbol	Unit	Description
N_0	[cfu]	Microbial concentration at the beginning of observation period
N_t	[cfu]	Microbial concentration at the end of observation period
t	[min]	Time
IF	[Number of decadic reduction levels]	Inactivation factor
k'		Modified reaction constant
[EtO]	[mg/ l] or [mmol / l]	Ethylene oxide concentration
D_{EtO}	[min]	Actual decadic reduction factor with specified [EtO]
D_{RefEtO}	mg/l EtO · min e.g.: 600 mg/l, 54°C	Decadic reference reduction factor

For steam sterilisation processes, a reaction of the first order takes place. This means that the reaction velocity of this process at a constant temperature depends only on the microbial count. The D value for describing the resistance in steam sterilisation processes has the unit [min]. Conversely, in the ethylene ox-

ide sterilisation process investigated in this study a reaction of the second order takes place. In ethylene oxide sterilisation the reaction velocity depends on, in addition to the microbial count, the EtO concentration. In this reaction the D value does not have the unit [min], but rather [mg EtO/l x min] or [mol EtO/l x min]. The D value is valid only for the experimental condition applicable while carrying out the assessment. If other ethylene oxide concentrations are used, the D value of the biological indicator may be calculated using the formula (3).

If the D_{EtO} value is known for an ethylene oxide concentration, it can be calculated to a reference D_{RefEto} value or to a D_{EtO} value with any ethylene concentration:

$$D_{EtO} = \frac{D_{Ref\ EtO}}{[EtO]} = \left[\frac{mg\ EtO \times min}{l} \times \frac{l}{mg\ EtO}\right] = [min] \qquad (3)$$

Using the D_{Eto} value makes it easier to use the equation (2) for calculating the inactivation factor for

$$IF = \frac{t\ [min]}{D_{EtO}\ [min]} \qquad (4)$$

In addition, with a relative humidity greater than 60% RH and a constant ethylene oxide concentration, a given D value can be calculated with the following formula from the known temperature T1 to another temperature T2, if [EtO] remains constant and the z value is known:

$$D_{EtO\ with\ T2} = D_{EtO\ with\ T1} \times 10^{(T1-T2)/z} \qquad (5)$$

An z value of 35.4 °C was ascertained in the experiments (see Figure 3).

References

1. Joslyn, L J: Gaseous Chemical Sterilization, aus: Block, S S: Disinfection, Sterilization, and Preservation. Philadelphia: Lippincott Williams & Wilkins, 2001: 337–359.
2. Caputo, R I and Rohn, K J: The Effects of EtO Sterilization variables on BI Performance. Medical Devices & Development 1982, 36–41.
3. Euronorm EN 866-2. Biologische Systeme für die Prüfung von Sterilisatoren und Sterilisationsverfahren. Teil 2: Spezielle Systeme für den Gebrauch von Ethylenoxid-Sterilisatoren. Beuth Verlag 1997.
4. Ernst, R R and Shull, J J: Ethylene Oxide Sterilization, I. Concentration and Temperature Effects. Soc. Am. Microb. 1962; 337–341.
5. Ernst, R R and Shull, J J: Ethylene oxide gaseous sterilization, II. Influence of Method of Humidification. Appl. Microbiol. 1969; 10: 342–344.
6. Gilbert, G L, Gambill, V M, Spinner, D R: Effect of moisture in ethylene oxide sterilization. Appl. Microbiol. 1964; 12: 496–509.

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Dr. Gudrun Westermann, ✆ +49 (0) 6122/7709-135
E-mail: ZentrSteril@mhp-verlag.de

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