

CHAPTER 5

Sterilization

INTRODUCTION

A FERMENTATION product is produced by the culture of a certain organism, or organisms, in a nutrient medium. If the fermentation is invaded by a foreign micro-organism then the following consequences may occur:

- (i) The medium would have to support the growth of both the production organism and the contaminant, resulting in a loss of productivity.
- (ii) If the fermentation is a continuous one then the contaminant may 'outgrow' the production organism and displace it from the fermentation.
- (iii) The foreign organism may contaminate the final product, e.g. single-cell protein where the cells, separated from the broth, constitute the product.
- (iv) The contaminant may produce compounds which make subsequent extraction of the final product difficult.
- (v) The contaminant may degrade the desired product; this is common in bacterial contamination of antibiotic fermentations where the contaminant would have to be resistant to the normal inhibitory effects of the antibiotic and degradation of the antibiotic is a common resistance mechanism, e.g. the degradation of β -lactam antibiotics by β -lactamase-producing bacteria.
- (vi) Contamination of a bacterial fermentation with phage could result in the lysis of the culture.

Avoidance of contamination may be achieved by:

- (i) Using a pure inoculum to start the fermentation, as discussed in Chapter 6.

- (ii) Sterilizing the medium to be employed.
- (iii) Sterilizing the fermenter vessel.
- (iv) Sterilizing all materials to be added to the fermentation during the process.
- (v) Maintaining aseptic conditions during the fermentation.

The extent to which these procedures are adopted is determined by the likely probability of contamination and the nature of its consequences. Some fermentations are described as 'protected' — that is, the medium may be utilized by only a very limited range of micro-organisms, or the growth of the process organism may result in the development of selective growth conditions, such as a reduction in pH. The brewing of beer falls into this category; hop resins tend to inhibit the growth of many micro-organisms and the growth of brewing yeasts tends to decrease the pH of the medium. Thus, brewing worts are boiled, but not necessarily sterilized, and the fermenters are thoroughly cleaned with disinfectant solution but are not necessarily sterile. Also, the precautions used in the development of inoculum for brewing are far less stringent than, for example, in an antibiotic fermentation. However, the vast majority of fermentations are not 'protected' and, if contaminated, would suffer some of the consequences previously listed. The approaches adopted to avoid contamination will be discussed in more detail, apart from the development of aseptic inocula which is considered in Chapter 6 and the aseptic operation and containment of fermentation vessels which are discussed in Chapters 6 and 7.

MEDIUM STERILIZATION

As pointed out by Corbett (1985), media may be sterilized by filtration, radiation, ultrasonic treatment, chemical treatment or heat. However, for practical

reasons, steam is used almost universally for the sterilization of fermentation media. The major exception is the use of filtration for the sterilization of media for animal-cell culture — such media are completely soluble and contain heat labile components making filtration the method of choice. Filtration techniques will be considered later in this chapter. Before the techniques which are used for the steam sterilization of culture media are discussed it is necessary to discuss the kinetics of sterilization. The destruction of micro-organisms by steam (moist heat) may be described as a first-order chemical reaction and, thus, may be represented by the following equation:

$$-\frac{dN}{dt} = kN \quad (5.1)$$

where N is the number of viable organisms present, t is the time of the sterilization treatment, k is the reaction rate constant of the reaction, or the specific death rate.

It is important at this stage to appreciate that we are considering the total number of organisms present in the volume of medium to be sterilized, *not* the concentration — the minimum number of organisms to contaminate a batch is one, regardless of the volume of the batch. On integration of equation (5.1) the following expression is obtained:

$$\frac{N_t}{N_0} = e^{-kt} \quad (5.2)$$

where N_0 is the number of viable organisms present at the start of the sterilization treatment, N_t is the number of viable organisms present after a treatment period, t .

On taking natural logarithms, equation (5.2) is reduced to:

$$\ln \left(\frac{N_t}{N_0} \right) = -kt \quad (5.3)$$

The graphical representations of equations (5.1) and (5.3) are illustrated in Fig. 5.1, from which it may be seen that viable organism number declines exponentially over the treatment period. A plot of the natural logarithm of N_t/N_0 against time yields a straight line, the slope of which equals $-k$. This kinetic description makes two predictions which appear anomalous:

- An infinite time is required to achieve sterile conditions (i.e. $N_t = 0$).
- After a certain time there will be less than one viable cell present.

Thus, in this context, a value of N_t of less than one is considered in terms of the probability of an organism surviving the treatment. For example, if it were pre-

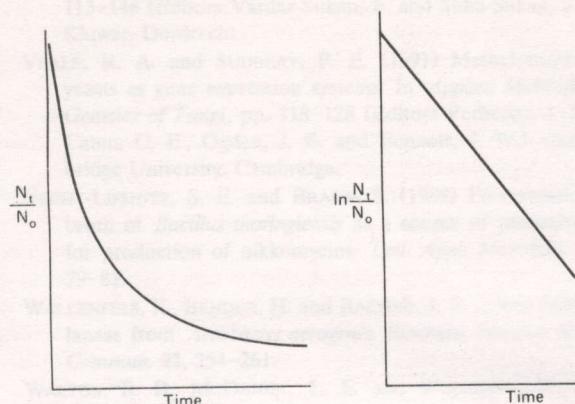


FIG. 5.1. Plots of the proportion of survivors and the natural logarithm of the proportion of survivors in a population of micro-organisms subjected to a lethal temperature over a time period.

dicted that a particular treatment period reduced the population to 0.1 of a viable organism, this implies that the probability of one organism surviving the treatment is one in ten. This may be better expressed in practical terms as a risk of one batch in ten becoming contaminated. This aspect of contamination will be considered later.

The relationship displayed in Fig. 5.1 would be observed only with the sterilization of a pure culture in one physiological form, under ideal sterilization conditions. The value of k is not only species dependent, but dependent on the physiological form of the cell; for example, the endospores of the genus *Bacillus* are far more heat resistant than the vegetative cells. Richards (1968) produced a series of graphs illustrating the deviation from theory which may be experienced in practice. Figures 5.2a, 5.2b and 5.2c illustrate the effect of the time of heat treatment on the survival of a population of bacterial endospores. The deviation from an immediate exponential decline in viable spore number is due to the heat activation of the spores, that is the induction of spore germination by the heat and moisture of the initial period of the sterilization process. In Fig. 5.2a the activation of spores is significantly more than their destruction during the early stages of the process and, therefore, viable numbers increase before the observation of exponential decline. In Fig. 5.2b activation is balanced by spore death and in Fig. 5.2c activation is less than spore death.

Figures 5.3a and 5.3b illustrate typical results of the sterilization of mixed cultures containing two species with different heat sensitivities. In Fig. 5.3a the population consists mainly of the less-resistant type where the

FIG. 5.2a. Activation of spores.

FIG. 5.2b. Activation treatment compensated by

FIG. 5.2c. In a sterilization sated by the

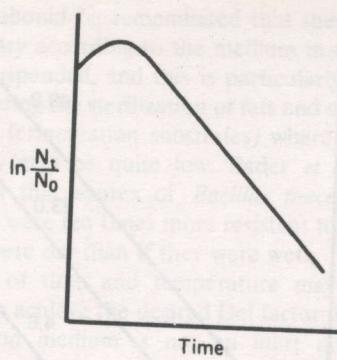


FIG. 5.2a. Initial population increase resulting from the heat activation of spores in the early stages of a sterilization process (Richards, 1968).

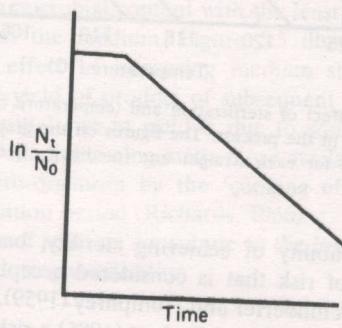


FIG. 5.2b. An initial stationary period observed during a sterilization treatment due to the death of spores being completely compensated by the heat activation of spores (Richards, 1968).

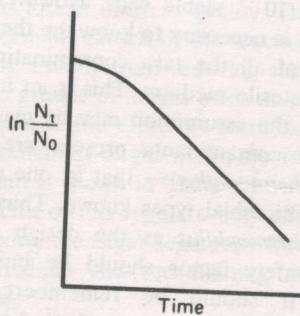


FIG. 5.2c. Initial population decline at a sub-maximum rate during a sterilization treatment due to the death of spores being compensated by the heat activation of spores (Richards, 1968).

initial decline is due principally to the destruction of the less-resistant cell population and the later, less rapid decline, is due principally to the destruction of the more resistant cell population. Figure 5.3b represents the reverse situation where the more resistant type predominates and its presence disguises the de-

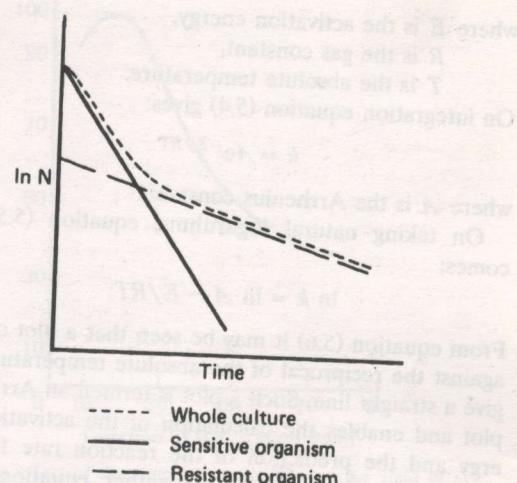


FIG. 5.3a. The effect of a sterilization treatment on a mixed culture consisting of a high proportion of a very sensitive organism (Richards, 1968).

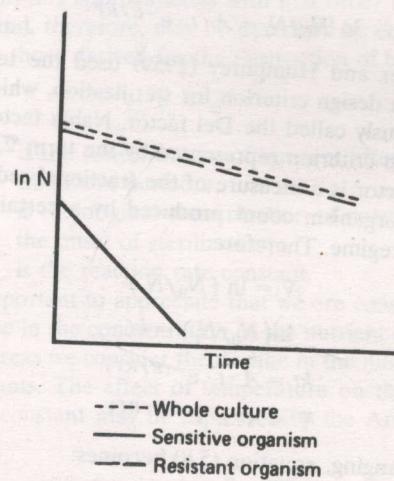


FIG. 5.3b. The effect of a sterilization treatment on a mixed culture consisting of a high proportion of a relatively resistant organism (Richards, 1968).

crease in the number of the less resistant type.

As with any first-order reaction, the reaction rate increases with increase in temperature due to an increase in the reaction rate constant, which, in the case of the destruction of micro-organisms, is the specific death rate (k). Thus, k is a true constant only under constant temperature conditions. The relationship between temperature and the reaction rate constant was demonstrated by Arrhenius and may be represented by the equation:

$$d \ln k / dT = E / RT^2 \quad (5.4)$$

where E is the activation energy,
 R is the gas constant,
 T is the absolute temperature.

On integration equation (5.4) gives:

$$k = Ae^{-E/RT} \quad (5.5)$$

where A is the Arrhenius constant.

On taking natural logarithms, equation (5.5) becomes:

$$\ln k = \ln A - E/RT. \quad (5.6)$$

From equation (5.6) it may be seen that a plot of $\ln k$ against the reciprocal of the absolute temperature will give a straight line. Such a plot is termed an Arrhenius plot and enables the calculation of the activation energy and the prediction of the reaction rate for any temperature. By combining together equations (5.3) and (5.5), the following expression may be derived for the heat sterilization of a pure culture at a constant temperature:

$$\ln N_0/N_t = A \cdot t \cdot e^{-E/RT} \quad (5.7)$$

Deindoerfer and Humphrey (1959) used the term $\ln N_0/N_t$ as a design criterion for sterilization, which has been variously called the Del factor, Nabla factor and sterilization criterion represented by the term ∇ . Thus, the Del factor is a measure of the fractional reduction in viable organism count produced by a certain heat and time regime. Therefore:

$$\nabla = \ln (N_0/N_t)$$

$$\text{but } \ln(N_0/N_t) = kt$$

$$\text{and } kt = A \cdot t \cdot e^{-(E/RT)}$$

$$\text{thus } \nabla = A \cdot t \cdot e^{-(E/RT)}. \quad (5.8)$$

On rearranging, equation (5.8) becomes:

$$\ln t = E/RT + \ln(\nabla/A). \quad (5.9)$$

Thus, a plot of the natural logarithm of the time required to achieve a certain ∇ value against the reciprocal of the absolute temperature will yield a straight line, the slope of which is dependent on the activation energy, as shown in Fig. 5.4. From Fig. 5.4 it is clear that the same degree of sterilization (∇) may be obtained over a wide range of time and temperature regimes; that is, the same degree of sterilization may result from treatment at a high temperature for a short time as from a low temperature for a long time.

This kinetic description of bacterial death enables the design of procedures (giving certain ∇ factors) for the sterilization of fermentation broths. By choosing a value for N_t , procedures may be designed having a

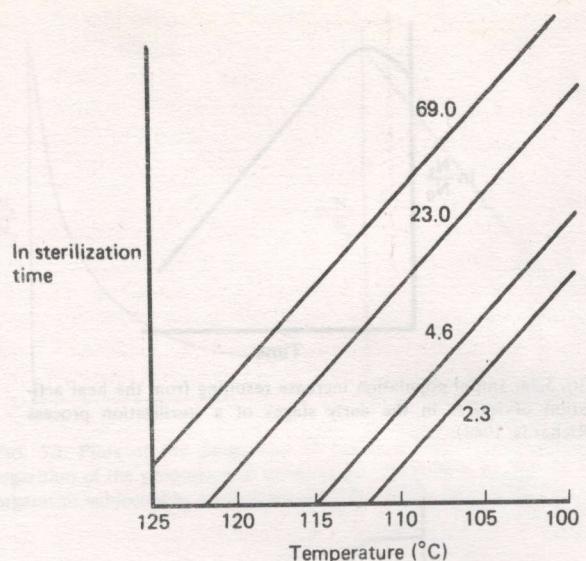


FIG. 5.4. The effect of sterilization and temperature on the Del factor achieved in the process. The figures on the graph indicate the Del factors for each straight line (modified after Richards, 1966).

certain probability of achieving sterility, based upon the degree of risk that is considered acceptable. According to Deindoerfer and Humphrey (1959), Richards (1968), Banks (1979) and Corbett (1985) a risk factor of one batch in a thousand being contaminated is frequently used in the fermentation industry — that is, the final microbial count in the medium after sterilization should be 10^{-3} viable cells. However, to apply these kinetics it is necessary to know the thermal death characteristics of all the taxa contaminating the fermenter and unsterile medium. This is an impossibility and, therefore, the assumption may be made that the only microbial contaminants present are spores of *Bacillus stearothermophilus* — that is, one of the most heat-resistant microbial types known. Thus, by adopting *B. stearothermophilus* as the design organism a considerable safety factor should be built into the calculations. It should be remembered that *B. stearothermophilus* is not always adopted as the design organism. If the most heat-resistant organism contaminating the medium ingredients is known, then it may be advantageous to base the sterilization process on this organism. Deindoerfer and Humphrey (1959) determined the thermal death characteristics of *B. stearothermophilus* spores as:

$$\text{Activation energy} = 67.7 \text{ kcal mole}^{-1}$$

$$\text{Arrhenius constant} = 1 \times 10^{36.2} \text{ second}^{-1}$$

However, it should be remembered that these kinetic values will vary according to the medium in which the spores are suspended, and this is particularly relevant when considering the sterilization of fats and oils (which are common fermentation substrates) where the relative humidity may be quite low. Bader *et al.* (1984) demonstrated that spores of *Bacillus macerans* suspended in oil were ten times more resistant to sterilization if they were dry than if they were wet.

A regime of time and temperature may now be determined to achieve the desired Del factor. However, a fermentation medium is not an inert mixture of components, and deleterious reactions may occur in the medium during the sterilization process, resulting in a loss of nutritive quality. Thus, the choice of regime is dictated by the requirement to achieve the desired reduction in microbial content with the least detrimental effect on the medium. Figure 5.5 illustrates the deleterious effect of increasing medium sterilization time on the yield of product of subsequent fermentations. The initial rise in yield is due to some components of the medium being made more available to the process micro-organism by the 'cooking effect' of a brief sterilization period (Richards, 1966).

Two types of reaction contribute to the loss of nutrient quality during sterilization:

- (i) *Interactions between nutrient components of the medium.* A common occurrence during sterilization is the Maillard-type browning reaction which results in discolouration of the medium as well as loss of nutrient quality. These reactions are normally caused by the reaction of carbonyl groups, usually from reducing sugars, with the amino groups of amino acids and proteins. An example of the effect of sterilization time on the availability of glucose in a corn-steep liquor medium is shown in Table 5.1 (Corbett, 1985). Problems of this type are normally resolved by sterilizing the sugar separately from the rest of the medium and recombining the two after cooling.
- (ii) *Degradation of heat labile components.* Certain vitamins, amino acids and proteins may be degraded during a steam sterilization regime. In extreme cases, such as the preparation of media for animal-cell culture, filtration may be used and this aspect will be discussed later in the chapter. However, for the vast majority of fermentations these problems may be resolved by the judicious choice of steam sterilization regime.

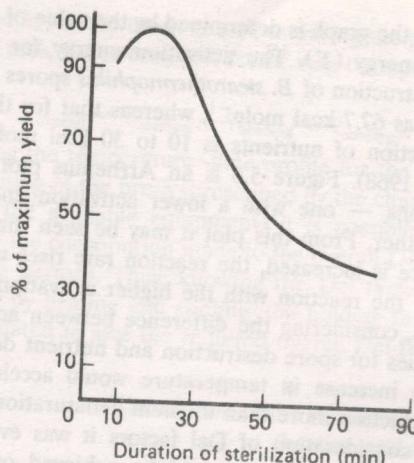


FIG. 5.5. The effect of the time of sterilization on the yield of a subsequent fermentation (Richards, 1966).

The thermal destruction of essential media components conforms approximately with first order reaction kinetics and, therefore, may be described by equations similar to those derived for the destruction of bacteria:

$$x_t/x_0 = e^{-kt} \quad (5.10)$$

where x_t is the concentration of nutrient after a heat treatment period, t ,
 x_0 is the original concentration of nutrient at the onset of sterilization,
 k is the reaction rate constant.

It is important to appreciate that we are considering the decline in the concentration of the nutrient component, whereas we consider the decline in the number of contaminants. The effect of temperature on the reaction rate constant may be expressed by the Arrhenius equation:

$$\ln k = \ln A - E/RT.$$

Therefore, a plot of the natural logarithm of the reaction rate against $1/T$ will give a straight line, slope $-(E/R)$. As the value of R , the gas constant, is fixed

TABLE 5.1. The effect of sterilization time on glucose concentration and product accretion rate in an antibiotic fermentation (Corbett, 1985)

Time at 121° (min)	Amount of added glucose remaining (%)	Relative accretion rate
60	35	90
40	46	92
30	64	100

the slope of the graph is determined by the value of the activation energy (E). The activation energy for the thermal destruction of *B. stearothermophilus* spores has been cited as 67.7 kcal mole⁻¹, whereas that for thermal destruction of nutrients is 10 to 30 kcal mole⁻¹ (Richards, 1968). Figure 5.6 is an Arrhenius plot for two reactions — one with a lower activation energy than the other. From this plot it may be seen that as temperature is increased, the reaction rate rises more rapidly for the reaction with the higher activation energy. Thus, considering the difference between activation energies for spore destruction and nutrient degradation, an increase in temperature would accelerate spore destruction more than medium denaturation.

In the consideration of Del factors it was evident that the same Del factor could be achieved over a range of temperature/time regimes. Thus, it would appear to be advantageous to employ a high temperature for a short time to achieve the desired probability of sterility, yet causing minimum nutrient degradation. Thus, the ideal technique would be to heat the fermentation medium to a high temperature, at which it is held for a short period, before being cooled rapidly to the fermentation temperature. However, it is obviously impossible to heat a batch of many thousands of litres of broth in a tank to a high temperature, hold for a short period and cool without the heating and cooling periods contributing considerably to the total sterilization time. The only practical method of materializing the objective of a short-time, high-temperature treatment is to sterilize the medium in a continuous stream. In the past the fermentation industry was reluctant to adopt continuous sterilization due to a number of disadvantages outweighing the advantage of nutrient

quality. The relative merits of batch and continuous sterilization may be summarized as follows:

Advantages of continuous sterilization over batch sterilization

- (i) Superior maintenance of medium quality.
- (ii) Ease of scale-up — discussed later.
- (iii) Easier automatic control.
- (iv) The reduction of surge capacity for steam.
- (v) The reduction of sterilization cycle time.
- (vi) Under certain circumstances, the reduction of fermenter corrosion.

Advantages of batch sterilization over continuous sterilization

- (i) Lower capital equipment costs.
- (ii) Lower risk of contamination — continuous processes require the aseptic transfer of the sterile broth to the sterile vessel.
- (iii) Easier manual control.
- (iv) Easier to use with media containing a high proportion of solid matter.

The early continuous sterilizers were constructed as plate heat exchangers and these were unsuitable on two accounts:

- (i) Failure of the gaskets between the plates resulted in the mixing of sterile and unsterile streams.
- (ii) Particulate components in the media would block the heat exchangers.

However, modern continuous sterilizers use double spiral heat exchangers in which the two streams are separated by a continuous steel division. Also, the spiral exchangers are far less susceptible to blockage. However, a major limitation to the adoption of continuous sterilization was the precision of control necessary for its success. This precision has been achieved with the development of sophisticated computerized monitoring and control systems resulting in continuous sterilization being very widely used and it is now the method of choice. However, batch sterilization is still used in many fermentation plants and, thus, it will be considered here before continuous sterilization is discussed in detail.

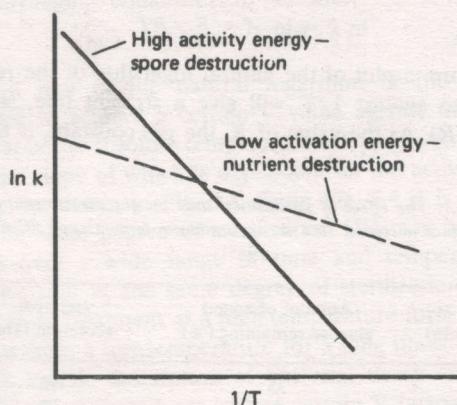


FIG. 5.6. The effect of activation energy on spore and nutrient destruction.

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THE DESIGN OF BATCH STERILIZATION PROCESSES

Although a batch sterilization process is less successful in avoiding the destruction of nutrients than a continuous one, the objective in designing a batch process is still to achieve the required probability of obtaining sterility with the minimum loss of nutritive quality. The highest temperature which appears to be feasible for batch sterilization is 121°C so the procedure should be designed such that exposure of the medium to this temperature is kept to a minimum. This is achieved by taking into account the contribution made to the sterilization by the heating and cooling periods of the batch treatment. Deindoefer and Humphrey (1959) presented a method to assess the contribution made by the heating and cooling periods. The following information must be available for the design of a batch sterilization process:

- (i) A profile of the increase and decrease in the temperature of the fermentation medium during the heating and cooling periods of the sterilization cycle.
- (ii) The number of micro-organisms originally present in the medium.
- (iii) The thermal death characteristics of the 'design' organism. As explained earlier this may be *Bacillus stearothermophilus* or an alternative organism relevant to the particular fermentation.

Knowing the original number of organisms present in the fermenter and the risk of contamination considered acceptable, the required Del factor may be calculated. A frequently adopted risk of contamination is 1 in 1000, which indicates that N_t should equal 10^{-3} of a viable cell. It is worth reinforcing at this stage that we are considering the total number of organisms present in the medium and *not* the concentration. If a specific case is considered where the unsterile broth was shown to contain 10^{11} viable organisms, then the Del factor may be calculated, thus:

$$\bar{Y} = \ln (10^{11}/10^{-3})$$

$$\begin{aligned} \bar{V} &= \ln 10^{14} \\ &= 32.2. \end{aligned}$$

Therefore, the overall Del factor required is 32.2. However, the destruction of cells occurs during the heating

and cooling of the broth as well as during the period at 121°C, thus, the overall Del factor may be represented as:

$$\bar{V}_{\text{overall}} = \bar{V}_{\text{heating}} + \bar{V}_{\text{holding}} + \bar{V}_{\text{cooling}}.$$

Knowing the temperature-time profile for the heating and cooling of the broth (prescribed by the characteristics of the available equipment) it is possible to determine the contribution made to the overall Del factor by these periods. Thus, knowing the Del factors contributed by heating and cooling, the holding time may be calculated to give the required overall Del factor.

Calculation of the Del factor during heating and cooling

The relationship between Del factor, the temperature and time is given by equation (5.8):

$$\bar{V} = A \cdot t \cdot e^{-(E/RT)}$$

However, during the heating and cooling periods the temperature is not constant and, therefore, the calculation of \bar{V} would require the integration of equation (5.8) for the time-temperature regime observed. Deindoefer and Humphrey (1959) produced integrated forms of the equation for a variety of temperature-time profiles, including linear, exponential and hyperbolic. However, the regime observed in practice is frequently difficult to classify, making the application of these complex equations problematical. Richards (1968) demonstrated the use of a graphical method of integration and this is illustrated in Fig. 5.7. The time axis is divided into a number of equal increments, t_1, t_2, t_3, \dots , etc., Richards suggesting 30 as a reasonable number.

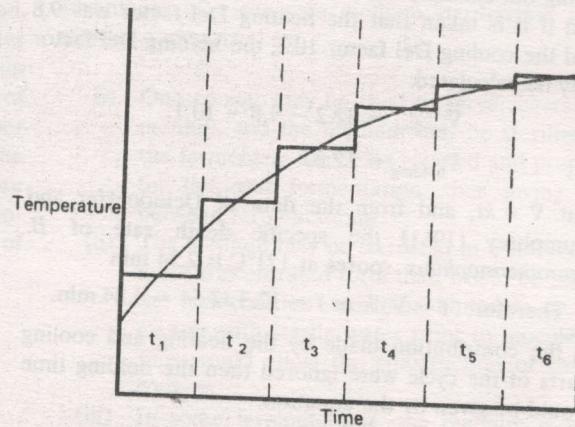


FIG. 5.7. The graphical integration method applied to the increase in temperature over a time period. t_1, t_2, \dots represent equal time intervals (Richards, 1968).

For each increment, the temperature corresponding to the mid-point time is recorded. It may now be approximated that the total Del factor of the heating-up period is equivalent to the sum of the Del factors of the mid-point temperatures for each time increment. The value of the specific death rate of *B. stearothermophilus* spores at each mid-point temperature may be deduced from the Arrhenius equation using the thermal death characteristic published by Deindoerfer and Humphrey (1959). The value of the Del factor corresponding to each time increment may then be calculated from the equations:

$$\nabla_1 = k_1 t,$$

$$\nabla_2 = k_2 t,$$

$$\nabla_3 = k_3 t,$$

etc.

The sum of the Del factors for all the increments will then equal the Del factor for the heating-up period. The Del factor for the cooling-down period may be calculated in a similar fashion.

Calculation of the holding time at constant temperature

From the previous calculations the overall Del factor, as well as the Del factors of the heating and cooling parts of the cycle, have been determined. Therefore, the Del factor to be achieved during the holding time may be calculated by difference:

$$\nabla_{\text{holding}} = \nabla_{\text{overall}} - \nabla_{\text{heating}} - \nabla_{\text{cooling}}$$

Using our example where the overall Del factor is 32.2 and if it is taken that the heating Del factor was 9.8 and the cooling Del factor 10.1, the holding Del factor may be calculated:

$$\nabla_{\text{holding}} = 32.2 - 9.8 - 10.1,$$

$$\nabla_{\text{holding}} = 12.3.$$

But $\nabla = kt$, and from the data of Deindoerfer and Humphrey (1961) the specific death rate of *B. stearothermophilus* spores at 121°C is 2.54 min⁻¹

Therefore, $t = \nabla/k$ or $t = 12.3/2.54 = 4.84$ min.

If the contribution made by the heating and cooling parts of the cycle were ignored then the holding time would be given by the equation:

$$t = \nabla_{\text{overall}}/k = 32.2/2.54 = 12.68 \text{ min.}$$

Thus, by considering the contribution made to the sterilization process by the heating and cooling parts of

the cycle a considerable reduction in exposure time is achieved

Richards' rapid method for the design of sterilization cycles

Richards (1968) proposed a rapid method for the design of sterilization cycles avoiding the time-consuming graphical integrations. The method assumes that all spore destruction occurs at temperatures above 100°C and that those parts of the heating and cooling cycle above 100° are linear. Both these assumptions appear reasonably valid and the technique loses very little in accuracy and gains considerably in simplicity. Furthermore, based on these assumptions, Richards has presented a table of Del factors for *B. stearothermophilus* spores which would be obtained in heating and cooling a broth up to (and down from) holding temperatures of 101–130°C, based on a temperature change of 1°C per minute. This information is presented in Table 5.2, together with the specific death rates for *B. stearothermophilus* spores over the temperature range. If the rate of temperature change is 1° per minute, the Del factors for heating and cooling may be read directly from the table; if the temperature change deviates from 1° per minute, the Del factors may be altered by simple proportion. For example, if a fermentation broth were heated from 100° to 121°C in 30 minutes and cooled from 121° to 100° in 17 minutes, the Del factors for the heating and cooling cycles may be determined as follows:

From Table 5.2, if the change in temperature had been 1° per minute, the Del factor for both the heating and cooling cycles would be 12.549. But the temperature change in the heating cycle was 21° in 30 minutes; therefore,

$$\text{Del}_{\text{heating}} = (12.549 \times 30)/21 = 17.93$$

and the temperature change in the cooling cycle was 21° in 17 minutes, therefore,

$$\text{Del}_{\text{cooling}} = (12.549 \times 17)/21 = 10.16.$$

Having calculated the Del factors for the heating and cooling periods the holding time at the constant temperature may be calculated as before.

The scale up of batch sterilization processes

The use of the Del factor in the scale up of batch sterilization processes has been discussed by Banks

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