Sterilization

Why we need sterilization

1. The medium would have to support the growth of both the production organism and the contaminant, resulting in a loss of productivity.

2. If the fermentation is a continuous one then the contaminant may 'outgrow' the production organism and displace it from the fermentation.

3. The foreign organism may contaminate the final product, e.g. single-cell protein where the cells, separated from the broth, constitute the product.

4. The contaminant may produce compounds which make subsequent extraction of the final product difficult.

5. 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 *b*-lactam antibiotics by b-lactamase-producing bacteria.

6. Contamination of a bacterial fermentation with phage could result in the lysis of the culture.

How to avoid contamination

(i) Using a pure inoculum to start the fermentation,

(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.

Medium sterilization

- Filtration, radiation, ultrasonic treatment, chemical treatment or heat.
- 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.

1. $\int a\,dx = ax + C$

2. $\int (u+v)\,dx = \int u\,dx + \int v\,dx$

3. $\int a\,f(x)\,dx = a\int f(x)\,dx$

4. $\int u^m\,du = \frac{u^{m+1}}{m+1} + C, \quad m \neq -1$

5. $\int \frac{du}{u} = \ln|u| + C$

6. $\int a^u\,du = \frac{a^u}{\ln a} + C, \quad a > 0,\ a \neq 1$

7. $\int e^u\,du = e^u + C$

8. $\int \sin u\,du = -\cos u + C$

DIFFERENTIALS

$$d\,ax = a\,dx$$

$$d(u + v) = du + dv$$

$$d\,uv = u\,dv + v\,du$$

$$d\frac{u}{v} = \frac{v\,du - u\,dv}{v^2}$$

$$d\,x^n = n\,x^{n-1}dx$$

$$dx^y = yx^{y-1}dx + x^y \log_e x\,dy$$

$$d\,e^x = e^x\,dx$$

$$d\,e^{ax} = a\,e^{ax}dx$$

$$d\,a^x = a^x \log_e a\,dx$$

$$d \log_e x = x^{-1}dx$$

$$d \log_a x = x^{-1} \log_a e\,dx$$

$$d\,x^x = x^x\,(1 + \log_e x)\,dx$$

Evaluating Log Expressions: General Rules

$\log_2 8$ *"2 raised to what power equals 8?"*

1) Set the log expression equal to x

$$\log_2 8 = x$$

2) Convert log to exponential form

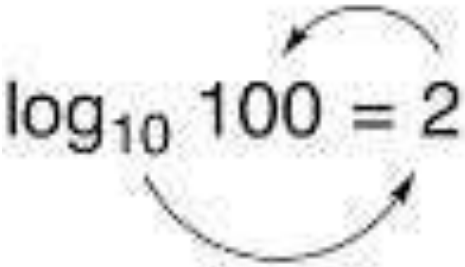
$$\log_2 8 = x \rightarrow 2^x = 8$$

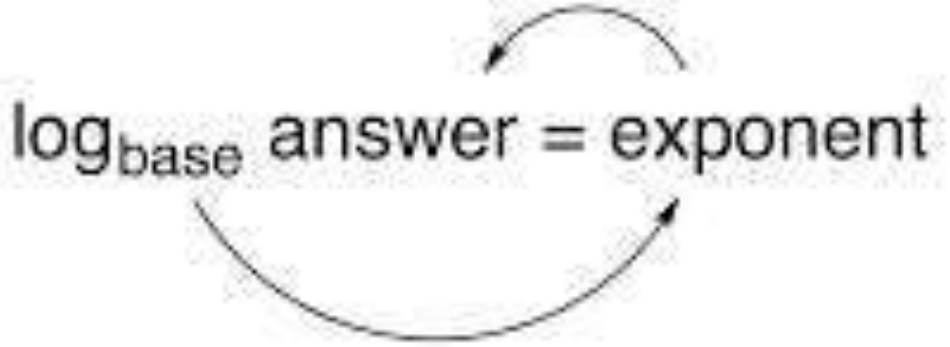
3) Solve the resulting exponential equation for x.

$$2^x = 8$$

$$2^x = 2^3$$

$$x = 3 \rightarrow \log_2 8 = 3$$





10^2 = 100

base^exponent = answer

Logs are circular!

$$\log_a(pq) = \log_a p + \log_a q$$

$$\log_a(\frac{p}{q}) = \log_a p - \log_a q$$

$$\log_a(p^n) = n \log_a p$$

$$\log_a a^x = a^{\log_a x} = x$$

Exponential Laws	Logarithm Laws
$x^a \cdot x^b = x^{a+b}$	$\log(ab) = \log(a) + \log(b)$
$\frac{x^a}{x^b} = x^{a-b}$	$\log\left(\frac{a}{b}\right) = \log(a) - \log(b)$
$(x^a)^b = x^{ab}$	$\log(a^b) = b \cdot \log(a)$
$x^{-a} = \frac{1}{x^a}$	$\log_x\left(\frac{1}{x^a}\right) = -a$
$x^0 = 1$	$\log_x 1 = 0$

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:

$$-\mathrm{d}N/\mathrm{d}t = kN \qquad (5.1)$$

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:

$$N_t/N_0 = \mathrm{e}^{-kt} \qquad (5.2)$$

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:

$$\ln\,(N_t/N_0) = -kt \qquad (5.3)$$

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/No* against time yields a straight line, the slope of which equals - *k.* This kinetic description makes two predictions which appear anomalous:

(i) An infinite time is required to achieve sterile conditions (i.e. N,. = 0).

(ij) After a certain time there will be less than one viable cell present.

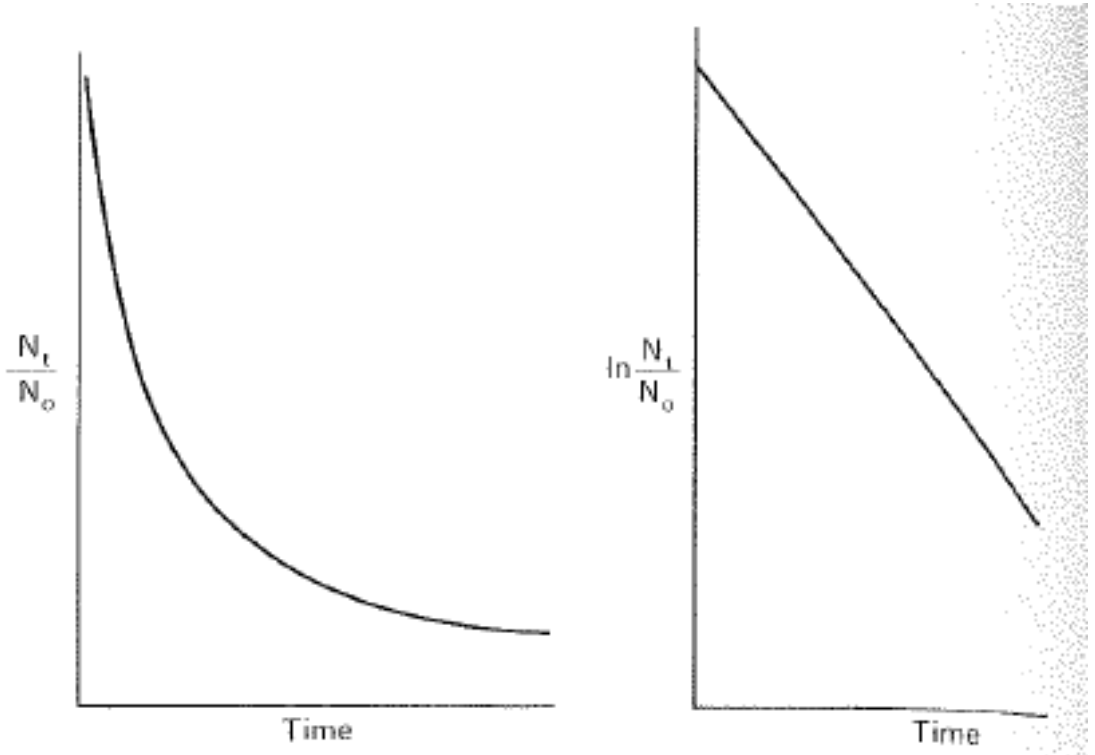


FIG. 5.1. Plots of the proportion of survivors and the natural logarithm of the proportion of survivors in a population of micro-organisms subjected to a lethal temperature over a time period.

- Thus, in this context, a value of *Nt* of less than one is considered in terms of the probability of an organism surviving the treatment.

- For example, if it were predicted 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.

- 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.

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
- Fig. 5.2c activation is less than spore death.

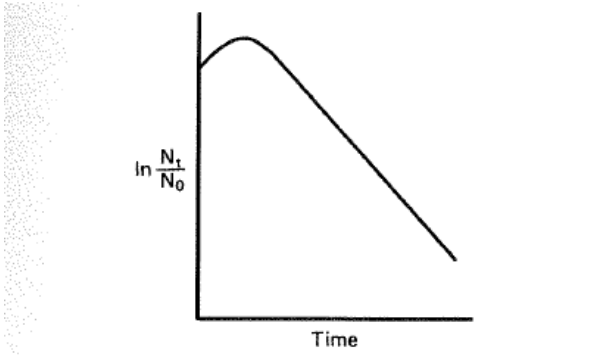


FIG. 5.2a. Initial population increase resulting from the heat activation of spores in the early stages of a sterilization process (Richards, 1968).

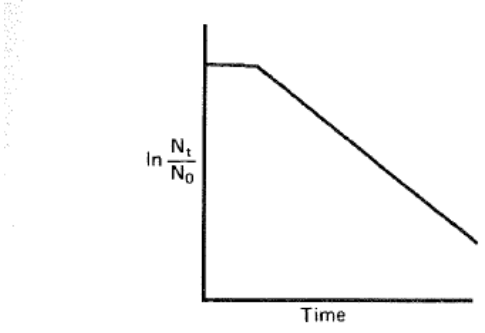


FIG. 5.2b. An initial stationary period observed during a sterilization treatment due to the death of spores being completly compensated by the heat activation of spores (Richards, 1968).

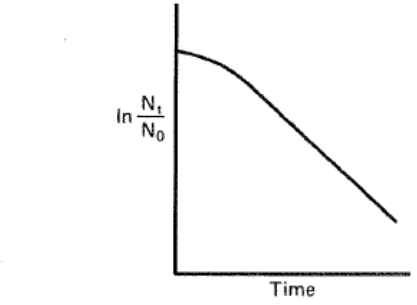


FIG. 5.2c. Initial population decline at a sub-maximum rate during a sterilization treatment due to the death of spores being compensated by the heat activation of spores (Richards, 1968).

- 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 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 decrease in the number of the less resistant type.

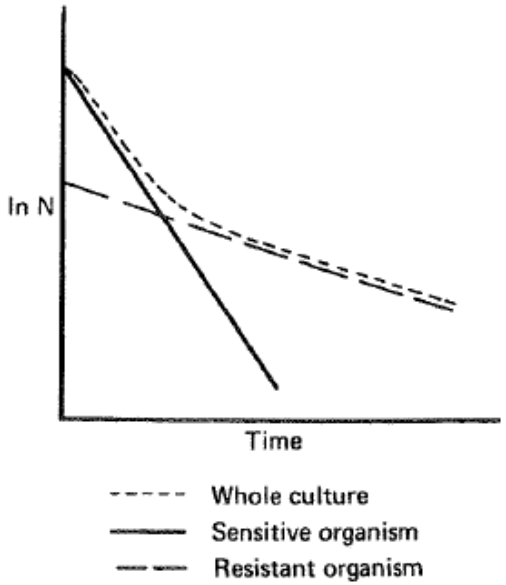


FIG. 5.3a. The effect of a sterilization treatment on a mixed culture consisting of a high proportion of a very sensitive organism (Richards, 1968).

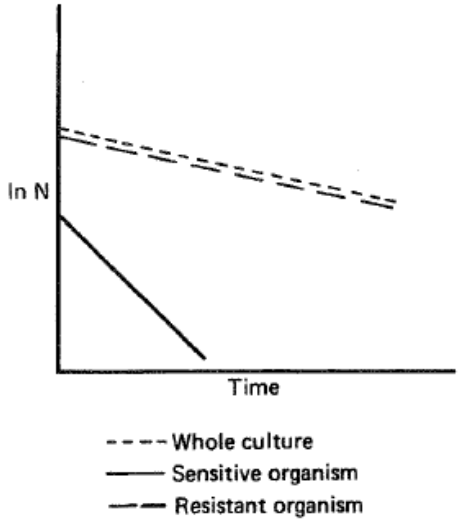


FIG. 5.3b. The effect of a sterilization treatment on a mixed culture consisting of a high proportion of a relatively resistant organism (Richards, 1968).

Arrhenius Equation
(continued)

$$k = Ae^{-Ea/RT}$$

$$\ln k = \frac{-Ea}{R}\frac{1}{T} + \ln A$$

- k = rate constant
- A = frequency factor (a measure of the probability of a favorable collision)
- E_a = activation energy
- T = temperature
- R = gas constant (8.31 J/molK)

Both *A* and *Ea* are specific to a given reaction

Temperature Effects

The Arrhenius Equation

$$k = Ae^{-Ea/RT}$$

where k is the rate constant at T

E_a is the activation energy

R is the energy gas constant

= 8.3145 J/(mol K)

T is the Kelvin temperature

A is the collision frequency factor

$$\ln k = \ln A - E_a/RT$$

$$\ln \frac{k_2}{k_1} = -\frac{E_a}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right)$$

Temperature: The Arrhenius Equation

- Svante Arrhenius developed this relationship among (1) the temperature (T), (2) the activation energy (E_a), and (3) the specific rate constant (k).

$$\ln k_1 = \ln A - \frac{E_a}{RT_1} \qquad \ln k_2 - \ln k_1 = \ln A - \ln A - \frac{E_a}{RT_2} - \left(-\frac{E_a}{RT_1}\right)$$

and

$$\ln k_2 = \ln A - \frac{E_a}{RT_2} \qquad \ln k_2 - \ln k_1 = \frac{E_a}{RT_1} - \frac{E_a}{RT_2}$$

- If the Arrhenius equation is written for two temperatures, T_2 and T_1 with $T_2 > T_1$.

$$\ln \frac{k_2}{k_1} = \frac{E_a}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

or

$$\ln \frac{k_2}{k_1} = \frac{E_a}{R}\left(\frac{T_2 - T_1}{T_2 T_1}\right)$$

- 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:

$$\mathrm{d} \ln k / \mathrm{d}T = E / RT^2 \qquad (5.4)$$

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

On integration equation (5.4) gives:

$$k = A\mathrm{e}^{-E/RT} \qquad (5.5)$$

where A is the Arrhenius constant.

On taking natural logarithms, equation (5.5) becomes

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

- From equation (5.6) it may be seen that a plot of In *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:

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

Deindoerfer and Humphrey (1959) used the term In ***No/*** $\mathbf{N_t}$ 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:

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

but $$\ln(N_0/N_t) = kt$$

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

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

On rearranging, equation (5.8) becomes:

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

- Thus, a plot of the natural logarithm of the time required to achieve a certain V' 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 (V') 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

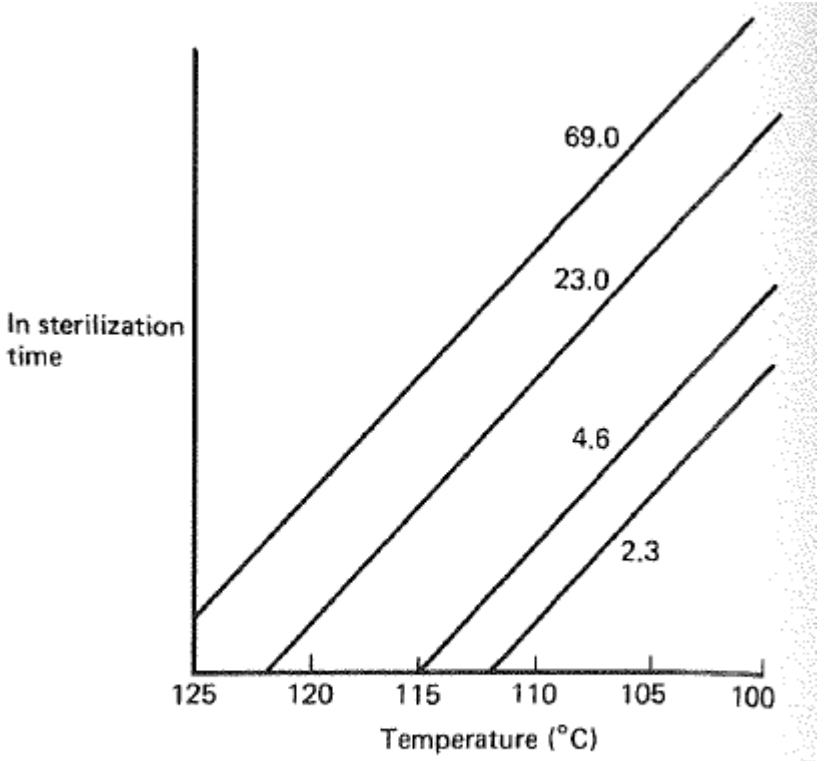


FIG. 5.4. The effect of sterilization and temperature on the Del factor achieved in the process. The figures on the graph indicate the Del factors for each straight line (modified after Richards, 1966).

- This kinetic description of bacterial death enables the design of procedures (giving certain V' factors) for the sterilization of fermentation broths.

- By choosing a value for ~, procedures may be designed having a 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 stearothelmophilus* - that is, one of the most heat-resistant microbial types known.

- Thus, by adopting *B. stearothennophilus* as the design organism a considerable safety factor should be built into the calculations.

- It should be remembered that *B. stearothennophilus* 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).

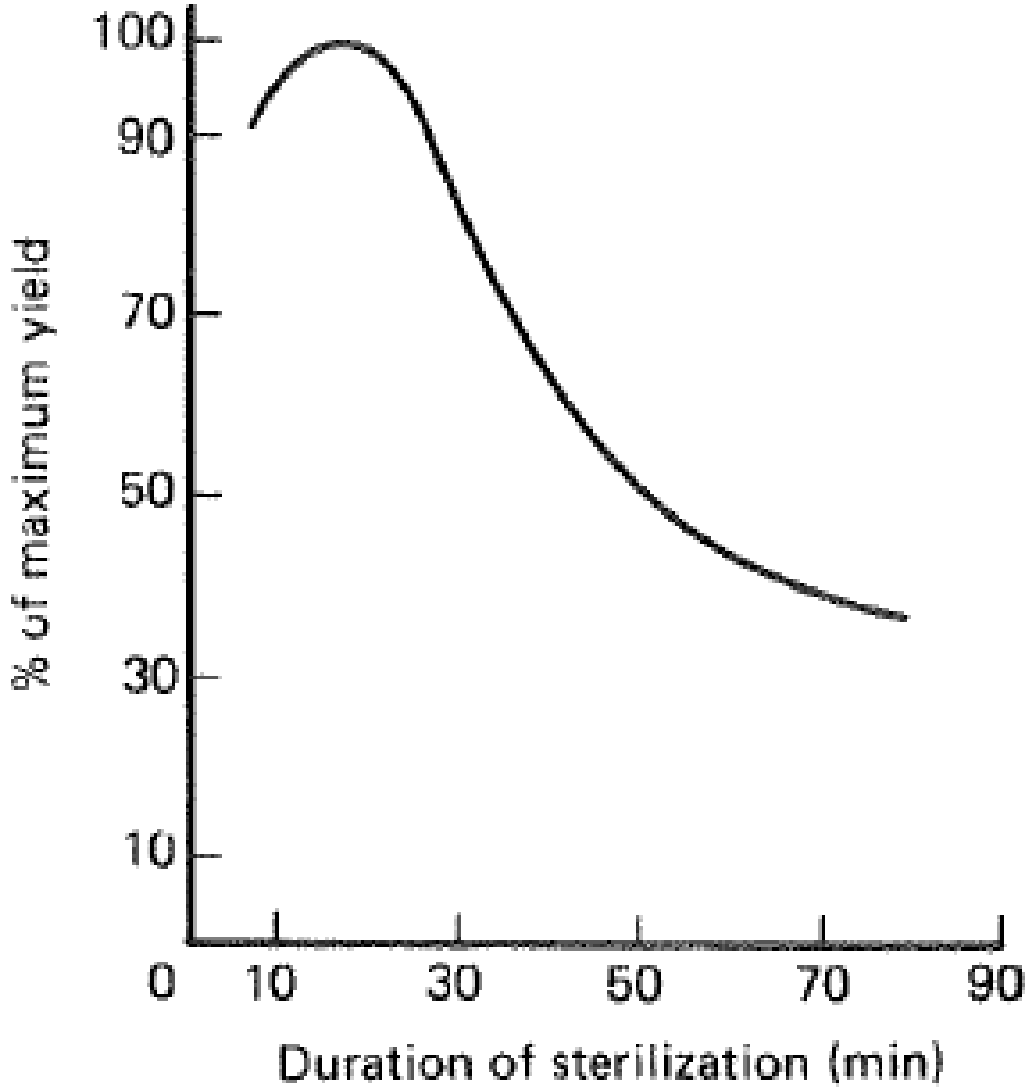


FIG. 5.5. The effect of the time of sterilization on the yield of a subsequent fermentation (Richards, 1966).

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

1. ***Interactions between nutrient components of the medium.*** A common occurrence during sterilization is the Maillard-type browning reaction which results in discoloration 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.

2. ***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.

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:

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

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 the slope of the graph is determined by the value of the activation energy *(E).*

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 activation energy for the thermal destruction of *B. stearothermophilus* spores has been cited as 67.7 kcal mole - \ 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.

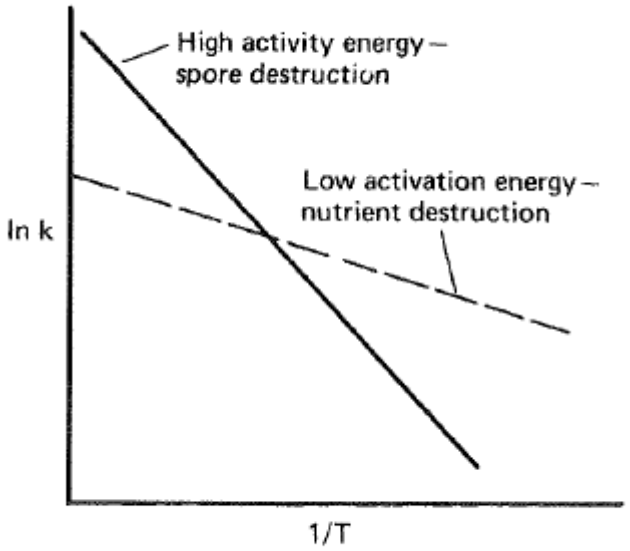


FIG. 5.6. The effect of activation energy on spore and nutrient destruction.

- 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 follow

Advantages of continuous sterilization over batch sterilization

- Superior maintenance of medium quality.
- Ease of scale-up
- Easier automatic control.
- The reduction of surge capacity for steam.
- The reduction of sterilization cycle time.
- Under certain circumstances, the reduction of fermenter corrosion.

Advantages of batch sterilization over continuous sterilization

- Lower capital equipment costs.
- Lower risk of contamination over continuous process processes require the aseptic transfer of the sterile broth to the sterile vessel.
- Easier manual control.
- 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.

The design of batch sterilization Processes

- The highest temperature which appears to be feasible for batch sterilization is 121°C so the process 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. Deindoerfer and Humphrey (1959) presented a method to assess the contribution made by the heating and cooling periods.

(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 ~ 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:

$$\nabla = \ln \left(10^{11}/10^{-3}\right)$$

$$\nabla = \ln 10^{14}$$

$$= 32.2.$$

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:

$$\nabla_{\text{overall}} = \nabla_{\text{heating}} + \nabla_{\text{holding}} + \nabla_{\text{cooling}}.$$

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):

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

- However, during the heating and cooling periods the temperature is not constant and, therefore, the calculation of V would require the integration of equation (5.8) for the time-temperature regime observed.

- Deindoerfer 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 l* , *t2* , *t3*, etc., Richards suggesting 30 as a reasonable number.

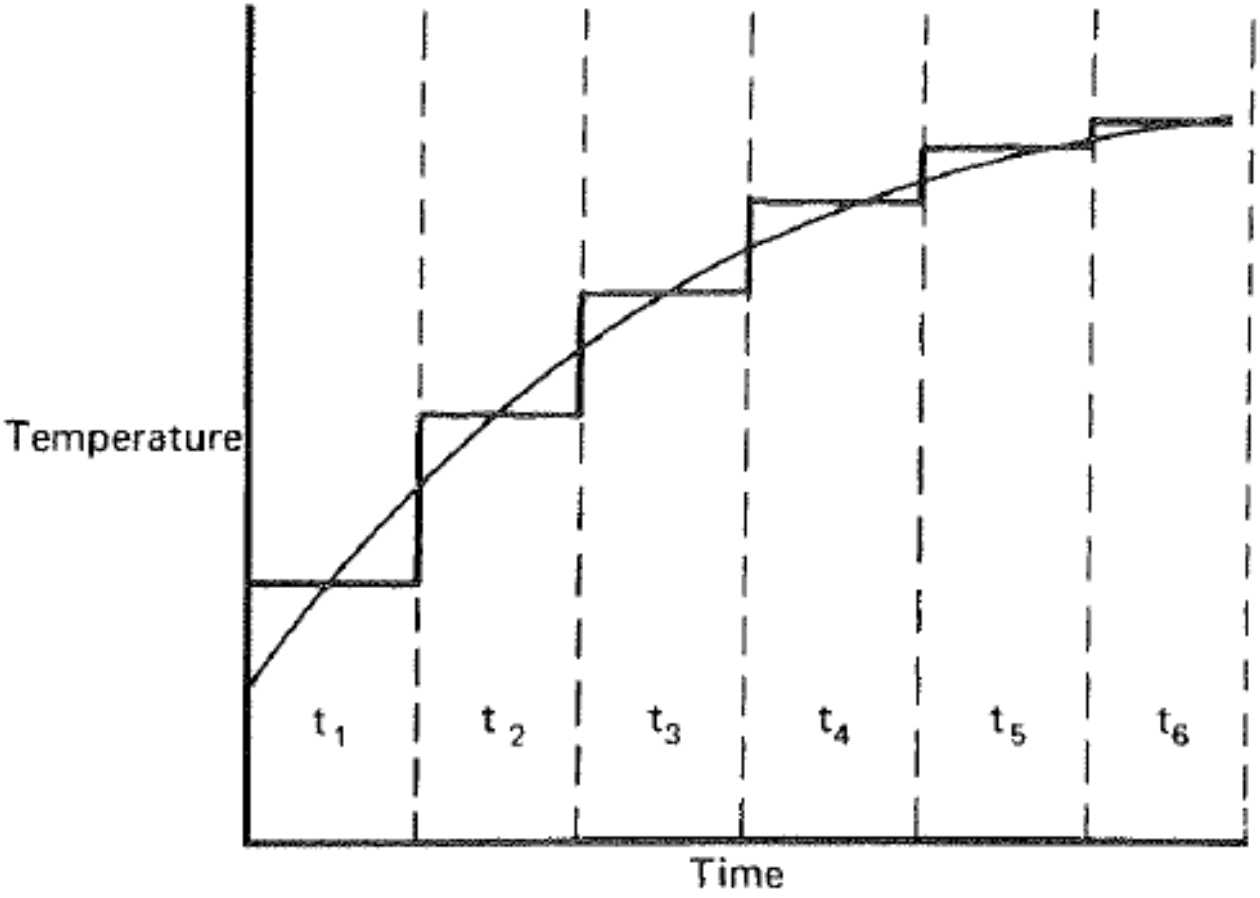


FIG. 5.7. The graphical integration method applied to the increase in temperature over a time period. t_1, t_2, etc. represent equal time intervals (Richards, 1968).

- 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:

$$\nabla_1 = k_1 t,$$
$$\nabla_2 = k_2 t,$$
$$\nabla_3 = k_3 t,$$
$$\text{etc.}$$

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 cycle

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:

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

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.

TABLE 5.2. *Del values for* B. stearothermophilus *spores for the heating-up period over a temperature range of 100 to 130°, assuming a rate of temperature change of 1° min^{-1} and negligible spore destruction at temperatures below 100°* (Richards, 1968)

T (°C)	k (min^{-1})	▽
100	0.019	—
101	0.025	0.044
102	0.032	0.076
103	0.040	0.116
104	0.051	0.168
105	0.065	0.233
106	0.083	0.316
107	0.105	0.420
108	0.133	0.553
109	0.168	0.720
110	0.212	0.932
111	0.267	1.199
112	0.336	1.535
113	0.423	1.957
114	0.531	2.488
115	0.666	3.154
116	0.835	3.989
117	1.045	5.034
118	1.307	6.341
119	1.633	7.973
120	2.037	10.010
121	2.538	12.549
122	3.160	15.708
123	3.929	19.638
124	4.881	24.518
125	6.056	30.574
126	7.506	38.080
127	9.293	47.373
128	11.494	58.867
129	14.200	73.067
130	17.524	90.591

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(1979).

- It should be appreciated by this stage that the Del factor does not include a volume term, i.e. absolute numbers of contaminants and survivors are considered, *not* their concentration.

- Thus, if the size of a fermenter is increased the initial number of spores in the medium will also be increased, but if the same probability of achieving sterility is required the final spore number should remain the same, resulting in an increase in the Del factor.

- For example, if a pilot sterilization were carried out in a 1000-dm^3 vessel with a medium containing 10^6 organisms cm^{-3} requiring a probability of contamination of 1 in 1000, the Del factor would be:

$$\nabla = \ln \{(10^6 \times 10^3 \times 10^3)/10^{-3}\}$$
$$= \ln (10^{12}/10^{-3})$$
$$= \ln 10^{15} = 34.5.$$

If the same probability of contamination were required in a 10,000-dm3 vessel using the same medium the Del factor would be:

$$\begin{aligned} \nabla &= \ln \{(10^6 \times 10^3 \times 10^4)/10^{-3}\} \\ &= \ln (10^{13}/10^{-3}) \\ &= \ln 10^{16} = 36.8. \end{aligned}$$

- Thus, the Del factor increases with an increase in the size of the fermenter volume. The holding time in the large vessel may be calculated by the graphical integration method or by the rapid method of Richards (1968), as discussed earlier, based on the temperature-time profile of the sterilization cycle in the large vessel.

- However, it must be appreciated that extending the holding time on the larger scale (to achieve the increased V factor) will result in increased nutrient degradation. Also, the contribution of the heating-up and cooling-down periods to nutrient destruction will be greater as scale increases. Maintaining the same nutrient quality on a small and a large scale can be achieved in batch sterilization only by compromising the sterility of the vessel, which is totally unacceptable.

- **Thus, the decrease in the yield of a fermentation when it is scaled up is often due to problems of nutrient degradation during batch sterilization and the only way to eradicate the problem is to sterilize the medium continuously.**

Methods of batch sterilization

The batch sterilization of the medium for a fermentation may be achieved either in the fermentation vessel or in a separate mash cooker. Richards (1966) considered the relative merits of *in situ* medium sterilization and the use of a special vessel. The major advantages of a separate medium sterilization vessel may be summarized as:

(i) One cooker may be used to serve several fermenters and the medium may be sterilized as the fermenters are being cleaned and prepared for the next fermentation, thus saving time between fermentations.

(ii) The medium may be sterilized in a cooker in a more concentrated form than would be used in the fermentation and then diluted in the fermenter with sterile water prior to inoculation. This would allow the construction of smaller cookers.

(iii) In some fermentations, the medium is at its most viscous during sterilization and the power requirement for agitation is not alleviated by aeration as it would be during the fermentation proper. Thus, if the requirement for agitation during *in situ* sterilization were removed, the fermenter could be equipped with a less powerful motor. Obviously, the sterilization kettle would have to be equipped with a powerful motor, but this would provide sterile medium for several fermenters.

(iv) The fermenter would be spared the corrosion which may occur with medium at high temperature.

The major disadvantages of a separate medium sterilization vessel

(i) The cost of constructing a batch medium sterilizer is much the same as that for the fermenter.

(ii) If a cooker serves a large number of fermenters complex pipework would be necessary to transport the sterile medium, with the inherent dangers of contamination.

(iii) Mechanical failure in a cooker supplying medium to several fermenters would render all the fermenters temporarily redundant. The provision of contingency equipment may be prohibitively costly.

The design of continuous sterilization processes

The Del factor for the example sterilization was 45.7 and the following temperature time regimes were calculated to give the same Del factor:

Temperature	Holding time
130°	2.44 minutes
135°	51.9 seconds
140°	18.9 seconds
150°	2.7 seconds

- The most suitable indirect heat exchangers are of the double-spiral type which consists of two sheets of high-grade stainless steel which have been curved around a central axis to form a double spiral, as shown in Fig. 5.8.

- The ends of the spiral are sealed by covers. A full scale example is shown in Fig. 5.9. To achieve sterilization temperatures steam is passed through one spiral and medium through the other in countercurrent streams. Spiral heat exchangers are also used to cool the medium after passing through the holding coil.

- Incoming unsterile medium is used as the cooling agent in the first cooler so that the incoming medium is partially heated before it reaches the sterilizer and, thus, heat is conserved.

The major advantages of the spiral heat exchanger

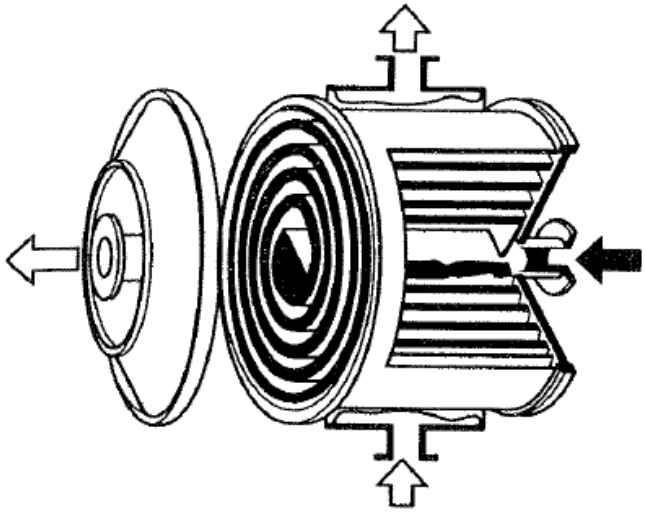


FIG. 5.8. A schematic representation of a spiral heat exchanger (Alfa-Laval Engineering Ltd, Brentford, Middlesex).

(i) The two streams of medium and cooling liquid, or medium and steam, are separated by a continuous stainless steel barrier with gasket seals being confined to the joints with the end plates. This makes cross contamination between the two streams unlikely.

(ii) The spiral route traversed by the medium allows sufficient clearances to be incorporated for the system to cope with suspended solids. The exchanger tends to be self-cleaning which reduces the risk of sedimentation, fouling and 'burning-on'.

The continuous steam injector injects steam directly into the unsterile broth. The advantages and disadvantages of the system have been summarized by Banks (1979):

1. **Very short (almost instantaneous) heating up times.**

2. **It may be used for media containing suspended solids.**

3. **Low capital cost.**

4. **Easy cleaning and maintenance.**

5. **High steam utilization efficiency.**

However, the disadvantages are:

(i) Foaming may occur during heating.

(ii) Direct contact of the medium with steam requires that allowance be made for condense dilution and requires 'clean' steam, free from anticorrosion additives.

In some cases the injection system is combined with flash cooling, where the sterilized medium is cooled by passing it through an expansion valve into a vacuum chamber.

Cooling then occurs virtually instantly. A flow chart of a continuous sterilization system using direct steam injection is shown in Fig. 5.10. In some cases a combination of direct and indirect heat exchangers may be used

- The most widely used continuous sterilization system is that based on the spiral heat exchangers and a typical layout is shown in Fig. 5.11.

- The plant is sterilized prior to sterilization of the medium by circulating hot water through the plant in a closed circuit. At the same time, the fermenter and the pipework between the fermenter and the sterilizer are steam sterilized.

- Heat conservation is achieved by cooling the sterile medium against cold, incoming unsterile medium which will then be partially heated before it reaches the sterilizer.

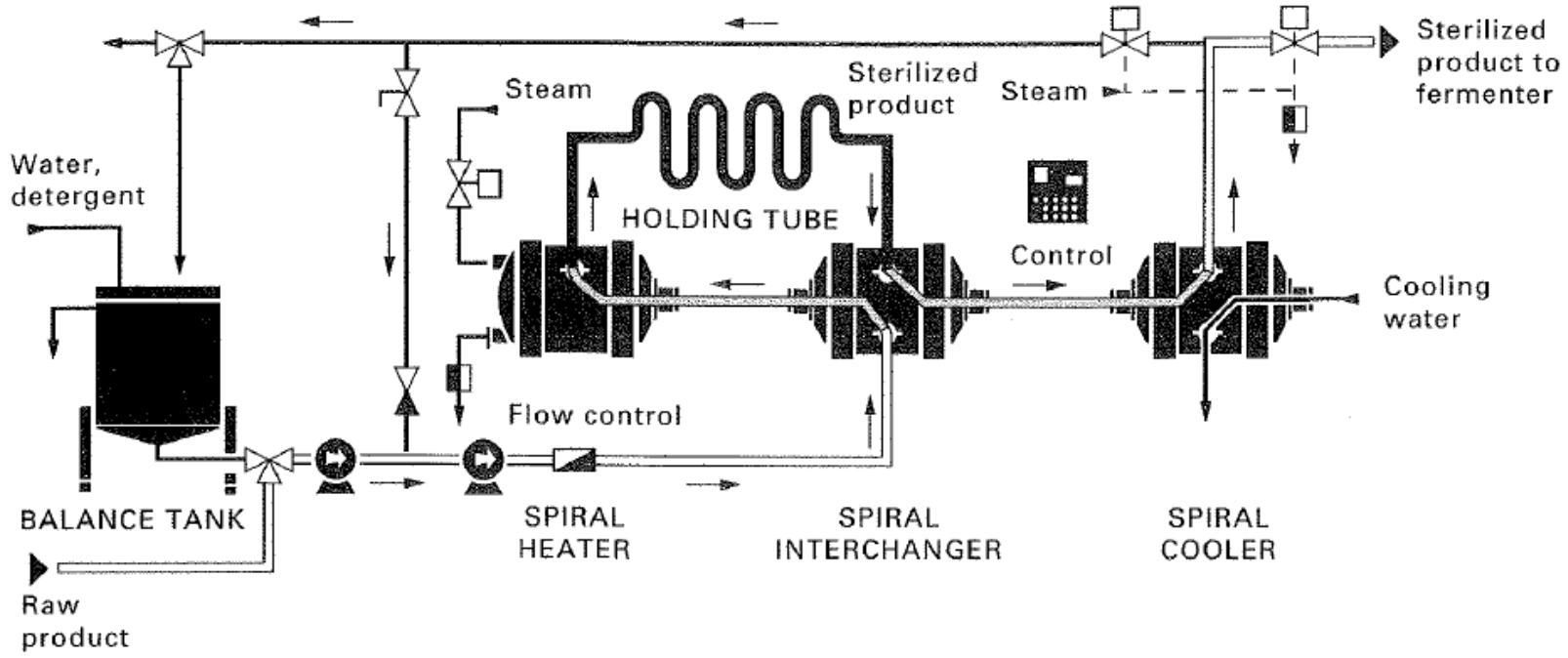
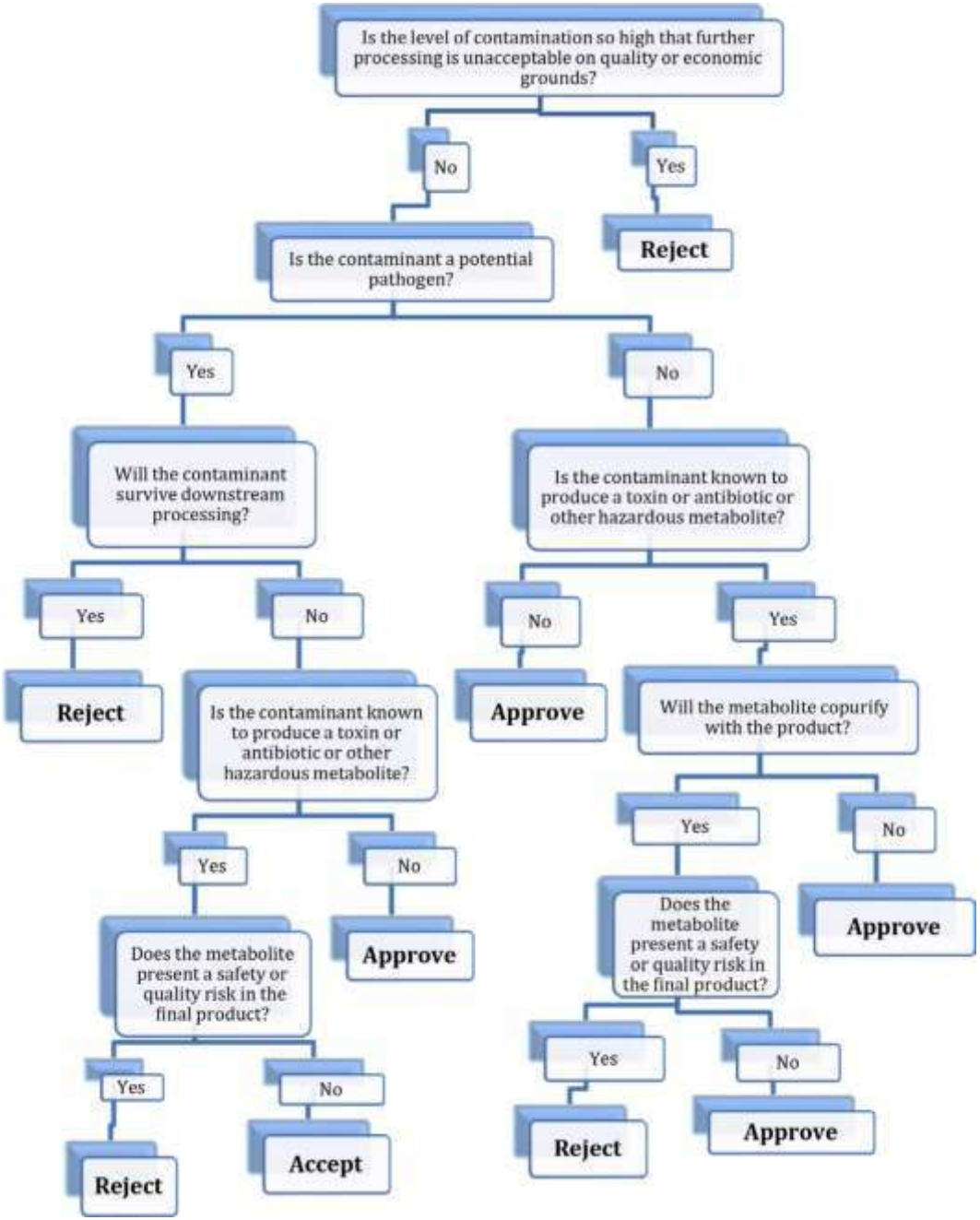


FIG. 5.11. Flow diagram of a typical continuous sterilization system employing spiral heat exchangers (Alfa-Laval Engineering Ltd., Brentford, Middlesex).

- The Del factor to be achieved in a continuous sterilization process has to be increased with an increase in scale, and this is calculated exactly as described in the consideration of the scale up of batch regimes.

- Thus, if the volume to be sterilized is increased from 1000 dm^3 to 10,000 dm^3 and the risk of failure is to remain at 1 in 1000 then the Del factor must be increased from 34.5 to 36.8.

- However, the advantage of the continuous process is that temperature may be used as a variable in scaling up a continuous process so that the increased V' factor may be achieved whilst maintaining the nutrient quality constant. Deindoerfer and Humphrey (1961) attempted to rationalize the choice of time-temperature regime by the use of a Nutrient Quality Criterion *(Q)*, based on similar logic to the Del factor:


Is the level of contamination so high that further processing is unacceptable on quality or economic grounds?
No
Yes
Reject
Is the contaminant a potential pathogen?
Yes
No
Will the contaminant survive downstream processing?
Is the contaminant known to produce a toxin or antibiotic or other hazardous metabolite?
Yes
No
No
Yes
Reject
Is the contaminant known to produce a toxin or antibiotic or other hazardous metabolite?
Approve
Will the metabolite copurify with the product?
Yes
No
Yes
No
Does the metabolite present a safety or quality risk in the final product?
Approve
Yes
No
Does the metabolite present a safety or quality risk in the final product?
Yes
No
Reject
Accept
Yes
No
Reject
Approve

$$Q = \ln (x_0/x_t) \qquad (5.11)$$

where x_0 is the concentration of essential heat labile nutrient in the original medium,

x_t is the concentration of essential heat labile nutrient in the medium after a sterilization time, t.

As considered earlier, the destruction of a nutrient may be considered a first-order reaction:

$$x_t/x_0 = e^{-kt}$$

where k is the reaction rate constant

or $$x_0/x_t = e^{kt}.$$

Thus, taking natural logarithms,

$$\ln (x_0/x_t) = kt.$$

Therefore $$Q = kt.$$

The relationship between k and absolute temperature is described by the Arrhenius equation:

$$k = A \cdot e^{-(E/RT)}$$

therefore, substituting for k:

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

Therefore, as for the Del factor equation, by taking natural logarithms, and rearranging, the following equation is obtained

$$\ln t = E / RT + \ln Q / A. \qquad (5.12)$$

- Thus, a plot of the natural logarithms of the time required to achieve a certain Q value against the reciprocal of the absolute temperature will yield a straight line, the slope of which is dependent on the activation energy; that is, a very similar plot to Fig. 5.5 for the Del factor relationship.

- If both plots were superimposed on the same figure, then a continuous sterilization performance chart is obtained. The example put forward by Deindoerfer and Humphrey (1961) is shown in Fig. 5.12.

- Thus, in Fig. 5.12 each line of a constant Del factor specifies temperature-time regimes giving the same fractional reduction in spore number and each line of a constant nutrient quality criterion specifies temperature-time regimes giving the same destruction of nutrient.

- By considering the effect of nutrient destruction on product yield, limits may be imposed on Fig. 5.12 indicating the nutrient quality criterion below which no further increase in yield is achieved (i.e. the nutrient is in excess) and the nutrient quality criterion at which the product yield is at its lowest (i.e. there is no nutrient remaining). Thus, from such a plot a temperature-time regime may be chosen which gives the required Del factor and does not adversely affect the yield of the process.

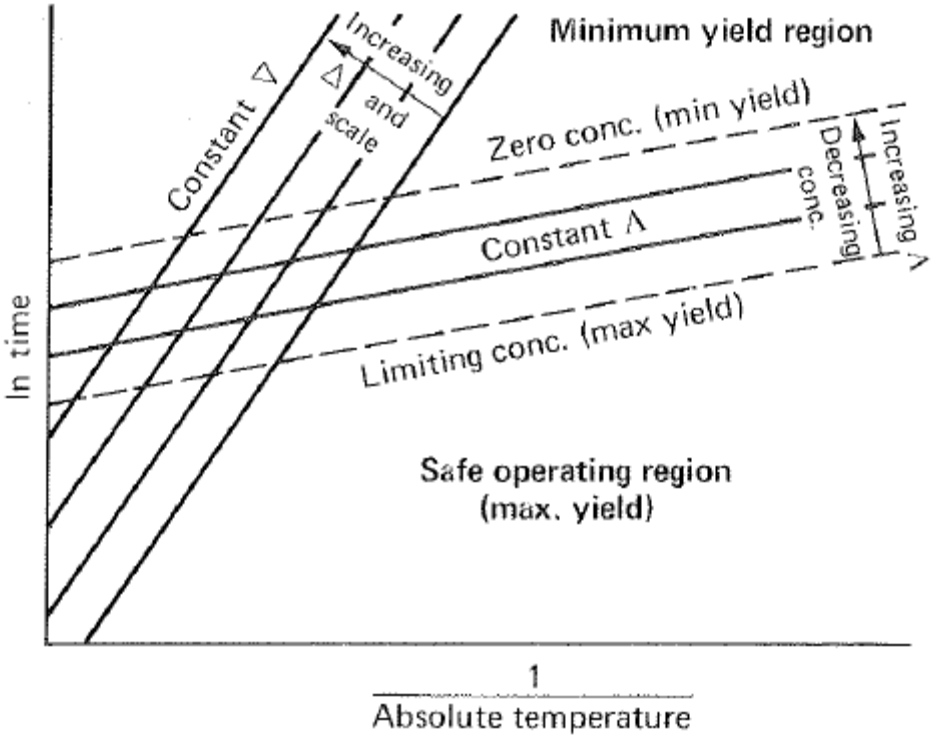


FIG. 5.12. Continuous sterilization performance chart (Deindoerfer and Humphrey, 1961).

Filter sterilization

Polytetrafluoroethylene (PTFE) is a synthetic fluoropolymer of tetrafluoroethylene that has numerous applications. The best known brand name of PTFE-based formulas is Teflon by Chemours.

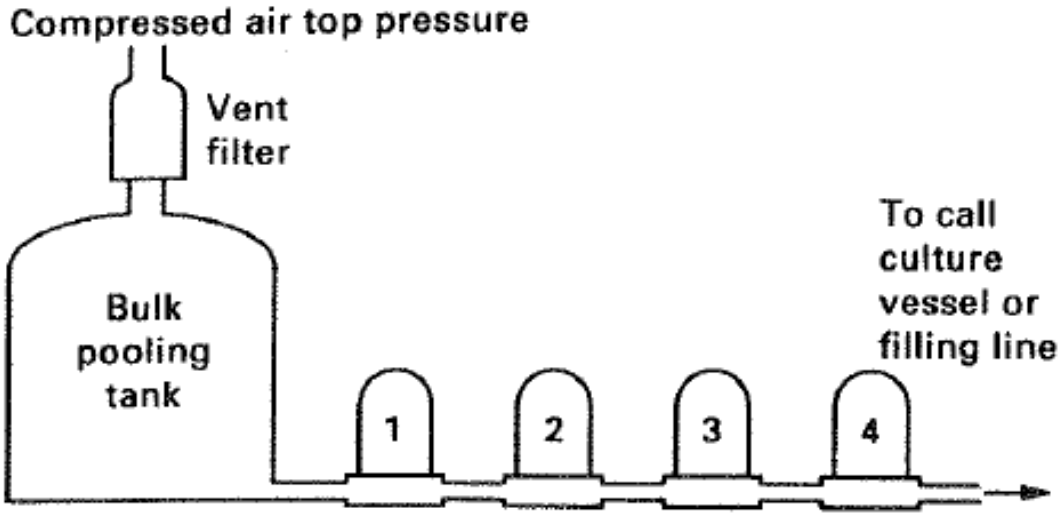


FIG. 5.14. Filtration system for the provision of sterile, mycoplasma free serum.
Filter 1. 5μm absolute rated prefilter for removal of coarse precipitates.
Filter 2. 0.5μm absolute rated prefilter for bulk bioburden removal.
Filter 3. 0.1μm absolute rated single layer prefilter for futher bioburden and endotoxin removal.
Filter 4. 0.1μm absolute rated double layer final filter for absolute sterility, mycoplasma removal and further endotoxin control.
(Pall Process Filtration Ltd., Portsmouth, U.K.)

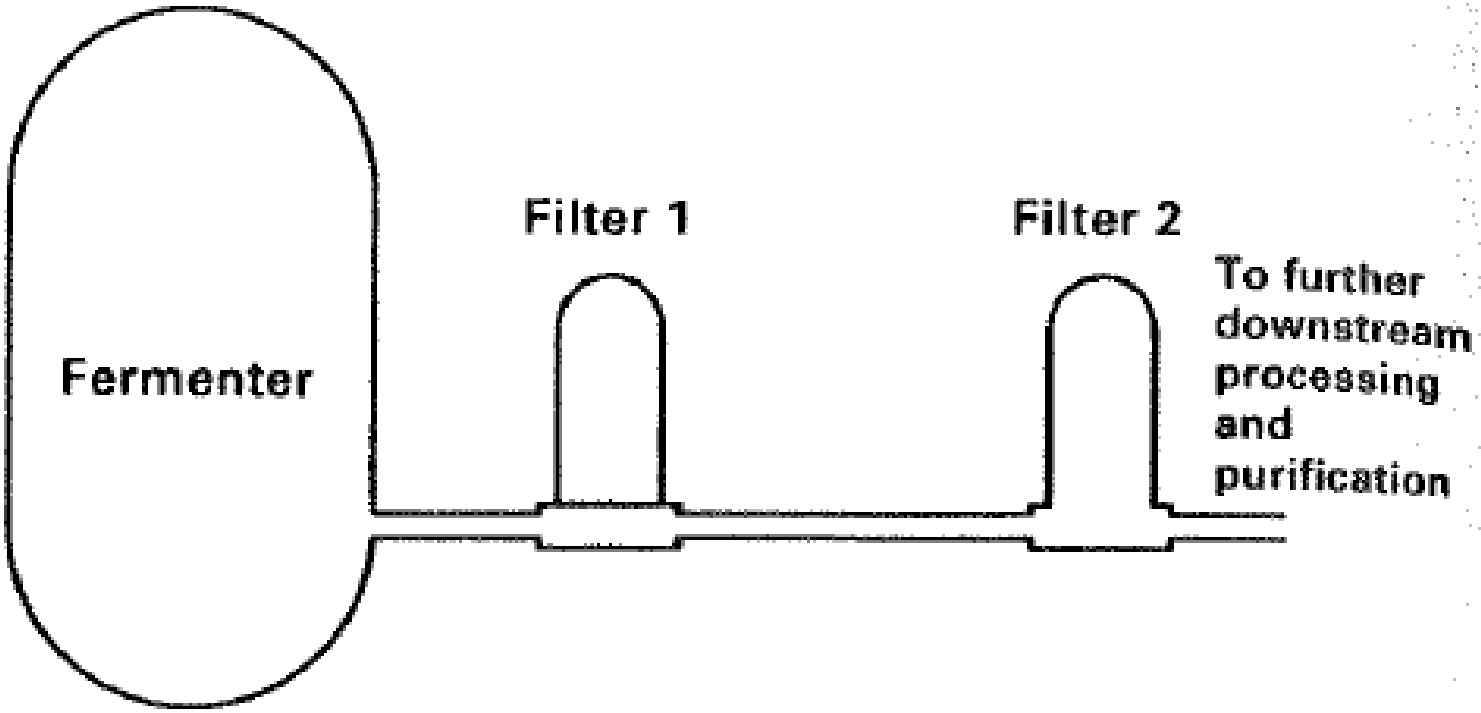


FIG. 5.15. Filtration system for the removal of cells and cell debris from an animal cell culture fermentation.
Filter 1. 1.0μm absolute rated prefilter for bulk cell and cell debris removal.
Filter 2. 0.2μm absolute rated single layer 'Bio-Inert' filter for final bioburden removal.
(Pall Process Filtration Ltd., Portsmouth, U.K.)

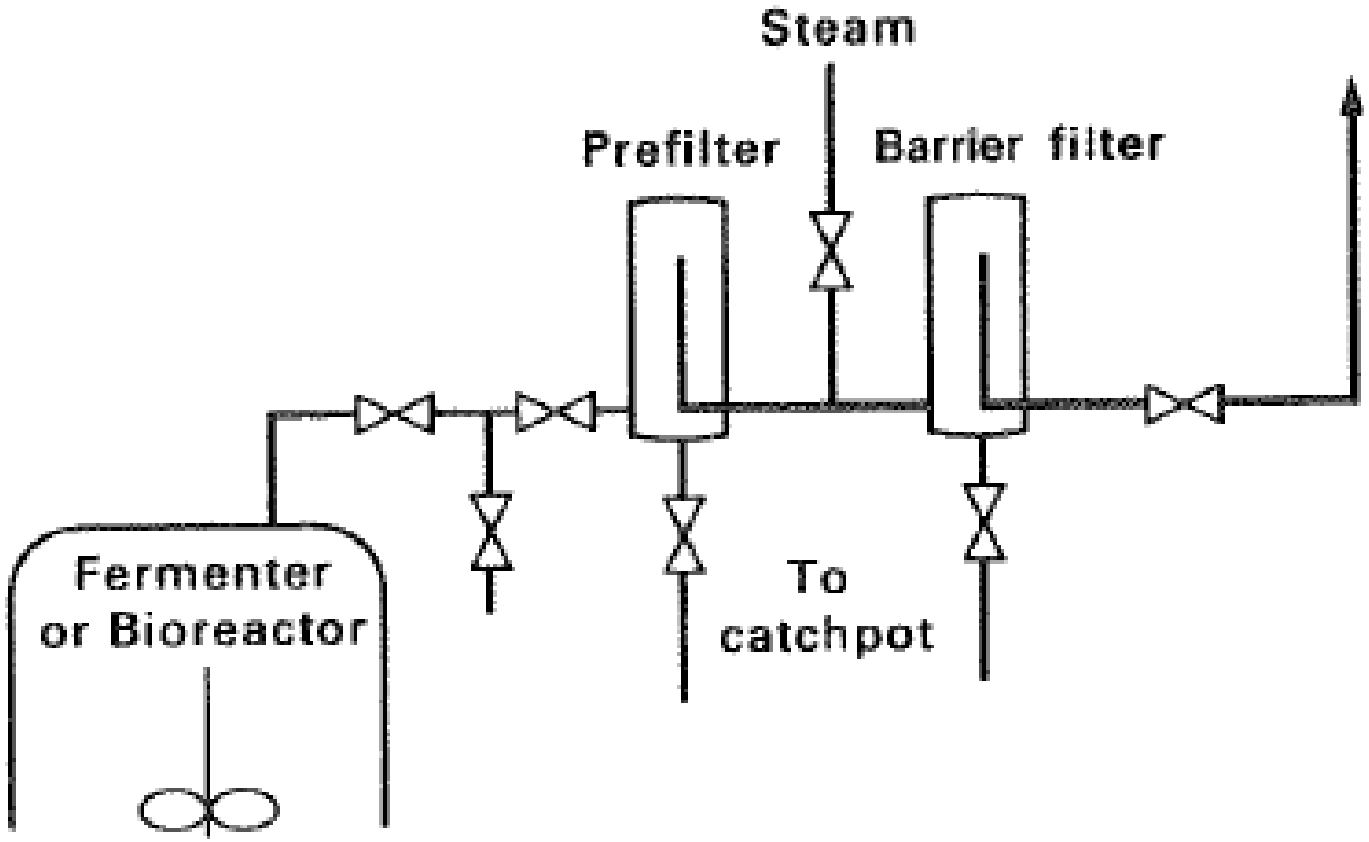


FIG. 5.18. Dual hydrophobic filter system for the sterilization of off-gas from a fermenter (Pall Process Filtration Ltd., Portsmouth, U.K.).

the concept *X90* the depth of filter
required to remove 90% of the total number of particles
entering the filter;

TABLE 5.3. X_{90} *values for the removal of a range of micro-organisms by a variety of filtration materials* (Humphrey, 1960; Rivierre, 1977)

Filter material	Diameter of fibres (μm)	Micro-organism	Air speed (cm s^{-1})	X_{90} (cm)
Glass wool	16	*Bacillus subtilis* spores	3	4
			15	9
			30	11.5
			150	1.5
			300	0.4
	18.5	*Serratia marcescens*	3.9	3.1
Glass fibre	8.5	*Escherichia coli*	3	0.4
			15	0.6
			30	0.7
			150	0.8
			300	1.1
Norite (15–30 mesh)	—	*Bacillus cereus* spores	1.4	1.7
			6.4	1.5
Activated carbon (4–8 mesh)		*Bacillus cereus* spores	18	1.7
			28.5	8.7

Equation (5.15), the log penetration relationship, is the same form as equation (5.3) in the derivation of thermal-death kinetics. In the case of heat sterilization the theory predicts that an infinite time is required to reduce the population to zero, whereas the theory of filtration predicts that a filter of infinite length is required to remove all organisms from an air stream. Thus, it is not surprising that the same approach is adopted in the design of filters and heat-sterilization cycles, in that an acceptable probability of contamination is determined. The probability of one fermentation in a thousand being contaminated is frequently used in filter design, as it is in the design of heat-sterilization cycles. Having arrived at an acceptable probability of contamination and determined the filtration characteristics (i.e. the value of K) of the material to be used, a filter may be designed to filter a certain volume of air containing a certain number of organisms; the following example illustrates the design calculation approach used by Richards (1967):

It is required to provide a 20-m^3 fermenter with air at a rate of 10 m^3 min^{-1} for a fermentation lasting 100 hours. From an investigation of the filter material to be used, the optimum linear air velocity was shown to be 0.15 m sec^{-1}, at which the value of K was 1.535 cm^{-1}. The dimensions of the filter may be calculated as follows:

The log penetration relationship states that:

$$\ln (N / N_0) = -Kx.$$

The air in the fermentation plant contained approximately 200 micro-organisms m^{-3}.
Therefore,

$$N_0 = \text{total amount of air provided} \times 200,$$

$$N_0 = 10 \times 60 \times 100 \times 200$$

$$= 12 \times 10^6 \text{ organisms.}$$

The acceptable degree of contamination is one in a thousand,
therefore $N = 10^{-3}$,

$$\ln \{10^{-3}/(12 \times 10^6)\} = -Kx,$$

$$\ln 8.33 \times 10^{-11} = -Kx,$$

$$\ln 8.33 \times 10^{-11} = -1.535x,$$

$$x = -23.21/-1.535 = 15.12 \text{ cm}.$$

Therefore, the filter to be used should be 15.12 cm long.
The cross-sectional area of the filter is given by the volumetric air flow rate divided by the linear air velocity:

$$\pi r^2 = 10/0.15 \times 60$$

where r is the radius of the filter

$$r = 0.59 \text{ m}.$$

Thus the filter to be employed should be 15.12 cm long and 0.59 m radius.

However, as Humphrey (1960) pointed out, the efficient operation of the filter is dependent on the supply of air at the optimum linear velocity. If the air velocity increases or decreases the value of K will decrease, resulting in a loss of filtration efficiency. Considering the example calculation, if the linear air velocity were to drop to 0.03 m sec^{-1}, then the value of K would decline to 0.2 cm^{-1}. The number of organisms which would enter the fermentation in 1 minute at this reduced air-flow rate would be as calculated below:

$$\ln (N/N_0) = -Kx.$$

At a linear air velocity of 0.03 m sec^{-1}, in 1 minute 0.03 $\times$ 60 $\times$ the cross-sectional area of the filter m^{-3} of air would enter the filter, i.e. 1.98 m^3. At a microbial contamination level of 200 organisms m^{-3} this means that 396 organisms would enter the filter in 1 minute. Thus:

$$\ln (N/396) = -0.2 \times 15.12,$$

$$N = 19.24.$$

Therefore, 19.24 organisms would have entered the fermenter in 1 minute at the decreased air-flow rate. If the filter had been designed to meet this contingency, then the length would have been:

$$\ln \left(10^{-3}/396\right) = -0.2x,$$

$$x = 64.4.$$

Thus a filter length of 64.4 cm would have been required to have maintained the same probability of contamination over the 1 minute of reduced air flow.

This example illustrates the hazards of attempting very precise design and the necessity to consider the reliability of ancillary equipment in any design calculation.

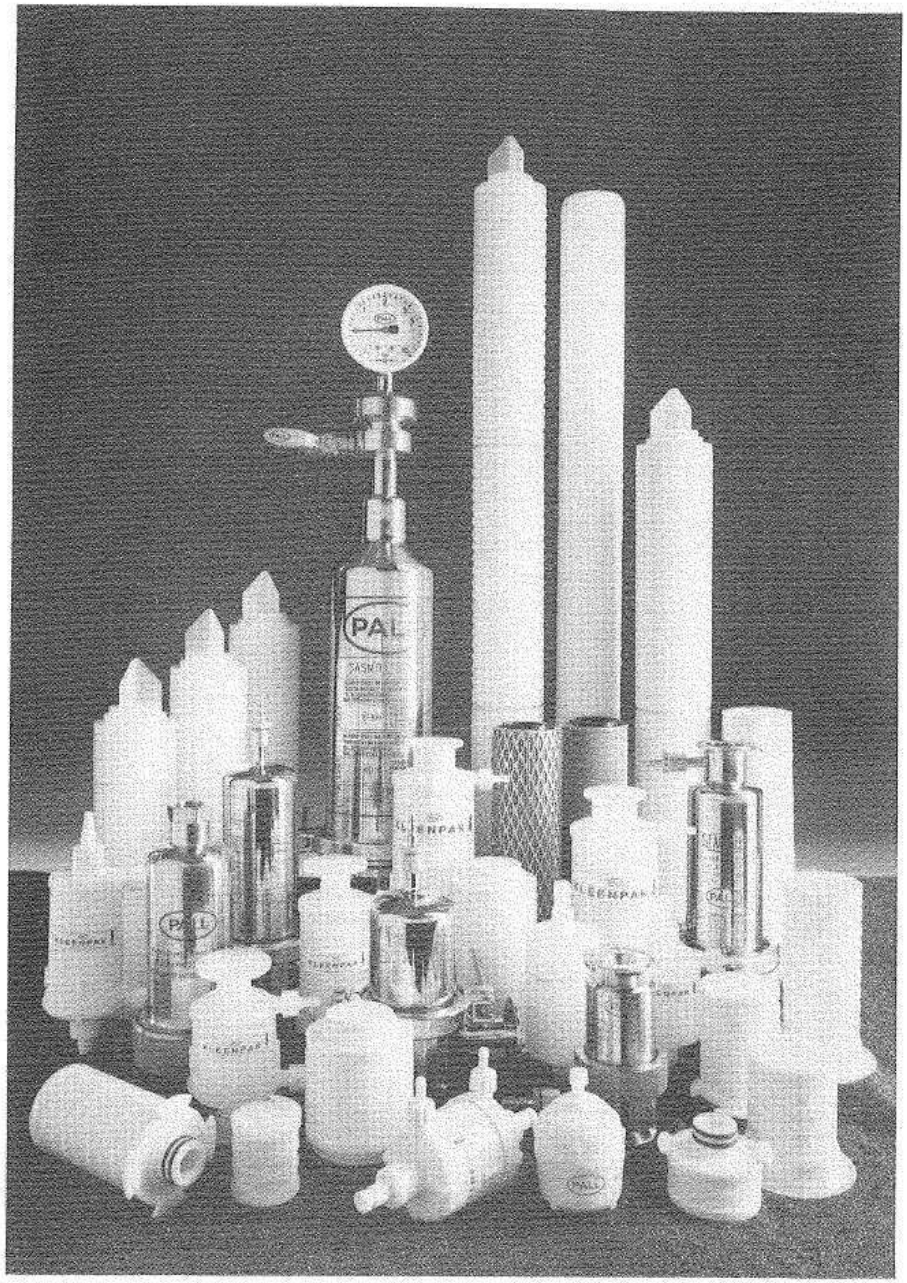


FIG. 5.16. A selection of absolute membrane filter cartridges and stainless steel housings (Pall Process Filtration, Portsmouth, U.K.).

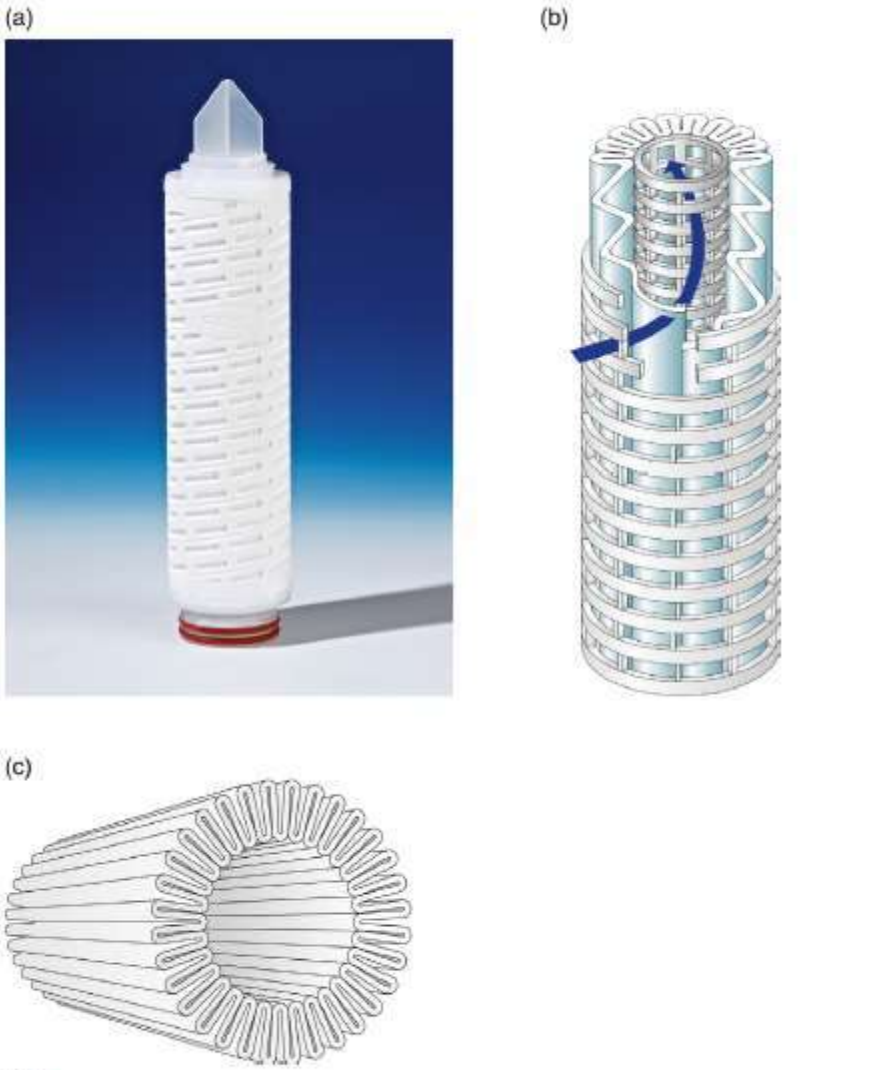


FIGURE 5.19

(a) A filter cartridge. (b) A schematic diagram showing the pleated membrane and supporting structure of a cartridge. (c) A schematic diagram showing the pleated membrane.

Pall Corporation, Portsmouth, UK

FIGURE 5.21 Examples of Stainless-Steel Cartridge Filter Housings