Development of an Industrial Biotechnology Process

Sterilization: Thermal

Dr. Kurt Eyer
kurt.eyer@epfl.ch
k.eyer@bioengineering.ch

Sterilize what?

Different phases:

- Gas sterilization
- Liquid sterilization
- Solids sterilization

Different locations:

- Space: storage, production, purification, formulation/packing suites
- Bioreactors
- DSP and other equipment

Sterilize how?

- Thermal
- Chemical
- Irradiation
- Barrier (filtration)

Thermal sterilization preferred, providing that materials and solutions can withstand elevated temperatures for sufficient periods

Death

The irreversible loss of the ability to multiply

Sterilization

The destruction or removal of all microorganisms

Sterility

Sterility means that no living microorganism is present

To reach sterility is a question about

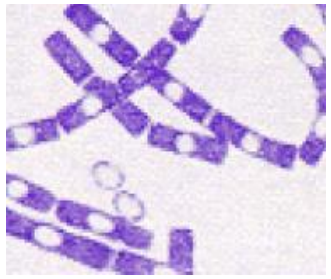
PROBABILITY

Sterilisation

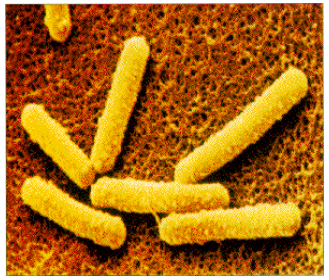
Mechanisms of heat inactivation of microorganisms

Two classes with respect to heat sensitivity

Bacterial endospores
(Bacillus, Clostridium)



Vegetative cells and spores
of other types: fungal spores



Kinetics of Heat Sterilisation

$$\frac{-dN}{dt} = k * N \quad [1]$$

k (min^{-1}) specific heat inactivation constant, also known as death rate constant

Integration:

$$\ln\left(\frac{N}{N_0}\right) = -k*t \quad [2]$$

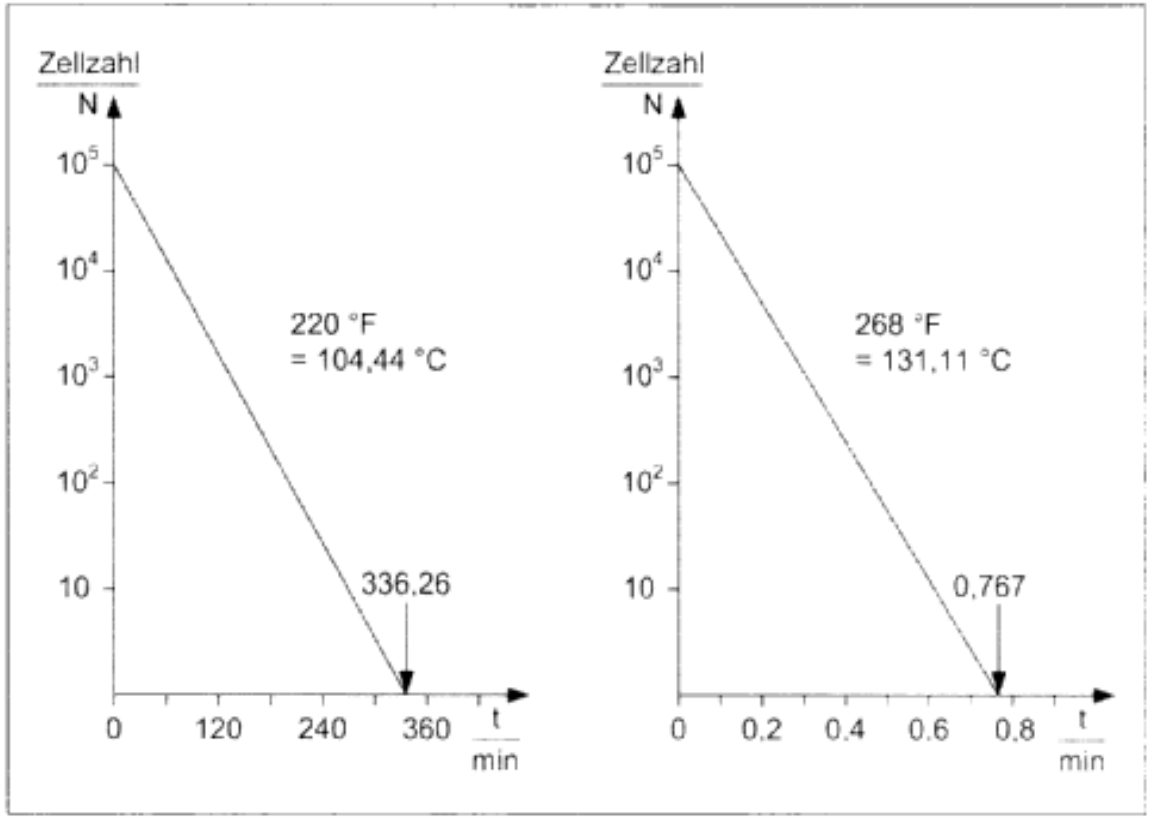
which can be rearranged to

$$\ln(N) = \ln(N_0) - kt \quad [3a]$$

Or $N = N_0 e^{-kt}$ [3b]

k: ***characteristic for a strain but depends also on physiological state, environmental conditions (pH, solids in medium, …temperature..)***

Kinetics of Heat Sterilisation



Inactivation of
B. stearothermophilus at
two different temperatures

Kinetics of Heat Sterilisation

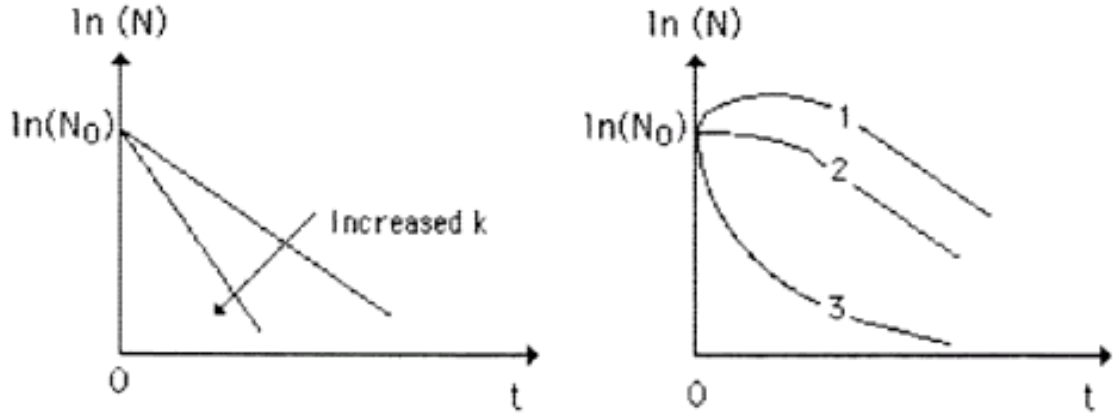


Fig 10.2 Heat inactivation curves. The left hand figure shows two inactivation curves with different death rate constants. The right hand figure shows some deviations from the model:

1. This form may be caused by super-dormant spores, which are activated by the first heat treatment and do not germinate unless they get this treatment;
2. This may be caused by delayed heat transfer in the experiment or it may be observed in samples that contain aggregates of cells, since analysis is made by viable count that gives number of colony forming units rather than number of cells. The viable count does then not decline until the last cell in an aggregate is killed;
3. Non-uniform heat resistance in the population, e.g. when the sample contains species with different thermal sensitivity.

Kinetics of Heat Sterilisation

The heat inactivation constant depends on temperature like most rate constants of chemical reactions. This is usually described by Arrhenius equation:

$$k = A * e^{-\Delta E/R*T} \qquad [4]$$

A (min^{-1}); E ($Jmol^{-1}$), R (~8.31Jmol $°K^{-1}$), T (°K)

$$\rightarrow \ln k = -\frac{\Delta E}{R} * \frac{1}{T} + \ln A \qquad [5]$$

Table 10.1 Examples of ΔE values for heat inactivation of spores and some chemical reactions.

Inactivation of	$-\Delta E$ (kJ mol^{-1})
B. subtilis spores	318
B. stearothermophilus spores	283
Cl. botulinum spores	343
Folic acid	70
d-panthotenyl alcohol	88
Cyanocobalamin	97
Thiamine HCl	92
Maillard reactions	≈125

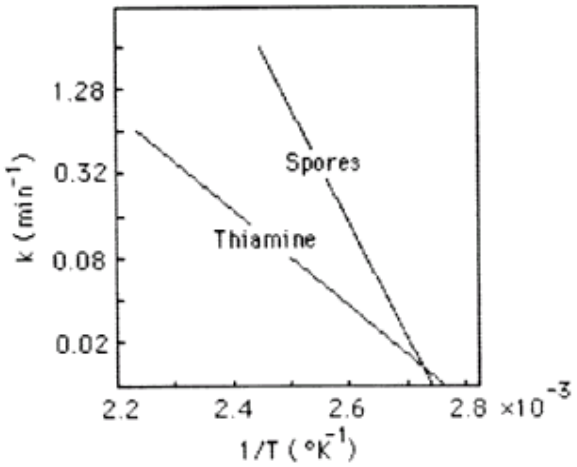


Fig 10.3 Arrhenius plots of inactivation of *B. stearothermophilus* spores and thiamine. Note that a temperature increase has a larger effect on the spore inactivation rate than on the vitamin inactivation rate.

$$ln\frac{1}{10} = -k_d \cdot t$$

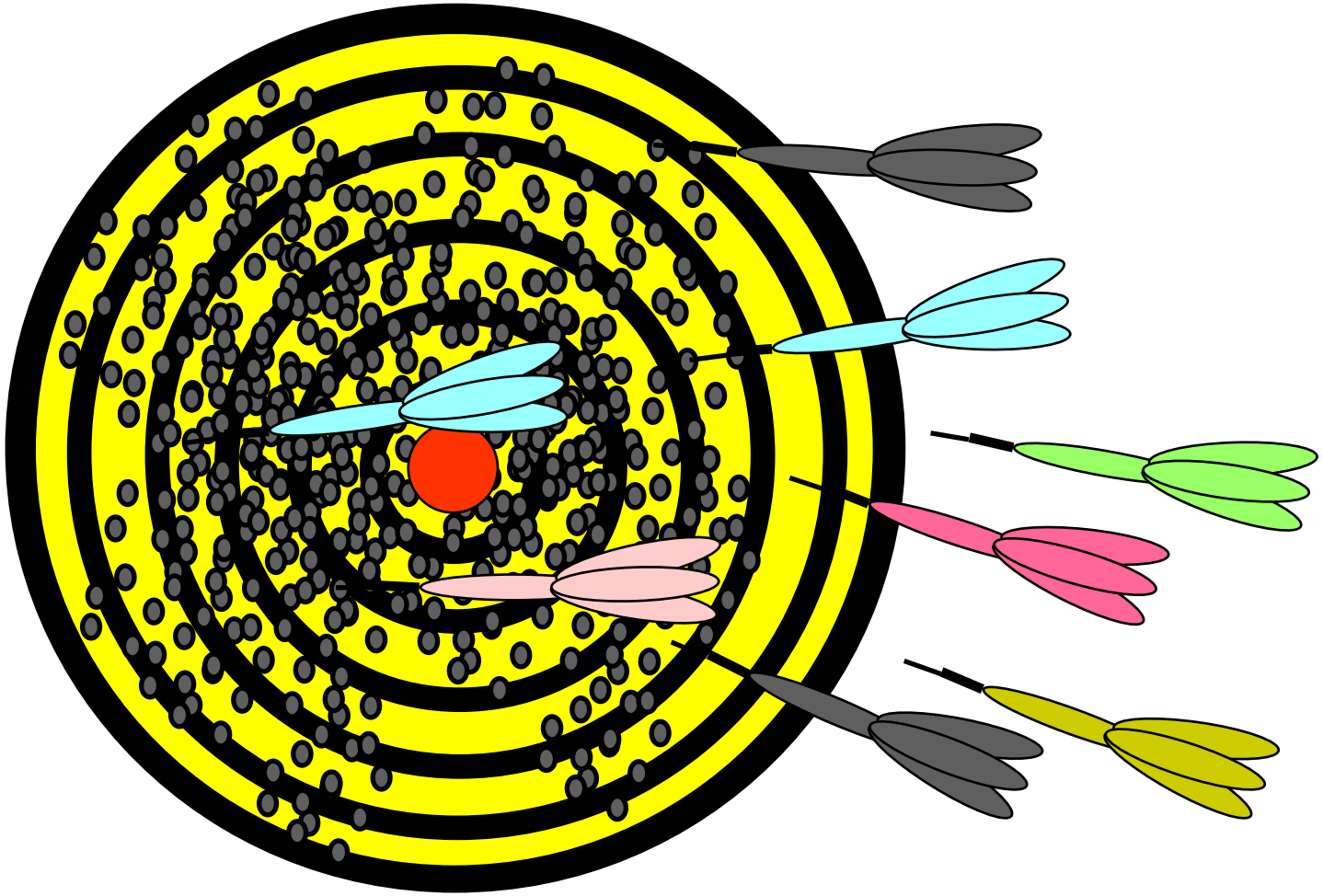
$$-2{,}303 = -k_d \cdot t$$

$$\boxed{t = D = \frac{2{,}303}{k_d}}$$

Killing Kinetics

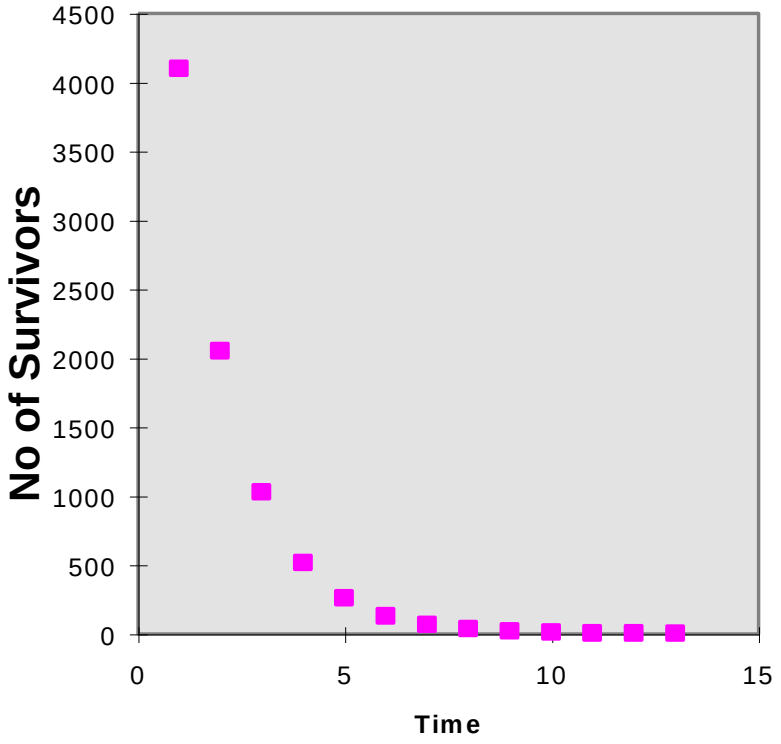
Time	Survivors	Death per unit time		Total death	Log Red
0	1 000 000	0		0	0
1	100 000	900 000	= 90%	900 000	1
2	10 000	90 000	= 90%	990 000	2
3	1 000	9 000	= 90%	999 000	3
4	100	900	= 90%	999 900	4
7	0,1	0,9	= 90%	999 999,90	7
8	0,01	0,09	= 90%	999 999,99	8

Killing Hypothesis

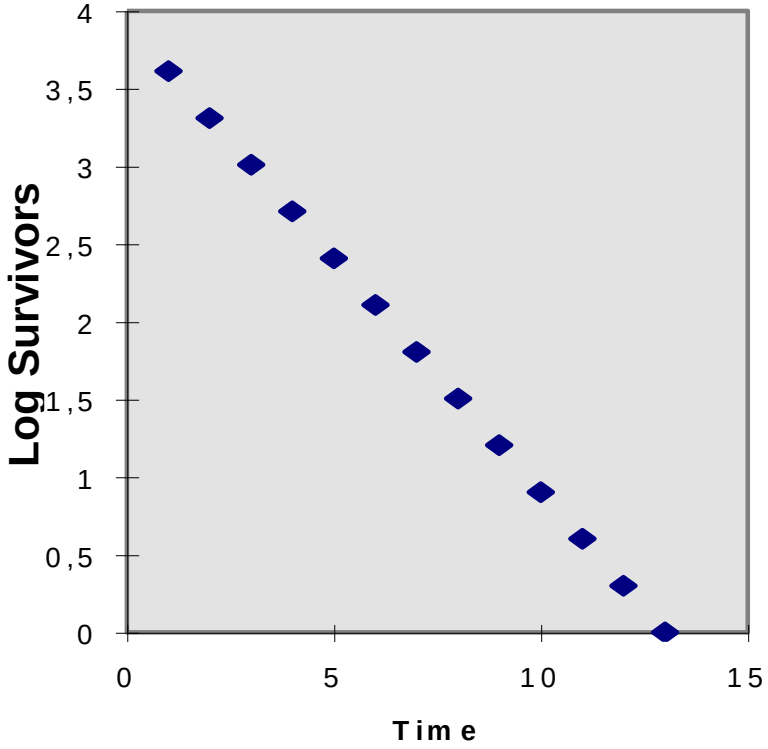


Graphs of Survival Curves

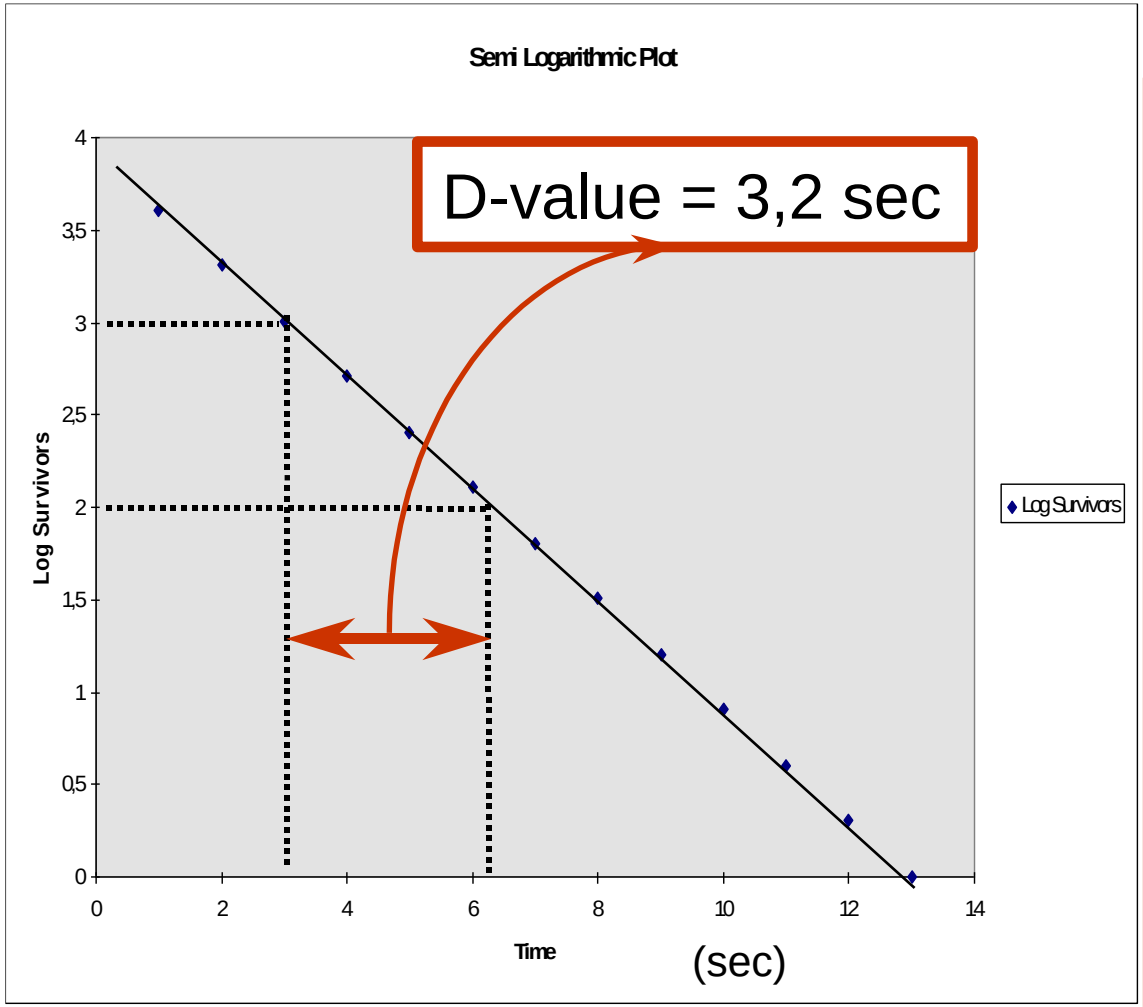
Arithmetic



Semilogarithmic



Decimal Reduction Value



D-value:

is the time required for a process to reduce the number of a specific organism by 90 % or with

1 Log Red or

1 D

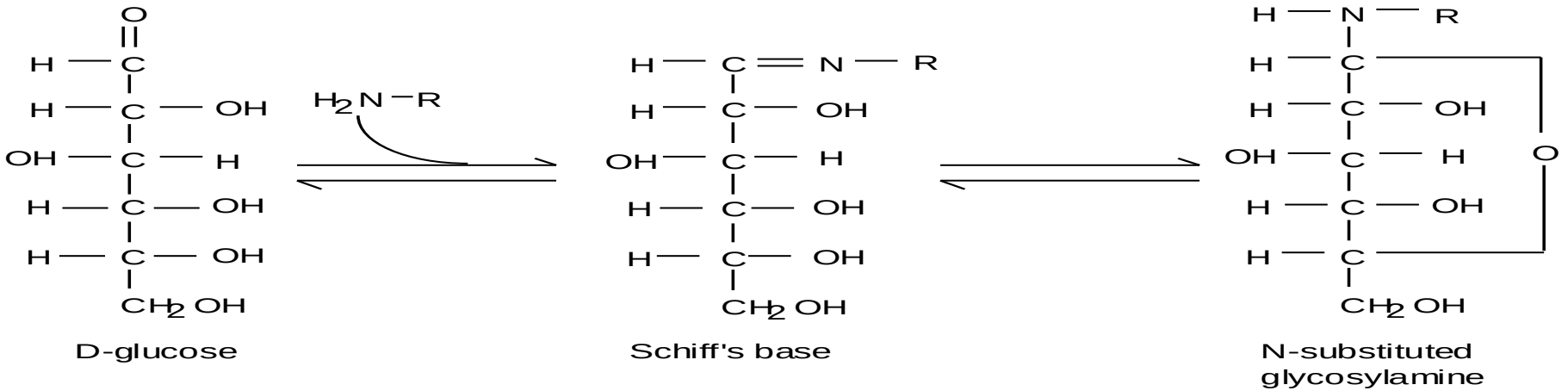
Explanation of D-value

The expression D_{65} = 1 min
means that the number of organisms is reduced with 1 D when treated one minute at a processing temperature of 65 ^{0}C.

Two (2) minutes exposure results in 2D.

1D = 90	% reduction = 1 Log Red
2D = 99	% reduction = 2 Log Red
3D = 99,9	% reduction = 3 Log Red
4D = 99,99	% reduction = 4 Log Red

Maillard Reaction



Reaction of sugars upon heating;
especially in presence of
salts, ammonia or proteins

Bioprocess sterilization

- Cell culture media contains heat labile components: sugar, amino acids, hormones and growth factors and sterilized by microfiltration
- Reactor and peripherals (acid/ base, medium reservoirs and piping) sterilized by heat
- DSP equipment usually sterilized chemically (0.1-2 M NaOH or acid)
- All production equipment requires CIP and SIP protocols and analytical methods for validation

Kinetics of Heat Sterilisation

The heat inactivation constant depends on temperature like most rate constants of chemical reactions. This is usually described by Arrhenius equation:

$$k = A * e^{-\Delta E/R*T} \qquad [4]$$

A (min^{-1}); E ($Jmol^{-1}$), R (~8.31Jmol $°K^{-1}$), T (°K)

$$\rightarrow \ln k = -\frac{\Delta E}{R} * \frac{1}{T} + \ln A \qquad [5]$$

Table 10.1 Examples of ΔE values for heat inactivation of spores and some chemical reactions.

Inactivation of	$-\Delta E$ (kJ mol^{-1})
B. subtilis spores	318
B. stearothermophilus spores	283
Cl. botulinum spores	343
Folic acid	70
d-panthotenyl alcohol	88
Cyanocobalamin	97
Thiamine HCl	92
Maillard reactions	≈125

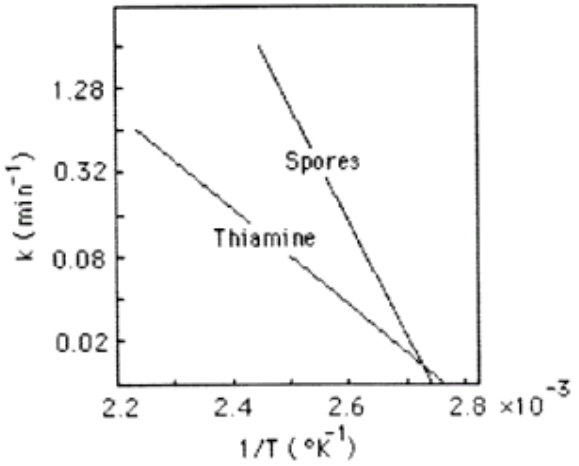


Fig 10.3 Arrhenius plots of inactivation of *B. stearothermophilus* spores and thiamine. Note that a temperature increase has a larger effect on the spore inactivation rate than on the vitamin inactivation rate.

Example 1: Thermal death kinetics

The number of viable spores of a new strain of *Bacillus subtilis* is measured as a function of time at various temperatures

	Number of spores at:			
Time [min]	T=85°C	T=90°C	T=110°C	T=120°C
0.0	2.40×10^9	2.40×10^9	2.40×10^9	2.40×10^9
0.5	2.39×10^9	2.38×10^9	1.08×10^9	2.05×10^7
1.0	2.37×10^9	2.30×10^9	4.80×10^8	1.75×10^5
1.5	-	2.29×10^9	2.20×10^8	1.30×10^3
2.0	2.33×10^9	2.21×10^9	9.85×10^7	-
3.0	2.32×10^9	2.17×10^9	2.01×10^7	-
4.0	2.28×10^9	2.12×10^9	4.41×10^6	-
6.0	2.20×10^9	1.95×10^9	1.62×10^5	-
8.0	2.19×10^9	1.87×10^9	6.88×10^3	-
9.0	2.16×10^9	1.79×10^9	-	-

a) Determine the activation energy for thermal death of *B. subtilis* spores
b) What is the specific death constant at 100°C
c) Estimate the time required to kill 99% of spores in a sample at 100°C

Calculation of sterilisation time

According to the model for heat inactivation $\ln(N) = \ln(N_0) - kt$ it is not possible to calculate the time needed to reach sterility.

Thus, a sterility criterion, ∇, has to be defined:

$$\nabla = \ln\left(\frac{N_0}{N_f}\right) \qquad [6]$$

N_f: final number of organisms

∇: is also called ***Del factor*** or the ***design criterion often also mentioned as*** S_L

Estimation of sterilisation time F, using equation [2]

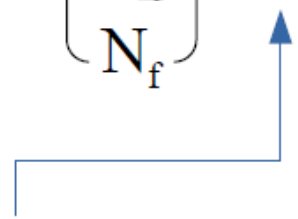
$$F_T = \frac{\nabla}{k} \qquad [7a]$$

This sterilisation time depends also in the temperature applied since k is a function of temperature.

Some help:

$$\nabla = \ln\left(\frac{N_0}{N_f}\right)$$

$$\ln\left(\frac{N_0}{N_f}\right) = k\,t$$



$$k = A * e^{-\Delta E/R*T}$$

$$\nabla = A\ e^{-\Delta E/R*T}\,t$$ *is true for T constant*

Therefore:

$$\nabla = A \int_0^t e^{-\Delta E/R*T} dt$$

General Guidelines

Volumes < 100 L $\nabla = 25 - 50$

Volume > 100 L $\nabla = 50 - 200$

Example 2: Inactivation of *B. subtilis* spores

For the inactivation of *B. subtilis* spores is $A = 9.5 \times 10^{37}$ min^{-1} and $E = 287.4$ kJ/mol. Calculate the holding time for a liquid enriched with spores at 115°C, so that a death ratio of 10^6 will be reached.

Example 3: Sterilisation of a bioreactor

In a bioreactor 10000 L of medium was sterilised at 120°C. The time/ temperature profile for this process is summarised in the following table:

Practical comments:

- Sterilisation effect below 100°C can be neglected (only ca. 2% on total lethality)
- Heating and cooling rates are considered constant With 1°C/min

115	79	0.483
120	91	1.47
120	101	1.47
115	104	0.483
110	107	0.154
100	114	0.0143

Calculate ∇ *ges (=* ∇*heating +* ∇*holding +* ∇*cooling)!*

Use the following table!

Example 3: Sterilisation of a bioreactor

Tabelle 2.3. Werte für k und ∇_{ges} aus Daten, die mit *B. stearothermophilus* gewonnen wurden. $A = 4,93 \cdot 10^{37}$; $E = 282,1$ kJ/mol

Temperatur, °C	k	Kumulativer Wert von ∇
100	0,0143	–
101	0,0182	0,0325
102	0,0232	0,0558
103	0,0296	0,0854
104	0,0376	0,1229
105	0,0477	0,171
106	0,0604	0,231
107	0,0765	0,308
108	0,0967	0,404
109	0,122	0,526
110	0,154	0,681
111	0,194	0,875
112	0,244	1,12
113	0,307	1,43
114	0,385	1,81
115	0,483	2,29
116	0,605	2,90
117	0,757	3,66
118	0,945	4,60
119	1,18	5,78
120	1,47	7,25
121	1,83	9,08
122	2,28	11,36
123	2,83	14,19
124	3,51	17,70
125	4,35	22,05
126	5,39	27,45
127	6,67	34,11
128	8,24	42,36
129	10,18	52,54
130	12,55	65,08

Comparison of two different volumes

$$N_{0(1)} / N_{0(2)} = V_1 / V_2$$

$$N_{0(1)} / N = (N_{0(2)} / N) \cdot (V_1/V_2)$$

$$\ln (N_{0(1)}/N) = \ln (N_{0(2)}/N) + \ln(V_1/V_2)$$

Using eq. [6]

$$\nabla_1 = \nabla_2 + \ln(V_1/V_2)$$

Or $\nabla_1 = \nabla_2 + 2.3\log(V_1/V_2)$

V: volume; 1: bigger reactor, 2: smaller reactor

Example 4: Scaling-up sterilisation

For a 10 L laboratory bioreactor a sterilisation criteria of $\nabla = 50$ is sufficient. Which is the minimum sterilisation criteria, if the process is scaled-up to a 1000 L reactor?

Bioprocess sterilization

- Cell culture media contains heat labile components: sugar, amino acids, hormones and growth factors and sterilized by microfiltration
- Reactor and peripherals (acid/base, medium reservoirs and piping) sterilized by heat
- DSP equipment usually sterilized chemically (0.1-2 M NaOH or acid)
- All production equipment requires CIP and SIP protocols and analytical methods for validation