LESSON 3:
STERILIZATION

Sterilization

The basic principles of sterilization you have already studied in your lessons of mirobiology. Here we will consider specific aspects re-lating to the fermentation process.

In virtually all fermentation processes, it is man-datory for a cost-effective operation to have con-tamination-free seed cultures at all stages, from the preliminary culture to the production fer-menter. A bioreactor can be sterilized either by destroying the organisms with some lethal agent such as heat, radiation, or a chemical, or by re-moving the viable organisms by a physical pro-cedure such as filtration.

Microorganisms can be removed from fluids by mechanical methods, for example, by filtration, centrifugation, flotation, or electrostatically. They may also be destroyed by heat, chemical agents, or electromagnetic waves. Although cells may be disrupted and killed by mechanical abrasion on a small scale, this method is not satisfactory industrially. Similarly x-rays, beta rays, ultra-violet light, and sonic radiations, while useful for laboratory purposes are not applicable to the sterilization of large volume of fluids. Gamma rays, on the other hand, may prove useful, particularly in food industry.

The process of destroying a population gen-erally follows first-order kinetics. Using the ini-tial number of cells No/ml, the number of de-stroyed organisms N' at time t (min), and the surviving cells N, the death rate can be calculated as follows

$$\frac{dN}{dt} = k(No - N') = kN \qquad \text{————1}$$

(k is the specific death constant/min)

When integrated between No at time t = 0 and N at time t = t, the following equation is obtained

$$kt = \ln No/N'o \qquad \text{————2}$$

$$\ln \frac{N}{No} = -kt \qquad \text{————3}$$

The ratio of No/N is the inactivation factor, the ratio of N/No is the survival factor and the In of No/N=V is the design criterion, a parameter which encompasses the contaminatian level of the medium to be sterilized, No, and the desired sterility level, N.

In the above equations, k is a constant which expresses the specific death rate. It increases sharply with temperature and can be experimentally determined far an organism using equation 3.

If the experimentally determined In k value from this equation is plotted against the recip-rocal temperature value, a straight line should be obtained from which the k value can be calcu-lated for a desired temperature.

During fermentation the following points must be observed to ensure sterility:

- Sterility of the culture media
- Sterility of incoming and outgoing air

Sterilization of Culture Media

Nutrient media as initially prepared contain a variety of different vegetative cells and spores, derived from the constituents of the culture me-dium, the water, and the vessel. These must be eliminated by a suitable means before inocula-tion. A number of means are available for ster-ilization, but in practice for large-scale installa-tions, heat is the main mechanism used.

Heat Sterilization

This is the most useful method for the sterilization of nutrient media. A number of factors influence the success of heat steriliz-ation:

- the number and type of microorganisms present,
- the composition of the culture medium,
- the pH value,
- the size of the suspended par-ticles.

Vegetative cells are rapidly eliminated at relatively low temperatures, but for destruction of spores, temperatures of 121°C are needed.

Spores of Bacillus stearothermophilus are the most heat resistant. Therefore they are used as assay organisms for testing the various proce-dures used to sterilize equipment. Table 3.1 pro-vides data on the relationship between temper-ature, k value, and design criterion for this, organism. A list of steriliza-tion times and tem-peratures for various organisms is given in Table 3.2.

Radiation (UV, X *Rays, or y rays)* Although oc-casionally used in the food industry, these agents are not used in industrial fermentation.

Chemical methods Although a number of chem-ical disinfectants are known, they cannot be used to sterilize nutrient media because there is a risk that inhibition of the fermentation organisg: could occur from the residual chemical.

Table 3.1 Relationship of temperature, k-value and the design criterion in Bacillus stearothermophilus

rc	k(minCI)	V
100	0.019	
115	0.666	3.154
118	1.307	6.341
121	2.538	12.549
130	17.524	90.591
140	135.9	
150	956.1	

Table 3.2 Sterilization time and sterilization temperature of various groups of organisms

Cells	Sterilization time min	Sterilization temperature °C
Vegetative cells	5-10	60
Fungus spores/yeast spores	15	80
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Bacterial spores; general	5	121
Spores of *Bacillus stearothermophilus*	15	121

Mechanical Removal of Organisms

- Centrifugation, adsorption to ion exchange, adsorption to activated carbon, or filtration are possible.
- Filtration is the only method in pra-tical use. Filter sterilization is often used for a:. components of nutrient solutions which are heat -sensitive and would thus be denatured through the steam sterilization process normally used in industrial fermentation.
- Deep filters (plate filters) are some time used to filter complex nutrient solutions. The disadvantages of filtration are: 1) certain com-ponents of the nutrient solution may be adsorbed on the filter material, and 2) high pressures must be used (up to 5 bar), which are undesirable inindustrial practice.

Batch Sterilization

- Most nutrient media are presently sterilized in batch volumes in the bioreactor at 121°C.
- Ap-proximate sterilization times can be calculatee from the nature of the medium and the size
- Not only the nutrient media, but also the fittings, valves and electrodes of the fer-menter itself must be sterilized. Therefore, actual. sterilization times are significantly longer than calculated ones and must be empirically deter-mined for the specific nutrient solutions in thefermenter.
- One method of sterilization is to injecting n into the fermenter mantle or interior coils red sterilization.
- Another method is to inject steam into the nutrient solution itself. With direct steam injection, condensate accumulates in the fermenter and the volume of liquid increases during the sterilization process.
- It takes 2-3 s to reach the sterilization temperature of 121^{0}C. depending on the steam conduction and enter size. Once the proper temperature has , reached, another 20-60 minutes are required for the actual killing process, followed by cooling for about one hour.
- Another disadvantage of heat sterilization (and from the standpoint of microbiology the most significant shortcoming) is that heating, sterilization and cooling phases not only kill microorganisms but also se-verely alter nutrient solutions. Discoloration and changes in the pH value result from carameli-zation and Maillard reactions. Vitamins are destroyed and the quality of the culture medium deteriorates. The extent to which the subsequent fermentation is affected depends on the organism and the process.

Continuous Sterilization

- The two -main disadvantages of batch steriliza-tion just mentioned, culture medium damage and high energy consumption, can be largely avoided by. use of a continuous sterilization procedure.
- Although continuous sterilization is the logical preliminary step for continuous fermentations in industrial scale, it is also of value in batch fer-mentations, making greater yields. possible for the time and space allotted.
- The reason for this is because of the exponential relationship' be-tween death rate and temperature, making the time required for the complete elimination of life shorter if higher temperatures are used. While batch sterilization is carried out in 30-60 minutes at 121°C, continuous sterilization is normally ac-complished in 30-120 seconds at 140°C.
- The heating of culture media for continuous sterilization can be done either by injection of steam or by means of heat exchangers.
- Sterili-zation with steam injection is done by injecting steam into the nutrient solution. The temperature is raised quickly to 140°C and is maintained for 30-120 seconds.
- Due to the formation of con-densate, the nutrient solution becomes diluted; to correct this, the hot solution is pumped through an expansion valve into a vaporizer and the condensate is removed via vacuum pumps so that the sterilized nutrient solution has the same concentration after the cooling process as before.
- The disadvantage of this process is the sensitivity it exhibits to changes in the viscosity of the medium and to pressure variations.
- In the continuous process using heat exchan-gers (Figure 3.1), the nutrient solution in the first heat exchanger is preheated to 90-120°C within 20-30 seconds by the exiting previously steri-lized nutrient solution. Then in the second heat exchanger, it is heated indirectly with steam to 140°C. This temperature is maintained for 30-120 seconds in a holding pipe before it is placed in the first exchanger for preliminary cooling and then in a third exchanger for cooling to the tem-perature of the fermenter. The cooling phase is only 20-30 seconds.
- In the process using heat exchangers, 90% of the energy input is recovered.
- The disadvantage of this method is that with some nutrient solutions, insoluble salts (e.g., calcium calcium oxalate) are formed and crusts a the first heat exchanger, due to the perature differences between the nutrient solution and the cold incoming.
- Starch-containing solutions which viscous when heated are difficult to use in continuous sterilization processes.

The advantages of continuous sterilization of media are as follows:

1. Increase of productivity since the short period of exposure to heat minimizes damage to media constituents,
2. Better control of quality,

3. Leveling of the demand for process steam,
4. Suitability for automatic control;

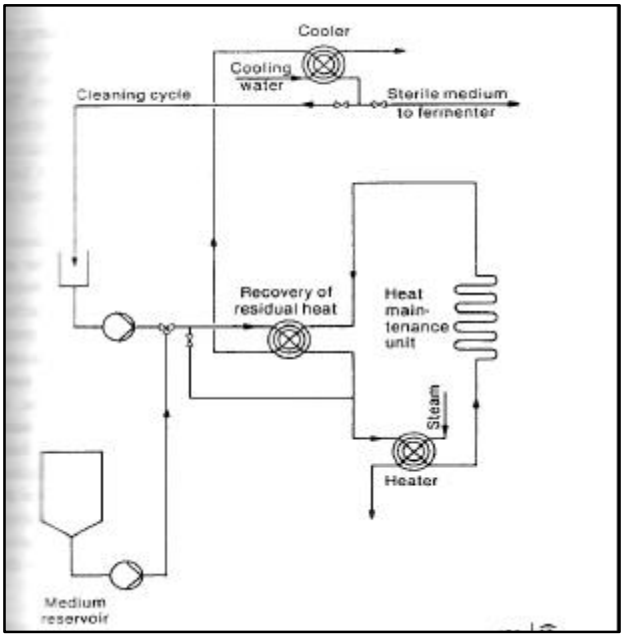

Figure 3.1: Continuous Sterilization by spiral heat exchanger

Sterilization of Fermentation Air

- Most industrial fermentations are operated under vigorous aeration and the air supplied to the fermenter must be sterilized.
- To prevent contamination of either the fermentation by air-borne.
- Microorganisms or the environment by aerosols generated within the fermenter, both air input and air exhaust ports have air filters attached.
- These fil-ters are designed to trap and contain microorganisms. Filters are made of glass fibre, mineral fibres, poly tetra-fluoroethylene (PTFE) or polyvinyl chloride (PYC), and must be steam sterilizable and easily changed.
- In some circumstances, particularly where pathogenic organisms are being grown, fermenter exhaust may also undergo dry heat sterilization (incineration) as an additional safety measure.
- methods available for sterilizing gases includee filtration, gas injection (ozone), gas scrubbing, radiation (UV), and heat.
- Of these_filtration and heat are practical at an in-dustrial scale
- Previously air was sterilized by passing it over electrically heated elements. Due to high cost of electricity this method has become obsolete.
- Filter sterilization by glass wool filters are used.Particles are trapped by a combination of physical effect which include inertial effects, blocking effect, diffusion, gravity separation and electrostatic attraction.The disadvantages are shrinkage and solidification during steam sterilization.
- Glass Fiber filter cartridges has replaced glass wool filter as these do not have the drawback mentioned.

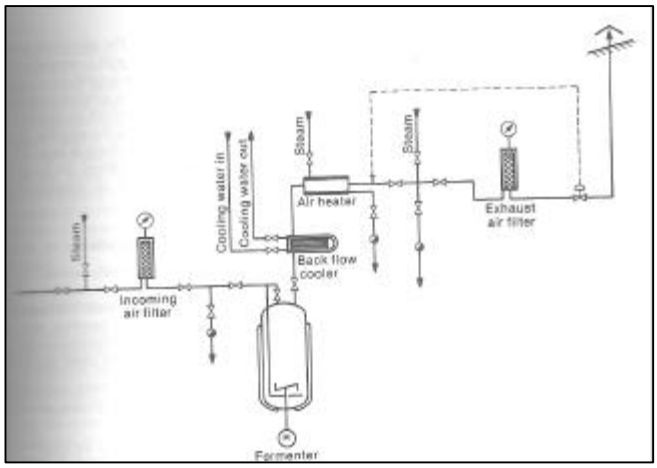

Figure 3.2: Installation of an air filter system in a fermenter

Media and Vessel Sterilization

Need of Sterilization

- For pilot-scale and industrial aseptic fermentations the fermenter' can be sterilized empty.
- The vessel is then filled with sterile medium, prepared in a batch or con-tinuous medium 'cooker' that may supply several fermentations.
- Alternatively, the fermenter is filled with formulated medium and the two are sterilized together in one operation.
- However, some industrial fermentation are not aseptic, but microbial contamination is still maintained at a minimum level by boiling or pasteuriza-tion of the media.
- Otherwise, a fast-growing contami-nant could outgrow the industrial microorganism, or at least utilize some valuable nutrients.
- Microbial contam-inants may also metabolize the target product, produce toxic compounds or secrete products that may block filters and interfere with downstream processing.
- If the contaminant is a bacteriophage it may lyses the culture, as can occur in fermentations involving lactic acid bacteria.

Process of Sterilization

- Small laboratory-scale fermenters of 1-5L capacity are usually filled with medium and then sterilized in a steam al1toclave.
- Here sterilization is normally per-formed using pressurized steam to attain a temperature of 121°C for 15 min.
- Care must be taken to avoid any pressure build-up inside the fermenter by venting without contaminating the contents.

- For pilot-scale -and industrial fermenters more rigorous sterilization is necessary, involving increased sterilization time and/or higher temperature.
- Normally, the aim is to provide sterilization conditions that give an acceptable proba-bility of contamination of 0.1 % (1 in 1000). Consequently, if the original number of microbial cells (No) is known, the Del factor (V =In N_o/N,) can be calculated). The value *pf* N" when a 0.1 % risk is adopted, will be 10-3 cells. A sterilization profile of a typical fermented is shown in Fig. 3.1. Destruction of cells occurs during heating (to 121°C in this case) and cooling (here from 121 °C to the fermentation operating temperature). Therefore the overall Del factor maybe represented as

V total = V heating + V holding + V cooling

Thus knowing both $V_{heating}$ and $V_{cooling}$ for a particu-lar fermentation system, the necessary holding time needed to achieve the required overall Del factor can be calculated. Alternatively, the Richards approximation can be used, which employs only that part of the curve above 100°C (Fig. 3.1). This assumes that:

1. heating and cooling between O°C and 100°C is unimportant for sterilization;
2. heating between 100°C and 121 °E; is at 1°C per minute, i.e. 20 min; and
3. cooling from 121°C to 100"C is at 1 °C per minute, i.e. 20 min.

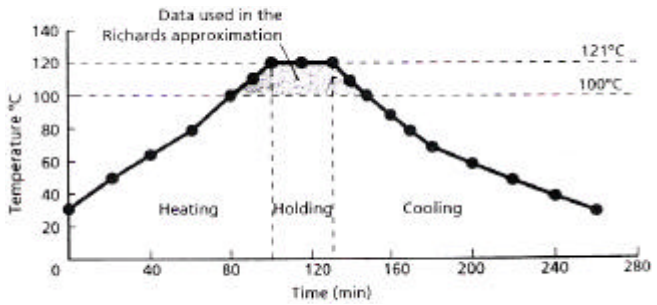

Figure 3.1: Sterilization Profile of a typical fermentation process

- In practice, steam sterilization involves passing the steam under pressure into the vessel jacket and/or inter-nal coils.
- Stream may also be injected directly into the headspace above the fermentation medium.
- This aids sterilization, but can result in media volume changes. Steam sterilization is effective and cheaper than dry heat sterilization.
- However, certain media constituents may be heat labile and destroyed by excessive heat, e.g. glucose, some vitamins and components of animal cell culture media. Such heat sensitive ingredients are often filter sterilized before use; alternatively; some can be heat sterilized with minimal degradation using a high temperature for a very short time, e.g. 140°C for 50s.
- This is usually a continuous operation where the holding time is controlled by the flow rate through the sterilizer and the material is then rapidly cooled in a heat exchanger.

Review Questions

1. Define sterilization?
2. Briefly describe the various techniques of sterilization used in industrial production processes?
3. Is there any role of antibacterial agents in media sterilization.

Notes