Pharmaceutical Microbiology (Theory)

Course code: BP308T

A Textbook for B. Pharm Semester III

As per the new Pharmacy Council of India (PCI) Syllabus 2026 (NEP 2020)

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Preface

This book presents the syllabus of Pharmaceutical Microbiology (BP308T) as a formal academic course text for the third-semester student of Bachelor of Pharmacy. Its purpose is to cover, to the depth the university examination requires, every topic the syllabus prescribes, in a clear and ordered academic register, so that a student who studies it carefully is equipped both to understand the subject and to answer the paper.

The treatment follows a consistent plan. Each chapter opens with a definition and an orientation, develops its subject through classification, mechanism, and application, and closes with a summary and a set of self-assessment questions in the pattern of the university paper, namely objective questions, short notes, long-answer questions with model answers, and, where the subject requires, numerical problems. The Indian Pharmacopoeia is taken as the governing compendium, with the British and United States Pharmacopoeias cited for comparison.

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Course Objectives

This course is designed to impart a fundamental knowledge on the science of microbiology and its applications in pharmacy. Upon completion of the course the student shall be able to:

1. understand the methods of identification, cultivation and preservation of various microorganisms;
2. understand the importance and implementation of sterilization in pharmaceutical processing and industry;
3. learn aseptic techniques and prevent the entry of microorganisms into the body and into pharmaceutical products;
4. carry out the microbiological standardization of pharmaceuticals;
5. understand the cell culture technique and its applications in the pharmaceutical industry and research.

Course Outcomes

Upon successful completion of this course, the student will be able to:

1. explain the basic concepts of microbiology and the role of microorganisms in the pharmaceutical field;
2. identify and differentiate microorganisms using staining and biochemical techniques;
3. evaluate the sources of microbial contamination and apply GMP-based methods for its control;
4. describe the microbial spoilage of pharmaceuticals and select suitable disinfectants, antiseptics and preservatives;
5. compare the methods of sterilization, assess their effectiveness and validation, and perform microbial limit and sterility tests in accordance with the pharmacopoeia.

Syllabus

Unit	Topics	No. of lectures
I	Introduction and role of microorganisms in the pharmaceutical industry. a) Fundamentals of microbiology: microorganisms and medicines; introduction to various microorganisms; microbial cultivation, isolation and enumeration; pharmaceutical importance of microorganisms. b) Introduction to the microscope (compound and electron). c) Identification of bacteria using staining (simple, Gram's, acid-fast) and biochemical tests (IMViC). d) Antibiotics produced by microorganisms (production and uses: streptomycin, cephalosporin).	09 Hours
II	Evaluation of microbiological contamination and spoilage. a) Sources and types of microbial contaminants. b) Control of microbial contamination during the manufacture of non-sterile and sterile dosage forms (including the aseptic area); control of atmosphere, water, raw material, facility, packaging and equipment. c) Microbiological spoilage of pharmaceuticals; factors affecting spoilage. d) Introduction to fermentation, its types and fermenters.	09 Hours
III	Microbial control and evaluation. a) Designing of the aseptic area; laminar flow equipment; clean area classification; biological safety level categories; methods of prevention. b) Disinfectants, antiseptics and preservatives, and their evaluation; factors affecting the antimicrobial activity of disinfectants.	09 Hours
IV	Sterilization procedures, assurance and evaluation. a) Physical, chemical, gaseous, radiation and mechanical methods of sterilization; advances in these methods. b) Evaluation of the efficiency of sterilization; validation of sterilization procedures and sterility indicators. c) Sterility assurance, bioburden determination, modelling for predicting microbial growth and death, and tests for bacteriostatic and bactericidal activity.	09 Hours

V	Microbiological quality control. a) Microbial limit tests and microbial assay (antibiotics, vitamins, amino acids). b) Sterility testing as per IP, BP and USP. c) Methods for monitoring water and air quality. d) In vitro cell cultures: general procedure and applications in the pharmaceutical industry and research.	09 Hours

How to use this book

The ten chapters correspond to the five units of the syllabus, each unit being treated in two chapters so that the material is divided into portions of a convenient size for study. The learning outcomes stated at the head of each chapter are drawn from the Course Outcomes of the syllabus, and each is both developed in the body and tested in the self-assessment.

The student is advised to read each chapter in three passes: first for the argument and the figures, which teach the same matter in words and in diagram; then for the summary and the quick-recall boxes, which consolidate it; and finally, before the examination, for the comparison tables and the self-assessment, which serve revision and practice. The figures are drawn for clarity in greyscale, the tables collect the comparisons the subject demands, and the consolidated formula sheet and glossary at the back gather the quantitative relationships and the definitions for ready reference.

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Chapter 1: Fundamentals of Microbiology and the Microbial World

Learning outcomes

After studying this chapter the student will be able to:

- Define microbiology and pharmaceutical microbiology and state the scope of the subject.
- Classify microorganisms into their major groups and state the defining features of each.
- Describe the structure of the bacterial cell and the structure and significance of the **bacterial endospore**.
- Explain the nutritional requirements of microorganisms, the classification of culture media, and the conditions of cultivation.
- Describe the isolation of **pure culture**s and the methods used to enumerate microorganisms.
- Interpret the microbial **growth curve**, calculate **generation time**, and state the pharmaceutical importance of microorganisms.

These outcomes serve Course Outcome CO1, the explanation of the basic concepts of microbiology and the role of microorganisms in pharmaceuticals.

1.1 Introduction

Microbiology is the branch of biological science that deals with microorganisms, the living forms too small to be seen clearly by the unaided eye and ordinarily requiring a microscope for their study. The discipline embraces their structure, classification, nutrition, growth, metabolism, genetics and their interactions with other organisms and with the environment. **Pharmaceutical microbiology** is the applied branch concerned with those aspects of microbiology that bear on the manufacture, quality and safe use of medicines. Its scope is twofold: the exclusion and control of microorganisms that contaminate, spoil, or render a medicine unsafe, and the exploitation of microorganisms in the manufacture of medicinal substances such as antibiotics, vitamins and vaccines.

The dimensions involved fix the character of the subject. A typical bacterium measures one to a few micrometres, a virus only tens to a few hundred nanometres, and these magnitudes lie far below the resolving power of the eye. Because such organisms cannot be seen on a finished product, their presence cannot be judged by inspection; it must be established by deliberate cultivation and measurement. This single fact, that the hazard is invisible and must be demonstrated rather than observed, organises the whole of pharmaceutical microbiology and recurs in every later chapter.

The relevance of the subject to pharmacy is direct. A non-sterile medicine must be kept within defined microbial limits and free of specified pathogens; a sterile medicine, such as an injection or

an ophthalmic preparation, must contain no viable microorganisms at all. Achieving and proving these states depends on an understanding of how microorganisms are built, how they grow, how they are killed, and how they are counted. The present chapter establishes that foundation. It defines the groups of microorganisms, describes the structure of the bacterial cell, and sets out the cultivation, isolation, enumeration and growth of microorganisms on which all subsequent control and testing rest.

1.2 Microorganisms and medicines

Microorganisms bear on medicine in two opposing ways, and pharmaceutical microbiology is the management of both.

In the first, harmful, role they act as contaminants and spoilage agents. Microorganisms gain entry to pharmaceutical products from raw materials, water, air, equipment, packaging and personnel; once present, they may degrade the active ingredient, spoil the dosage form, and, in a product administered by a vulnerable route, cause infection. The control of this hazard, through clean manufacture, sterilization and quality-control testing, occupies the greater part of the syllabus and of this book.

In the second, beneficial, role microorganisms are producers of medicinal substances. The metabolic versatility of microorganisms is exploited on an industrial scale to manufacture a wide range of pharmaceutical materials. The principal classes are the antibiotics (for example penicillin from *Penicillium chrysogenum*, streptomycin from *Streptomyces griseus*, and the cephalosporins from *Acremonium chrysogenum*), certain vitamins (such as vitamin B12 and riboflavin), several amino acids and organic acids (glutamic acid and citric acid among them), microbial enzymes, and, through recombinant DNA technology, therapeutic proteins such as human insulin. Vaccines, both the older attenuated and inactivated products and modern recombinant products, depend on the cultivation of microorganisms or of cultured cells.

The two roles are not contradictory but complementary, and they require the same body of knowledge applied in opposite directions: the conditions that must be denied to an unwanted contaminant are precisely those that must be supplied to a wanted producer. A consolidated account of the pharmaceutical products obtained from microorganisms is given in Section 1.10; the industrial process by which they are made, fermentation, is treated in Chapter 4.

1.3 Classification of microorganisms

Microorganisms are not a single natural group but a collection of diverse forms united only by their small size. The first and most important division is by cellular organisation, into the **prokaryote**s, the **eukaryote**s, and the **acellular agents**.

A prokaryote is a cell that lacks a true membrane-bound nucleus and membrane-bound organelles; its genetic material lies free in the **cytoplasm** as a single circular chromosome. The bacteria, including the **actinomycetes** and the cyanobacteria, are prokaryotes. A eukaryote is a cell possessing a true nucleus enclosed by a nuclear membrane and containing membrane-bound organelles such as mitochondria. The fungi, protozoa and algae are eukaryotes. The acellular agents, chiefly the viruses, are not cells at all; a virus is a particle of nucleic acid within a protein coat that can replicate only inside a living host cell. The major groups, their cell type, size range, and pharmaceutical relevance are set out in Table 1.1 and arranged as a chart in Figure 1.1.

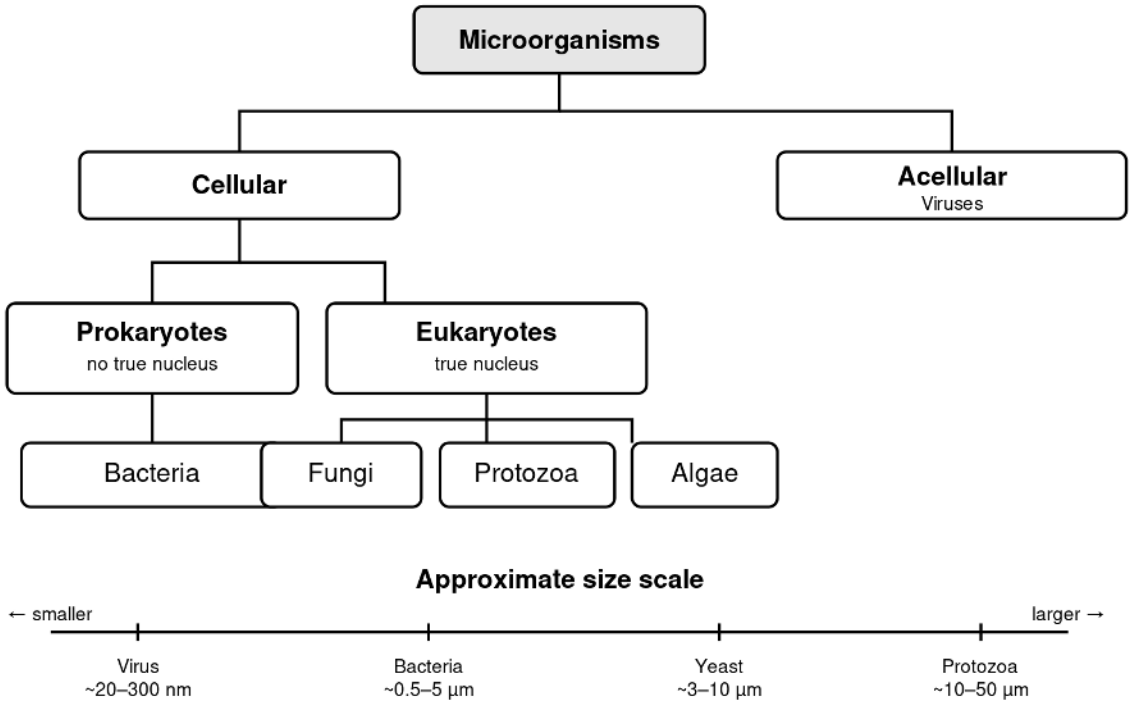


Figure 1.1 *The major groups of microorganisms, with an approximate size scale.*

Table 1.1: The major groups of microorganisms

Group	Cell type	Typical size	Cultivated on agar?	Principal pharmaceutical relevance
Bacteria	Prokaryote	0.5–5 µm	Yes	Commonest contaminant; producers of several antibiotics;

Group	Cell type	Typical size	Cultivated on agar?	Principal pharmaceutical relevance
				spore-formers resist sterilization
Fungi (**yeasts**, **moulds**)	Eukaryote	3–10 µm (yeasts); larger for moulds	Yes	Spoil low-moisture, acidic, high-sugar products; produce penicillin, cephalosporin, citric acid
Viruses	Acellular	20–300 nm	No (require host cells)	Grown in cell culture; central to vaccine manufacture
Protozoa	Eukaryote	10–50 µm	Some	A few are human pathogens (malaria, amoebic dysentery)
Algae	Eukaryote	Variable	Some	Source of agar and of certain pigments and supplements

1.3.1 Bacteria

Bacteria are unicellular prokaryotes and the most important organisms in pharmaceutical microbiology: they are at once the commonest contaminants of medicines and the source of several antibiotics. They are classified for descriptive purposes by their shape and arrangement. The principal morphological forms are: the **coccus**, a spherical cell; the **bacillus**, a rod-shaped cell; the **vibrio**, a short curved rod; the **spirillum**, a rigid spiral; and the **spirochaete**, a flexible spiral. Cocci are further described by the arrangement in which they remain after division: in pairs (diplococci), in chains (streptococci), in irregular clusters (staphylococci), in fours (tetrads), and in cubical

packets of eight (sarcinae). These forms are shown in Figure 1.2. The actinomycetes, a group of filamentous, branching bacteria that superficially resemble fungi, deserve separate mention because the genus *Streptomyces* is the natural source of the majority of clinically used antibiotics.

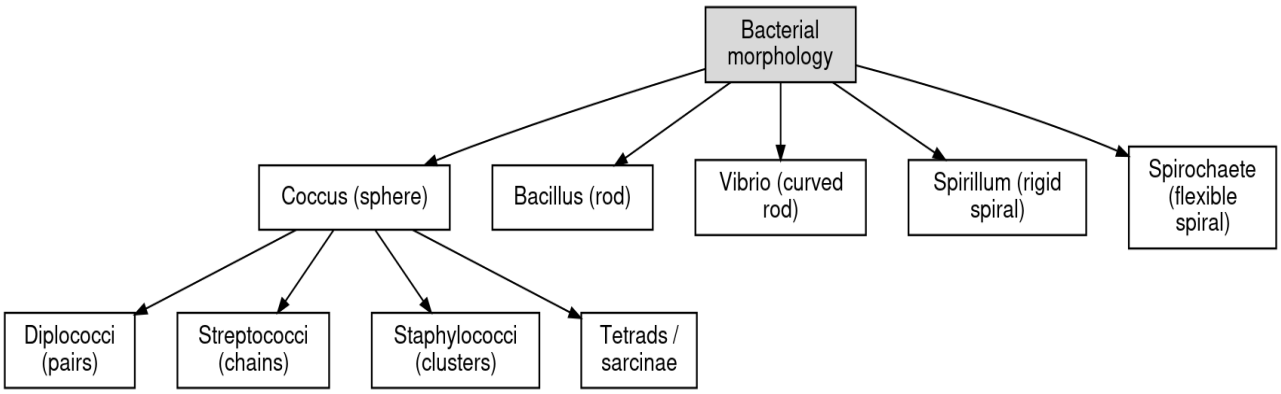


Figure 1.2 *Classification of bacteria by shape and arrangement.*

1.3.2 Fungi

Fungi are eukaryotic, non-photosynthetic organisms that obtain nutrients by absorption. The two forms relevant to pharmacy are the unicellular yeasts (for example *Saccharomyces cerevisiae* and the pathogen *Candida albicans*), which reproduce by budding, and the filamentous moulds (for example *Aspergillus* and *Penicillium* species), which grow as a mass of branching threads called a mycelium and reproduce by spores. Fungi tolerate low water activity, acidic pH, and high osmotic pressure far better than most bacteria, and they are therefore the characteristic spoilage organisms of syrups, creams and other products in which bacteria cannot grow. Several moulds are also industrially valuable producers, *Penicillium* of penicillin and *Aspergillus niger* of citric acid.

1.3.3 Viruses

A virus is an obligate intracellular parasite consisting of a core of nucleic acid, either DNA or RNA but never both, enclosed in a protein coat called the capsid, sometimes surrounded by a lipid envelope. Viruses possess no metabolic machinery of their own and can replicate only by subverting that of a host cell. They cannot therefore be grown on inanimate culture media; their propagation requires living cells, supplied as cell cultures, embryonated eggs, or laboratory animals. This requirement makes cell culture, treated in Chapter 10, central to the manufacture of viral vaccines.

1.3.4 Protozoa and algae

Protozoa are unicellular eukaryotes, mostly free-living in soil and water, that lack a **cell wall** and are typically motile. A minority are human pathogens of pharmaceutical interest as targets of chemotherapy, notably *Plasmodium* (malaria) and *Entamoeba histolytica* (amoebic dysentery). Algae are photosynthetic eukaryotes; their principal relevance to pharmacy is as the source of agar,

the polysaccharide gelling agent extracted from certain marine algae that makes solid culture media possible, and as a source of some pigments and nutritional supplements.

> **Quick recall.** Prokaryotes (bacteria) lack a true nucleus; eukaryotes (fungi, protozoa, algae) possess one; viruses are acellular and need a host cell. Bacterial shapes: coccus, bacillus, vibrio, spirillum, spirochaete. Actinomycetes (*Streptomyces*) supply most antibiotics; agar comes from algae.

1.4 Structure of the bacterial cell

Because bacteria dominate the subject, their structure is treated in detail. The components of a generalised bacterial cell are shown in Figure 1.3 and may be divided into those that are invariably present and those that are present in some species only.

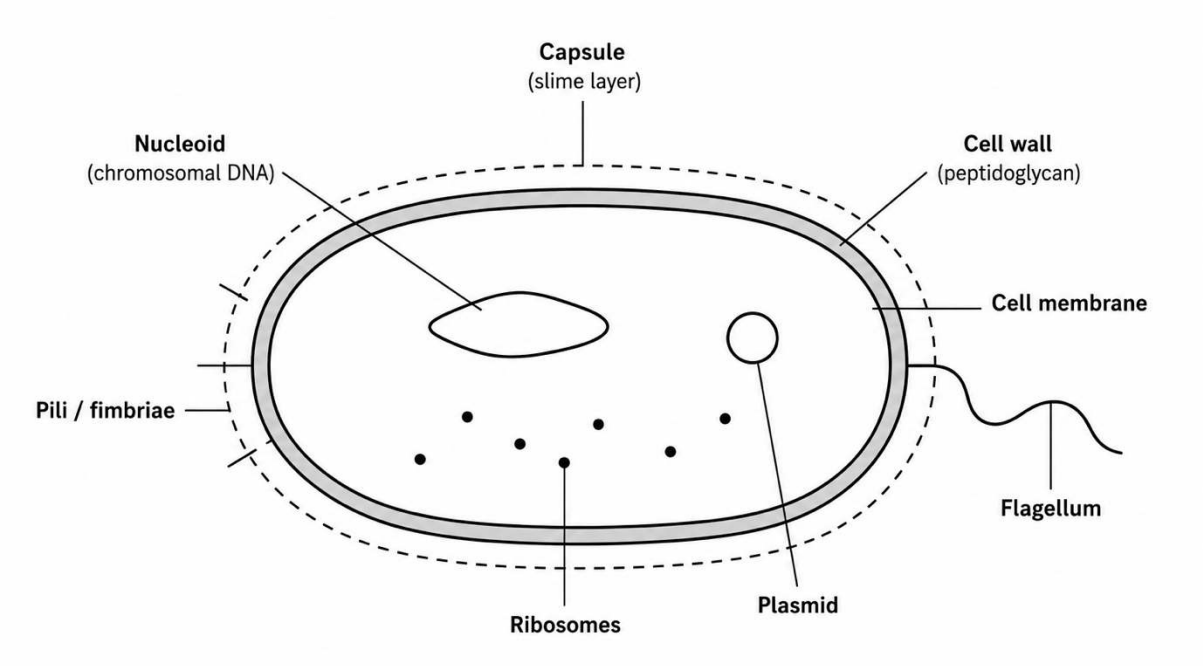


Figure 1.3 *Schematic of a generalised bacterial cell.*

The cell wall lies outside the cell membrane and is present in almost all bacteria. It gives the cell its characteristic shape and protects it against rupture by the high internal osmotic pressure. Its essential structural polymer is **peptidoglycan**, a macromolecule of glycan chains cross-linked by short peptides. The amount and arrangement of peptidoglycan, together with the presence or absence of an **outer membrane**, divide bacteria into the two great groups distinguished by the Gram stain, which is treated in Chapter 2 and summarised in Table 1.2. In the **Gram-positive** cell the wall is a thick, uniform layer of peptidoglycan. In the **Gram-negative** cell the peptidoglycan layer is thin and is overlaid by an additional outer membrane containing lipopolysaccharide, the lipid portion of which is the heat-stable endotoxin responsible for fever when such organisms or their fragments enter the bloodstream.

Table 1.2: Comparison of the Gram-positive and Gram-negative cell wall

Feature	Gram-positive	Gram-negative
Peptidoglycan layer	Thick	Thin
Outer membrane	Absent	Present (contains lipopolysaccharide)
Endotoxin (lipopolysaccharide)	Absent	Present
Gram reaction (final colour)	Purple (retains crystal violet)	Pink/red (takes safranin)
Representative genera	*Staphylococcus*, *Streptococcus*, *Bacillus*	*Escherichia*, *Pseudomonas*, *Salmonella*

The **cell (cytoplasmic) membrane** lies immediately beneath the wall. It is a selectively permeable lipid bilayer that controls the passage of substances into and out of the cell; lacking the organelles of the eukaryote, it also carries out functions such as respiration that in eukaryotes occur in mitochondria. Within the membrane lies the cytoplasm, which contains the **nucleoid**, the single circular chromosome that constitutes the essential genetic material; the **ribosomes**, the sites of protein synthesis, which in bacteria are of the 70S type and are an important target of several antibiotics; and frequently one or more **plasmids**, small circular molecules of DNA additional to the chromosome. Plasmids are of disproportionate importance because they often carry genes for antibiotic resistance and can be transferred between bacteria.

Several structures occur in some bacteria only. The **capsule** is a layer of polysaccharide slime external to the wall that resists desiccation, promotes adhesion to surfaces, and protects pathogenic species against the host defences; the capacity to adhere and to produce slime underlies the formation of biofilms on equipment and pipework, a recurring problem in manufacture. Flagella are long filaments responsible for motility. Pili (fimbriae) are short surface appendages concerned with adhesion and, in the case of the sex pilus, with the transfer of genetic material. The most resistant structure of all, the bacterial endospore, is treated separately because of its importance to sterilization.

1.5 The bacterial endospore

A bacterial endospore is a dormant, highly resistant resting body formed within the vegetative cell by certain Gram-positive genera, principally *Bacillus* (aerobic) and *Clostridium* (anaerobic), in

response to nutrient depletion or adverse conditions. One vegetative cell forms one endospore, so sporulation is a survival strategy rather than a means of multiplication. On the return of favourable conditions the spore germinates to give a single vegetative cell.

The extraordinary resistance of the endospore is the reason it dominates the design of sterilization processes. The resistance arises from several features acting together: a dehydrated core of very low water content; a large quantity of calcium dipicolinate, which stabilises the spore constituents; layers of specialised protein in the spore coat and the cortex; and protective small acid-soluble proteins that shield the spore DNA. As a result the endospore withstands heat, desiccation, radiation and many chemical agents that readily kill vegetative cells. The practical consequences are direct. A sterilization process must be designed to destroy the endospore, not merely the vegetative cell; the reference organisms used to challenge sterilizers are spore-formers (Chapter 8); and the persistence of spores in raw materials and on surfaces is a constant concern in manufacture. This single fact, that the spore is the hardest target, should be held in mind throughout the chapters on sterilization.

> **Quick recall.** The endospore is a dormant, heat- and chemical-resistant body formed by *Bacillus* and *Clostridium*. Its resistance (low water, calcium dipicolinate, protective coats) makes it the target every sterilization process must defeat.

1.6 Microbial nutrition and cultivation

To study, identify, count, or exploit a microorganism it must first be grown, and growth requires that the organism's nutritional and physical needs be supplied. Cultivation is therefore the practical foundation of the whole subject.

1.6.1 Nutritional requirements

Every microorganism requires a source of carbon, a source of energy, a source of nitrogen, various mineral elements, water, and, for some, specific growth factors such as vitamins or amino acids that the organism cannot synthesise for itself. Microorganisms are classified nutritionally by their carbon and energy sources. By carbon source they are autotrophs, which use carbon dioxide as the sole carbon source, or heterotrophs, which require organic carbon; almost all organisms of pharmaceutical interest are heterotrophs. By energy source they are phototrophs, which use light, or chemotrophs, which use chemical oxidation. The pathogens and the industrial producers of pharmaceutical importance are chemoheterotrophs that derive both carbon and energy from organic compounds.

1.6.2 Culture media

A **culture medium** is a preparation of nutrients used for the laboratory cultivation of microorganisms. A satisfactory medium supplies the required carbon, nitrogen, mineral and

growth-factor needs in available form, at a suitable pH and osmotic pressure, and is itself sterile before use. Media are classified on three independent bases (Figure 1.4).

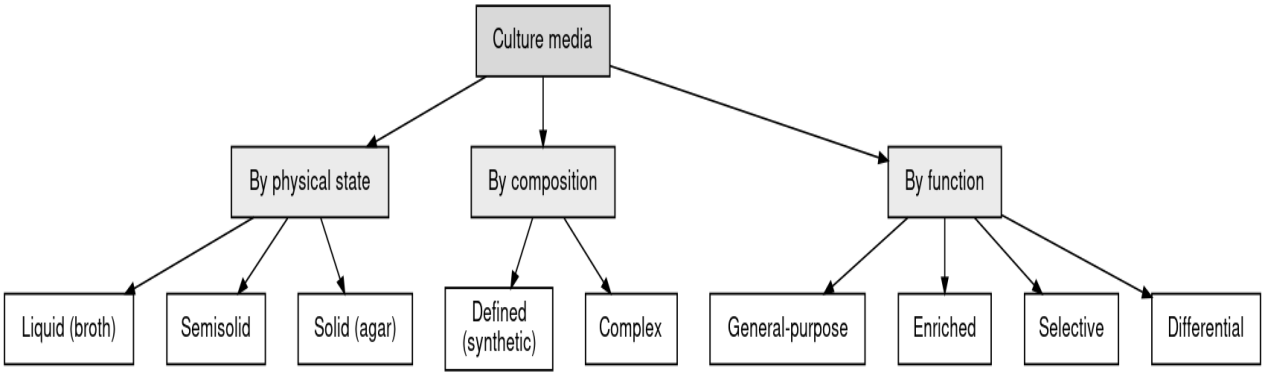


Figure 1.4 *Classification of culture media by physical state, composition, and function.*

By physical state, a medium is liquid, semisolid, or solid. A liquid medium (broth) contains no gelling agent and is used to grow organisms in bulk and to detect growth as turbidity. A solid medium is a broth gelled with about 1.5 to 2.0 per cent agar and is used to obtain isolated colonies, to maintain stock cultures, and to study **colony** characters. Agar is the preferred gelling agent for two reasons: it is not attacked by the great majority of microorganisms, and it melts near 100 degrees Celsius but does not resolidify until cooled to about 40 degrees Celsius, so inoculation is possible at a temperature the organism survives. A semisolid medium contains a reduced quantity of agar and is used to demonstrate motility.

By chemical composition, a medium is defined or complex. A defined (synthetic) medium is one in which every constituent is known in both identity and quantity. A complex medium contains ingredients of variable and incompletely known composition, such as peptone, beef extract, and yeast extract, which supply a rich mixture of nutrients; most routine media are complex.

By functional type, media are described as general-purpose, enriched, selective, differential, or transport. A general-purpose medium (for example nutrient agar) supports a wide range of organisms. An enriched medium has an added rich supplement such as blood or serum for fastidious organisms. A selective medium contains an agent, such as a dye, a bile salt, or a raised salt concentration, that suppresses unwanted organisms and allows the wanted one to grow. A differential medium contains an indicator that allows different organisms to be distinguished by a visible change, such as a colour shift around the colony. The student need not memorise individual recipes, but must know the purpose of each functional class, because the choice of medium determines whether a count is accurate or a contaminant detected, and the same classes reappear in the microbial limit tests of Chapter 9.

1.6.3 Physical conditions of cultivation

Growth depends on physical conditions as well as on nutrients. The principal factors are temperature, pH and the gaseous atmosphere. By optimum temperature, organisms are classified as psychrophiles (cold-loving), mesophiles (optimum about 25 to 40 degrees Celsius, the group that includes most human pathogens and contaminants), and thermophiles (heat-loving). By requirement for oxygen, organisms are classified as obligate aerobes, which require oxygen; obligate anaerobes, for which oxygen is toxic; facultative anaerobes, which grow with or without oxygen; and microaerophiles, which require oxygen at reduced tension. Most media are adjusted to a near-neutral pH for bacteria and to an acidic pH for fungi, a difference exploited in the media used to count bacteria and fungi separately.

1.7 Isolation of pure cultures

A natural sample, such as water, a raw material, or a swab, contains a mixture of organisms, whereas study and identification require a pure culture, a population descended from a single cell. The principle of isolation is to separate the cells of the mixture so widely on a solid medium that each gives rise to a discrete, visible colony, a colony being by convention the progeny of a single viable cell or clump. A pure culture is then obtained by picking a single well-separated colony.

Three techniques achieve this separation. In the streak-plate method, a loopful of the sample is drawn across the surface of an agar plate in a series of strokes that progressively dilute the inoculum, so that the final strokes deposit individual cells that grow into isolated colonies. In the pour-plate method, successive dilutions of the sample are mixed with molten agar and poured into plates, so that colonies develop both on and within the medium as separated points. In the spread-plate method, a small measured volume of a dilution is spread evenly over the surface of a set agar plate with a sterile spreader. The pour-plate and spread-plate methods are also the basis of the **viable count**, considered next.

1.8 Enumeration of microorganisms

The estimation of the number of microorganisms in a sample is fundamental to pharmaceutical microbiology, because limits and specifications are expressed as numbers and because a claim of contamination or of cleanliness has meaning only when quantified. Methods of enumeration fall into two classes, and the distinction between them is the point most often tested.

A viable count measures only those cells able to grow and divide. The standard method is the plate count. The sample is diluted in a series of known steps (a serial dilution), a known volume of an appropriate dilution is plated by the pour-plate or spread-plate method, the plates are incubated, and the colonies are counted on a plate bearing a statistically reliable number, conventionally taken as 30 to 300. The count in the original sample is obtained by multiplying the colony number by the dilution factor and dividing by the volume plated, and is expressed in **colony-forming units (CFU)**

per millilitre or per gram. The term colony-forming unit, rather than cell, acknowledges that a colony may arise from a clump as well as from a single cell. The strength of the viable count is that it counts only living organisms; its limitations are that it requires incubation and so gives a delayed result, and that clumped cells are counted once.

A **total count** measures all cells present, whether living or dead. It is performed by direct microscopic counting in a ruled counting chamber (a haemocytometer) or by an automated particle counter. Its advantage is speed; its limitation is that it cannot distinguish living from dead cells and so overstates the viable population, particularly after a process that has killed cells without removing them. Turbidimetry, the measurement of the light scattered by a suspension, gives a rapid indirect estimate of total biomass once calibrated against a count, and is used to follow growth in liquid culture and in the turbidimetric microbial assay of Chapter 9.

> **Worked example 1.1.** A water sample is diluted ten-fold three times in succession to give a final dilution of 1 in 1000. One millilitre of this dilution is plated and yields 180 colonies after incubation. Calculate the viable count of the original sample.
> Dilution factor = 10 × 10 × 10 = 1000. Count in original sample = (colonies × dilution factor) ÷ volume plated = (180 × 1000) ÷ 1 mL = 180 000 CFU/mL, that is 1.8×10^5 CFU/mL. The raw figure of 180 colonies is only the number on the diluted plate; the reported result is the concentration in the undiluted sample.

1.9 The microbial growth curve

When a liquid medium is inoculated with a small number of bacteria and the viable count is followed with time, the population passes through a characteristic sequence of phases. A plot of the logarithm of the viable count against time, the growth curve, shows four phases (Figure 1.5).

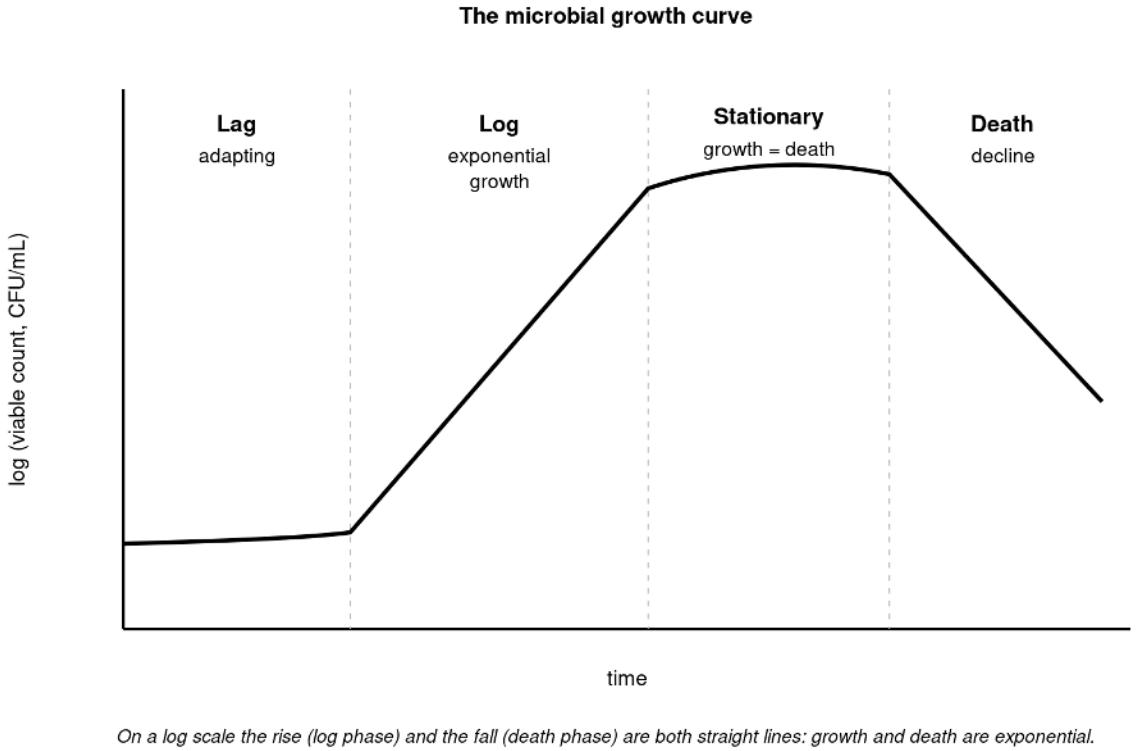


Figure 1.5 *The four phases of the microbial growth curve.*

In the lag phase the viable count does not increase. The cells are metabolically active but are adapting to the medium and are synthesising the enzymes and intermediates needed for growth; no division occurs until this preparation is complete. In the logarithmic (exponential) phase the cells divide at a constant maximum rate, the population doubling at a fixed interval called the generation time, and the count rises as a straight line on the logarithmic plot. Cells in this phase are physiologically uniform and most susceptible to antibiotics, which is why this phase is used for physiological study. In the stationary phase the rate of division equals the rate of death, and the viable count remains constant; the plateau is reached because nutrients are exhausted and toxic products accumulate. Spore-forming bacteria characteristically sporulate during this phase. In the death (decline) phase the viable count falls as cells die faster than they are replaced, frequently at a constant exponential rate, so that the decline is a straight line on the logarithmic plot.

The exponential character of both growth and death is fundamental. During exponential growth the number of cells doubles in each generation time, so that the population increases as a geometric progression; during the death phase the population falls by a constant fraction in each equal interval. The exponential nature of microbial death is the basis of the decimal reduction time and of the entire quantitative treatment of sterilization in Chapter 8, where it leads to the conclusion that sterility can be expressed only as a probability.

The generation time is obtained from the exponential phase. If a population grows from an initial number N0 to a number N after n divisions, then $N = N0 \times 2^n$, since the number doubles at each

division. The number of generations over a time interval t is n = t ÷ g, where g is the generation time, and g is found by rearrangement.

> **Worked example 1.2.** A culture grows from 1×10^3 to 1×10^6 cells in 5 hours during the exponential phase. Calculate the number of generations and the generation time.
> From $N = N0 \times 2^n$: $2^n = N \div N0 = 10^6 \div 10^3 = 1000$. Therefore $n = \log_2(1000) = \log(1000) \div \log(2) = 3 \div 0.301 = 9.97$, that is approximately 10 generations. Generation time g = t ÷ n = 5 hours ÷ 10 = 0.5 hours, or 30 minutes.

1.10 Pharmaceutical importance of microorganisms

The pharmaceutical importance of microorganisms may now be drawn together. On the harmful side, microorganisms contaminate and spoil medicines and cause infection, and their control is the dominant theme of Chapters 3 to 10. On the beneficial side, they are the source of a wide range of medicinal products, summarised in Table 1.3, manufactured by the process of fermentation described in Chapter 4. In addition, microorganisms serve as test organisms in the microbial assay of antibiotics and vitamins (Chapter 9) and in the evaluation of disinfectants (Chapter 6), and they provide the model systems on which much of molecular biology and biotechnology is founded.

Table 1.3: Representative pharmaceutical products obtained from microorganisms

Product	Class	Producing organism
Penicillin	Antibiotic	*Penicillium chrysogenum* (mould)
Streptomycin	Antibiotic (aminoglycoside)	*Streptomyces griseus* (actinomycete)
Cephalosporin C	Antibiotic (β-lactam)	*Acremonium chrysogenum* (mould)
Citric acid	Organic acid	*Aspergillus niger* (mould)
Glutamic acid, lysine	Amino acids	*Corynebacterium glutamicum*
Vitamin B12, riboflavin	Vitamins	Bacteria and fungi
Human insulin	Recombinant protein	Engineered *Escherichia coli* or yeast

1.11 Summary

Microbiology is the study of microorganisms, and pharmaceutical microbiology applies it to the manufacture, quality and safe use of medicines, with the dual aim of excluding harmful organisms and exploiting useful ones. Microorganisms are classified by cellular organisation into prokaryotes (bacteria), eukaryotes (fungi, protozoa, algae), and acellular viruses, each group having defining features and characteristic pharmaceutical relevance. The bacterial cell is bounded by a peptidoglycan wall whose structure underlies the Gram division and, in Gram-negative organisms, carries the endotoxin; within lie the nucleoid, the 70S ribosomes, and frequently resistance-bearing plasmids. The bacterial endospore, formed by *Bacillus* and *Clostridium*, is the most resistant of all microbial structures and is the target every sterilization process must defeat.

Cultivation requires that nutritional needs (carbon, nitrogen, minerals, growth factors) and physical conditions (temperature, pH, oxygen) be supplied on a culture medium, classified by physical state, chemical composition, and function. Pure cultures are isolated by the streak-, pour-, and spread-plate methods, and microorganisms are enumerated by a viable count (living cells, in CFU) or a total count (living and dead). The growth curve shows lag, logarithmic, stationary and death phases; growth and death are exponential, a fact that underlies the determination of generation time and the quantitative treatment of sterilization. Microorganisms are, finally, the source of antibiotics, vitamins, amino acids, and therapeutic proteins, and the test organisms of microbiological quality control. The next chapter takes up the microscope and the staining and biochemical methods by which these organisms are observed and identified.

1.12 Self-assessment

A. Multiple-choice questions

1. A prokaryotic cell is characterised by the absence of:

(a) a cell wall (b) ribosomes (c) a true membrane-bound nucleus (d) cytoplasm. Answer: (c).

2. The gelling agent used in solid culture media is obtained from:

(a) bacteria (b) algae (c) protozoa (d) viruses. Answer: (b).

3. The bacterial endospore is formed characteristically by the genera:

(a) *Staphylococcus* and *Streptococcus* (b) *Escherichia* and *Pseudomonas* (c) *Bacillus* and *Clostridium* (d) *Candida* and *Aspergillus*. Answer: (c).

4. A viable count is reported in:

(a) cells per mL (b) colony-forming units per mL (c) grams per litre (d) optical density units. Answer: (b).

5. During which phase of the growth curve is the generation time measured?

(a) lag (b) logarithmic (c) stationary (d) death. Answer: (b).

6. The heat-stable endotoxin of Gram-negative bacteria is a component of the:

(a) capsule (b) outer membrane lipopolysaccharide (c) peptidoglycan (d) flagellum. Answer: (b).

B. Short-answer questions (2 to 5 marks)

Q1. Define pharmaceutical microbiology and state its scope.

Pharmaceutical microbiology is the applied branch of microbiology concerned with the aspects of microorganisms that affect the manufacture, quality and safe use of medicines. Its scope is twofold: the control and exclusion of microorganisms that contaminate, spoil, or render medicines unsafe, and the exploitation of microorganisms in the manufacture of medicinal substances such as antibiotics, vitamins and vaccines.

Q2. Distinguish between a prokaryotic and a eukaryotic cell, with examples.

A prokaryotic cell lacks a true membrane-bound nucleus and membrane-bound organelles, its genetic material lying free as a single circular chromosome; bacteria are prokaryotes. A eukaryotic cell possesses a true nucleus enclosed by a nuclear membrane and contains membrane-bound organelles; fungi, protozoa and algae are eukaryotes. Marks are awarded for the nuclear distinction, the organelle distinction, and a correct example of each.

Q3. Classify culture media on the basis of functional type.

By function media are: general-purpose, which supports a wide range of organisms (nutrient agar); enriched, with a rich supplement such as blood for fastidious organisms; selective, with an agent that suppresses unwanted organisms; differential, with an indicator that distinguishes organisms by a visible change; and transport, for preserving a sample in transit. Each class is defined by its purpose.

Q4. Why is the bacterial endospore important in pharmaceutical sterilization?

The endospore is the most resistant microbial structure and withstands heat, desiccation, radiation and many chemicals because of its dehydrated core, calcium dipicolinate content, and protective coats. A sterilization process must therefore be designed to destroy the spore, not merely the vegetative cell, and spore-formers are used as the biological indicators that challenge sterilizers.

Q5. Differentiate a viable count from a total count.

A viable count measures only cells able to grow and divide, typically by the plate count, expressed in CFU; it counts living cells but is slow and counts clumps once. A total count measures all cells, living and dead, by direct microscopy or an instrument; it is rapid but cannot distinguish living from dead cells. The viable count is used to judge a real microbial load, the total count where speed matters and viability does not.

C. Long-answer questions (10 marks)

Q1. Describe the structure of a typical bacterial cell with a labelled diagram, and state the function of each component.

A complete answer reproduces Figure 1.3 with the cell wall, cell membrane, cytoplasm, nucleoid, ribosomes, plasmid, capsule, flagella and pili labelled, and gives the function of each: the wall (peptidoglycan) for shape and protection and as the basis of the Gram division; the membrane for selective permeability and respiration; the nucleoid as the essential genetic material; the 70S ribosomes for protein synthesis and as an antibiotic target; plasmids as carriers of resistance genes; the capsule for protection, adhesion and biofilm formation; flagella for motility; and pili for adhesion and genetic transfer.

Q2. Explain the microbial growth curve and derive the generation time, with a worked example.

A complete answer draws the four-phase curve (lag, logarithmic, stationary, death) on a logarithmic axis, explains the physiological state of the cells in each phase, derives the generation time from $N = N0 \times 2^n$ and $g = t \div n$, and works a numerical example such as Worked Example 1.2. Credit is given for stating that growth and death are exponential and noting the link to sterilization kinetics. (High-yield: the growth curve is among the most frequently examined topics in this unit.)

D. Numerical problem

A suspension is diluted 1 in 10^4 and 0.1 mL of the dilution is spread on a plate to give 250 colonies. Calculate the viable count per millilitre of the original suspension.

Count = (colonies × dilution factor) ÷ volume plated = $(250 \times 10^4) \div 0.1 = 250 \times 10^4 \times 10 = 2.5 \times 10^7$ CFU/mL.

References

1. Pelczar MJ, Chan ECS, Krieg NR. Microbiology: Concepts and Applications. New York: McGraw-Hill.
2. Prescott LM, Harley JP, Klein DA. Microbiology. New York: McGraw-Hill.
3. Denyer SP, Hodges NA, Gorman SP, editors. Hugo and Russell's Pharmaceutical Microbiology. 8th ed. Oxford: Wiley-Blackwell; 2011.
4. Indian Pharmacopoeia. Ghaziabad: Indian Pharmacopoeia Commission. [latest edition]

Chapter 2: Microscopy, Staining, Identification and Microbial Antibiotics

Learning outcomes

After studying this chapter the student will be able to:

- Describe the principles, construction and uses of the compound light microscope and the electron microscope, and distinguish magnification from resolution.
- Explain the principles of staining and describe simple, Gram's, and acid-fast staining with the mechanism of each.
- Describe the biochemical identification of bacteria, with particular reference to the **IMViC** tests.
- Define an **antibiotic** and outline the general process of its production by **fermentation**.
- Describe the production and therapeutic uses of streptomycin and of the cephalosporins.

These outcomes serve Course Outcomes CO1 and CO2, the explanation of basic concepts and the identification and differentiation of microorganisms by staining and biochemical techniques.

2.1 Introduction

The identification of a microorganism proceeds from what can be seen of it to what can be measured of its activity. Because microorganisms lie below the resolving power of the unaided eye, the microscope is the first instrument of the subject, revealing size, shape and arrangement. Cells are almost colourless, so a stain is applied to make them visible and, with a differential method, to divide them into groups by the chemistry of their walls. Two organisms that look and stain alike are then separated by their metabolism, read through biochemical tests. This chapter follows that sequence, from microscopy through staining to biochemical identification, and closes with the reverse application of the same microbiology, the use of microorganisms to manufacture antibiotics, with streptomycin and the cephalosporins as the prescribed examples.

2.2 The microscope

A microscope is an instrument that produces an enlarged image of an object too small to be seen by the unaided eye. Two properties define its performance, and the distinction between them is fundamental. **Magnification** is the degree to which the apparent size of the object is increased. **Resolution** (resolving power) is the smallest distance by which two points may be separated and still be distinguished as two; it sets the real limit of useful magnification, because enlarging an image beyond the resolution merely produces a larger blur. Resolution is governed by the wavelength of the radiation used to form the image: the shorter the wavelength, the finer the

resolution. The two principal instruments of microbiology, the compound light microscope and the electron microscope, differ chiefly in this respect, and are compared in Table 2.1 and Figure 2.1.

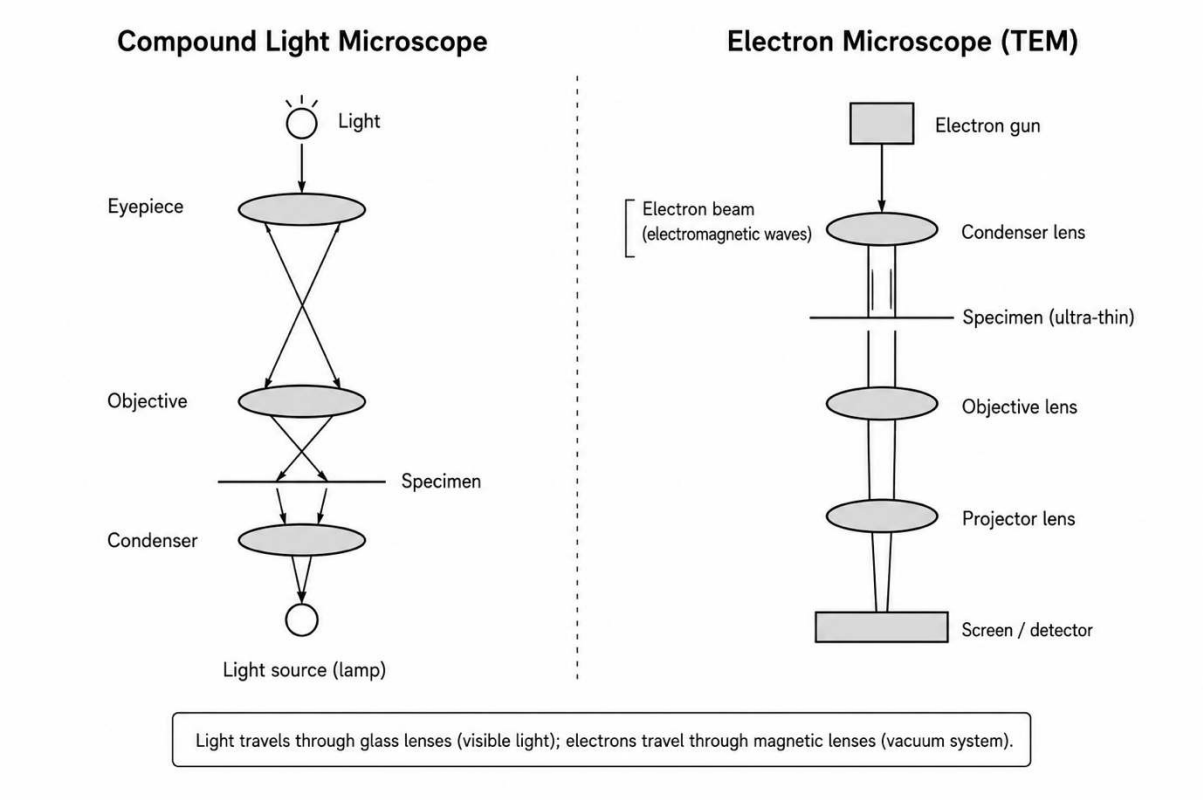


Figure 2.1 *The compound light microscope compared with the electron microscope.*

2.2.1 The compound light microscope

The compound light microscope forms its image with visible light and two systems of glass lenses arranged in series. The **objective lens**, close to the specimen, forms a real magnified image, which the **eyepiece** (ocular) lens further magnifies for the eye. The total magnification is the product of the objective and eyepiece magnifications; an objective of 100× used with an eyepiece of 10× gives a total of 1000×, the usual working magnification for bacteria. Light from a source below the stage is concentrated on the specimen by the **condenser**.

The parts of the instrument are conventionally divided into the mechanical and the optical. The mechanical parts comprise the base and the pillar that support the instrument, the stage on which the slide is held, the body tube, and the coarse and fine adjustment knobs by which the specimen is brought into focus. The optical parts comprise the light source or mirror, the condenser with its iris diaphragm, which controls the cone of light reaching the specimen, the objective lenses mounted on a revolving nosepiece, and the eyepiece. A working knowledge of these parts is expected, because the quality of an image depends as much on correct use of the condenser and diaphragm as on the lenses themselves.

The resolution of the light microscope is limited by the wavelength of visible light, which is a few hundred nanometres, to about 0.2 micrometres. This is sufficient to show the size, shape and arrangement of bacteria but not their internal fine structure, and it is insufficient to show viruses at all. To realise the full resolution at the highest magnification, the highest-power objective is used as an **oil-immersion objective**, the space between the specimen and the lens being filled with immersion oil of the same refractive index as glass, so that light which would otherwise be refracted away is gathered into the lens. Several specialised forms of the light microscope extend its use to unstained and living preparations. The dark-field microscope illuminates the specimen obliquely so that it appears bright against a dark background, and is valuable for thin, motile organisms such as spirochaetes that are difficult to stain. The phase-contrast microscope converts small differences in refractive index within a living cell into differences in brightness, allowing internal structures to be seen without staining. The fluorescence microscope uses ultraviolet light to excite fluorescent dyes (fluorochromes) bound to the specimen, and is the basis of immunofluorescence techniques used to detect specific organisms. These forms share the optical principles of the compound microscope and differ only in the manner of illumination.

2.2.2 The electron microscope

The electron microscope forms its image with a beam of electrons in place of light and with electromagnetic lenses in place of glass. Because the effective wavelength of a beam of accelerated electrons is far shorter than that of visible light, the resolution is improved by about a thousand-fold, to the order of 0.2 nanometres, so that viruses and the internal structures of cells become visible. Two types are distinguished. The **transmission electron microscope (TEM)** passes electrons through an ultra-thin section of the specimen to reveal internal structure. The **scanning electron microscope (SEM)** scans a beam across the surface of the specimen to produce a three-dimensional image of its exterior. The principal limitation follows from the method: the specimen must be examined in a vacuum, and is therefore dead and specially prepared, so a living organism can never be observed. The light microscope, of lower resolution, retains the unique ability to examine living cells. In pharmaceutical and biological work the electron microscope is used to determine the morphology of viruses, which lie below the resolution of the light microscope, to study the fine structure of bacterial cells and their surfaces, and to examine the structure of macromolecules and particulate materials. Specimen preparation is exacting: the material must be fixed, dehydrated, and either sectioned ultra-thin for the transmission instrument or coated with a thin film of metal for the scanning instrument, and these steps are a source of artefact that the microscopist must allow for.

Table 2.1: Comparison of the compound light microscope and the electron microscope

Feature	Compound light microscope	Electron microscope
Source of illumination	Visible light	Beam of electrons
Lenses	Glass	Electromagnetic
Resolution	about 0.2 µm	about 0.2 nm
Useful magnification	up to about 1000–1500×	up to several hundred thousand ×
Specimen	May be living	Dead, in vacuum, specially prepared
Shows	Shape and arrangement of bacteria	Viruses; fine internal and surface structure

> **Quick recall.** Magnification enlarges; resolution determines what detail can be distinguished and is set by wavelength. The light microscope resolves to about 0.2 µm and can view living cells; the electron microscope resolves to about 0.2 nm but the specimen must be dead and in vacuum.

2.3 Staining

A stain is a coloured compound used to impart colour to a microorganism so that it may be seen and studied under the microscope. Staining is necessary because microbial cells are nearly transparent and have a refractive index close to that of their surroundings. Most stains are salts in which the coloured ion, the chromophore, is either positively charged (a basic stain, such as methylene blue, **crystal violet**, or **carbol fuchsin**) or negatively charged (an acidic stain, such as eosin or nigrosin). Because the bacterial surface is negatively charged, basic stains, whose coloured ion is positive, are the ones generally taken up by the cell and are therefore the most widely used. Before staining, a film of the organisms is dried on a slide and heat-fixed, which kills and attaches the cells and makes them more receptive to the stain. Staining methods are of two broad kinds: simple staining, which uses a single stain to reveal morphology, and differential staining, which uses more than one reagent to distinguish groups of organisms.

2.3.1 Simple staining

In simple staining a single basic stain is applied to the fixed film for a short time and then washed off. The whole cell is coloured uniformly, and the preparation reveals the size, shape and arrangement of the organism but does not distinguish one type from another. Simple staining is used when only the morphology is required.

2.3.2 Gram's staining

Gram's stain, devised by Hans Christian Gram, is the most important differential stain in bacteriology, because it divides almost all bacteria into two great groups, the Gram-positive and the Gram-negative, that differ in cell-wall structure and, consequently, in many properties including antibiotic susceptibility. The procedure comprises four steps, shown in Figure 2.2:

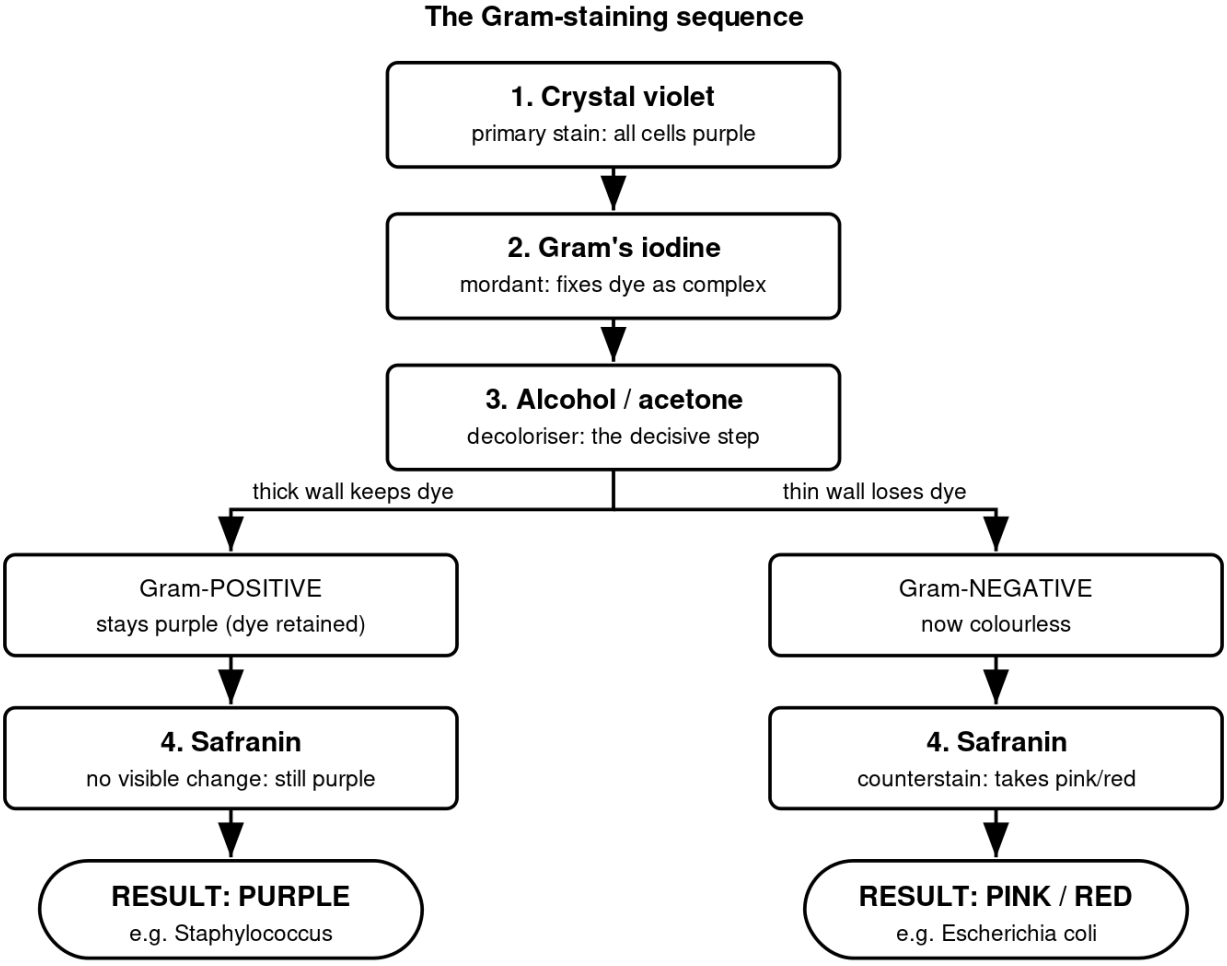


Figure 2.2 *The Gram-staining sequence and its two outcomes.*

1. The fixed film is flooded with the primary stain, crystal violet, which colours all cells purple.
2. **Gram's iodine** is applied as a mordant; it combines with the crystal violet to form a larger crystal violet–iodine complex within the cell that is less easily removed.
3. The film is treated briefly with a **decolouriser**, alcohol or acetone. This is the decisive and most error-prone step.
4. The film is counterstained with **safranin**, a red stain.

The result depends entirely on the cell wall. The Gram-positive cell, with its thick peptidoglycan layer, is dehydrated by the decolouriser; the pores of the peptidoglycan close, the crystal violet–iodine complex is retained, and the cell remains purple, the safranin having no visible effect. The Gram-negative cell has only a thin peptidoglycan layer beneath a lipid-rich outer membrane; the decolouriser dissolves the outer membrane and the thin peptidoglycan cannot retain the complex, so the cell is decolourised and then takes up the safranin to appear pink. The common error to guard against is over-decolourisation, which removes the complex even from Gram-positive cells and causes them to be reported, falsely, as Gram-negative; the timing of the decolourising step is therefore critical. The structural basis of the difference is summarised in Table 1.2 of Chapter 1.

2.3.3 Acid-fast staining

A small but important group of bacteria, chiefly the genus *Mycobacterium*, the cause of tuberculosis and leprosy, resists ordinary staining because the cell wall contains a high proportion of a waxy lipid, **mycolic acid**, which repels aqueous stains. The acid-fast stain, also called the Ziehl–Neelsen method, is designed to drive a stain through this barrier and then test whether the cell retains it against acid. The primary stain, carbol fuchsin, is applied and the slide is heated to soften the wax and allow penetration. The film is then treated with **acid-alcohol** as the decolouriser, and counterstained with methylene blue. Cells that resist decolourisation by acid and retain the red carbol fuchsin are termed acid-fast and appear red; cells that are decolourised take up the counterstain and appear blue. *Mycobacterium tuberculosis* therefore appears as red rods against a blue background in a stained sputum film. ### 2.3.4 Special stains

Beyond the routine differential stains, several special stains demonstrate particular structures. In **negative staining** the background is coloured with an acidic stain such as nigrosin while the cell itself is left unstained, so that the cell stands out as a clear shape; the method avoids heat fixation and is used for cells difficult to stain and to demonstrate the **capsule**, which appears as a clear halo around the cell. The **endospore stain** (the Schaeffer–Fulton method) uses malachite green driven into the resistant spore by heat, with safranin as a counterstain for the vegetative cell, so that spores appear green within red cells; it demonstrates the position and shape of the endospore, features of diagnostic value in the spore-forming genera. Flagella stains, which deposit a mordant to thicken the flagella enough to be seen, complete the list of special methods a student should recognise. These stains are required less often than the Gram and acid-fast methods but are part of the standard repertoire of bacterial identification.

> **Quick recall.** Simple stain: one basic stain, morphology only. Gram's stain: crystal violet → iodine (mordant) → alcohol (decolouriser) → safranin; purple is Gram-positive (thick peptidoglycan), pink is Gram-negative (thin wall, outer membrane). Acid-fast (Ziehl–Neelsen): carbol fuchsin with heat → acid-alcohol → methylene blue; red is acid-fast (*Mycobacterium*).

2.4 Biochemical identification: the IMViC tests

Two bacteria may share both morphology and Gram reaction yet differ in metabolism, and the final separation of such organisms rests on biochemical tests, which detect the enzymes an organism possesses by the products it forms. The most important set in the present syllabus is the group used to differentiate the enteric (intestinal) Gram-negative bacilli, especially to distinguish *Escherichia coli*, an indicator of faecal pollution, from the related *Enterobacter* and *Klebsiella*. The set is known by the acronym IMViC, in which the letters denote four tests; the lower-case "i" is inserted only for pronunciation. The four tests and their interpretation are set out below and as a chart in Figure 2.3.

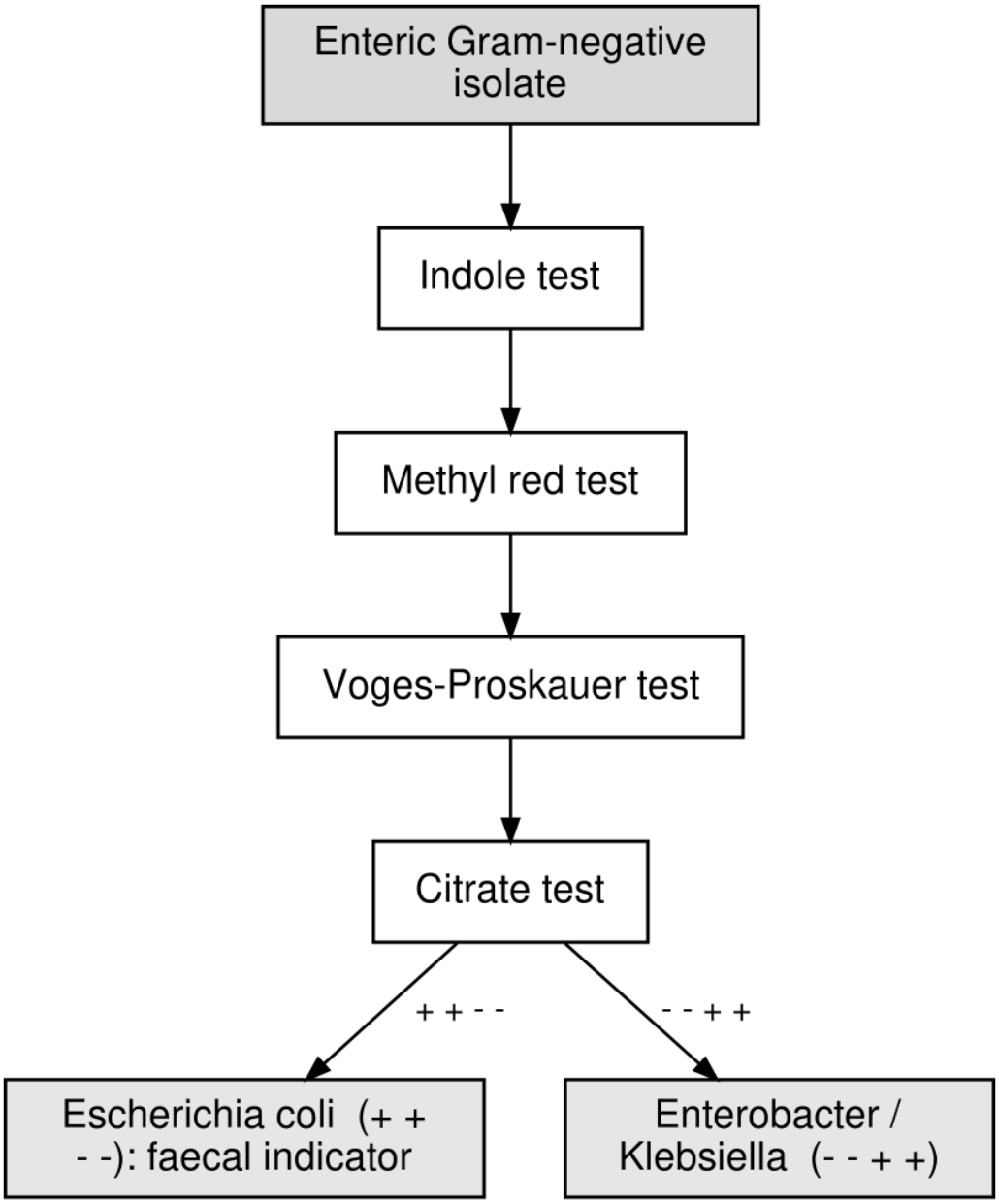


Figure 2.3 *The IMViC scheme separating Escherichia coli from Enterobacter and Klebsiella.*

The **indole test** detects the enzyme tryptophanase, which splits the amino acid tryptophan to release indole. After growth in a tryptophan-containing medium, Kovác's reagent is added; a red ring indicates indole and a positive result.

The **methyl red test** detects organisms that ferment glucose by the mixed-acid pathway, producing enough stable acid to lower the pH below about 4.4. The indicator methyl red is added to the culture; a red colour, persisting at this low pH, is a positive result.

The **Voges–Proskauer test** detects the alternative, butylene-glycol, pathway of glucose fermentation, in which the neutral product acetoin is formed. On addition of the appropriate reagents a red colour develops if acetoin is present. The methyl red and Voges–Proskauer tests examine two mutually alternative fermentation pathways, so an organism is generally positive for one and negative for the other.

The **citrate test** determines whether an organism can use citrate as its sole source of carbon. On Simmons citrate medium a positive organism grows and raises the pH, turning the indicator from green to blue.

The diagnostic value of the set lies in a clean and frequently examined contrast: *Escherichia coli* gives the pattern positive, positive, negative, negative (indole +, methyl red +, Voges–Proskauer −, citrate −), whereas the *Enterobacter–Klebsiella* group gives the reciprocal pattern negative, negative, positive, positive. Four inexpensive tests thus separate an organism that signals faecal contamination from one that does not, a distinction applied directly in the water testing of Chapter 10. The patterns are tabulated in Table 2.2.

Table 2.2: Typical IMViC reactions of two enteric bacteria

Test	*Escherichia coli*	*Enterobacter aerogenes* / *Klebsiella*
Indole	+	−
Methyl red	+	−
Voges–Proskauer	−	+
Citrate	−	+

> **Quick recall.** IMViC = Indole, Methyl red, Voges–Proskauer, Citrate. *Escherichia coli* gives + + − −; the *Enterobacter–Klebsiella* group gives − − + +. The methyl red and Voges–Proskauer tests examine two alternative glucose-fermentation pathways, so their results are usually opposite, and the overall contrast separates a faecal indicator from a non-indicator.

Several other biochemical tests are in routine use for the identification of the Gram-positive cocci and other organisms, and the student should recognise the commonest. The **catalase test** detects the enzyme catalase, which breaks down hydrogen peroxide with effervescence; it distinguishes the catalase-positive staphylococci from the catalase-negative streptococci. The **coagulase test** detects the enzyme coagulase, which clots plasma, and identifies the pathogen *Staphylococcus aureus* among the staphylococci. The **oxidase test** detects cytochrome c oxidase and helps to separate the oxidase-positive *Pseudomonas* from the oxidase-negative enterobacteria. The **urease test** detects the hydrolysis of urea and is used in the identification of *Proteus* and certain other genera. Sugar-fermentation tests, which record acid and gas production from individual sugars, complete the routine battery. These tests, used in combination, allow an isolate to be identified to genus and species, and they share with the IMViC set the principle that an organism is recognised by the enzymes it possesses.

In modern laboratories the individual tube tests are increasingly replaced by miniaturised and automated systems. Commercial identification strips carry a row of dehydrated biochemical substrates that are inoculated together, incubated, and read as a pattern of reactions converted to a numerical profile that identifies the organism from a database. Fully automated instruments perform the inoculation, incubation, and reading, and combine identification with antibiotic-susceptibility testing. The principle is unchanged from the manual tests, namely the recognition of an organism by its metabolic reactions, but the throughput and reproducibility are far greater, which matters in a pharmaceutical quality-control laboratory testing many samples to a defined procedure.

2.5 Antibiotics produced by microorganisms

The microbiology of the preceding sections is applied in reverse in the manufacture of antibiotics, where the metabolic capacity of the microorganism is used to produce the drug. An antibiotic is, in the classical definition, a substance produced by a microorganism that, in low concentration, inhibits or kills other microorganisms; the term is now extended to include semi-synthetic and synthetic analogues of such substances. Antibiotics may be classified by chemical structure (for example **β-lactam**s, **aminoglycoside**s, tetracyclines, macrolides), by spectrum of activity (broad or narrow), or by mode of action (inhibition of cell-wall synthesis, of protein synthesis, of nucleic acid synthesis, or of membrane function). The two examples prescribed by the syllabus, streptomycin and the cephalosporins, illustrate two structural classes and two modes of action.

Classification by mode of action is the most instructive for the pharmacy student, because it links the drug to the bacterial target it attacks. Five principal modes are recognised. Inhibitors of cell-wall synthesis, which include the β-lactams (penicillins and cephalosporins) and glycopeptides such as vancomycin, prevent the cross-linking of peptidoglycan and are selectively toxic because the human cell has no wall. Inhibitors of protein synthesis, which include the aminoglycosides (such as streptomycin), tetracyclines, macrolides, and chloramphenicol, bind the bacterial 30S or

50S ribosomal subunit, which differs from the human 80S ribosome. Inhibitors of nucleic acid synthesis include the fluoroquinolones, which inhibit DNA gyrase, and rifampicin, which inhibits bacterial RNA polymerase. Inhibitors of folate synthesis, the sulphonamides and trimethoprim, block the synthesis of the folate that bacteria, unlike humans, must make for themselves. Agents that disrupt the cell membrane, such as the polymyxins, complete the scheme. The principle that unites the useful drugs is **selective toxicity**: each acts on a structure or pathway present in the bacterium but absent or different in the host.

2.5.1 General process of antibiotic production

Most antibiotics are manufactured by fermentation, the large-scale controlled cultivation of the producing microorganism, treated in detail in Chapter 4. The general sequence, shown in Figure 2.4, is as follows. A high-yielding strain of the producer is maintained as a stock culture and grown up in stages, through shake flasks and seed tanks, to provide a large, active inoculum (inoculum development). This is transferred to the production fermenter, a sterilised, aerated, stirred vessel of many thousands of litres, in which the organism is grown under controlled temperature, pH and aeration while it secretes the antibiotic into the medium. The fermentation is followed by **downstream processing**, the recovery and purification of the antibiotic by filtration, solvent extraction, and crystallisation, and finally by formulation. Antibiotics are characteristically **secondary metabolites**, compounds not required for the growth of the producing organism itself and formed most actively after the phase of rapid growth, in contrast to the primary metabolites (such as amino acids) that are products of normal growth. For this reason the fermentation is managed in two stages, building cell mass first and then holding the culture in the conditions that favour product formation. The yield obtained from a wild strain is far too low for economic manufacture, so the producing strains used in industry are the result of prolonged **strain improvement** by mutation and selection, which has raised the yield of penicillin, for example, several thousand-fold over the original isolate. The medium, too, is optimised, often using inexpensive substrates such as corn-steep liquor as a nitrogen source. These principles of the fermentation process are developed in Chapter 4.

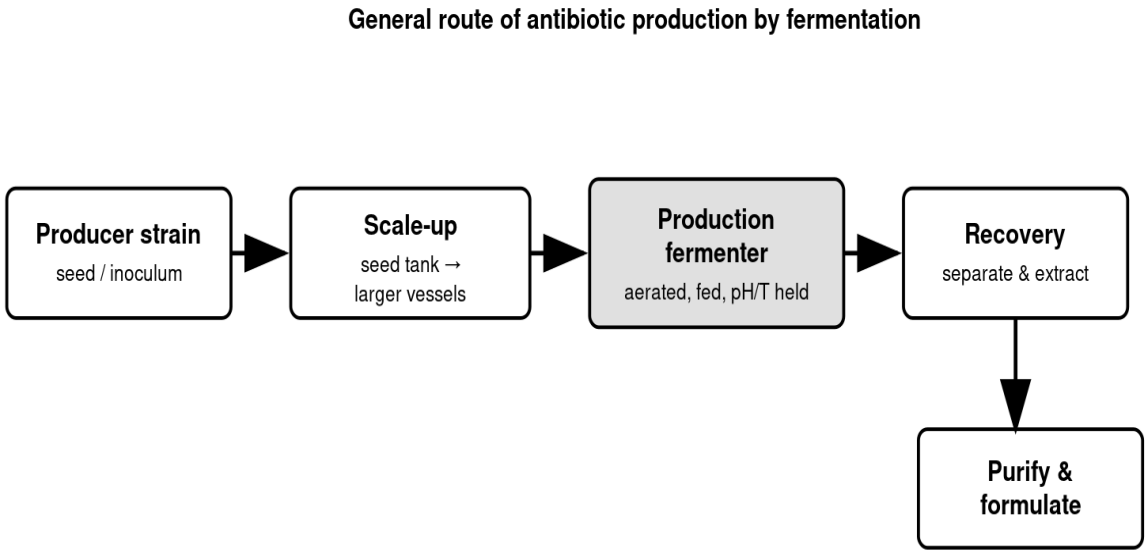


Figure 2.4 *General route of antibiotic production by fermentation.*

2.5.2 Streptomycin

Streptomycin was isolated in 1943 from the soil actinomycete *Streptomyces griseus* by Waksman and his colleagues, and was the first antibiotic effective against tuberculosis. It is the first member of the aminoglycoside class, a group of water-soluble, basic antibiotics composed of amino sugars joined by glycosidic linkages. It is produced by submerged fermentation of *S. griseus* and recovered from the broth by adsorption and purification.

Streptomycin acts by inhibiting bacterial protein synthesis: it binds irreversibly to the 30S subunit of the bacterial ribosome, which causes misreading of the genetic code and arrest of synthesis, an action that is bactericidal. Its spectrum includes several Gram-negative bacilli and, importantly, *Mycobacterium tuberculosis*; it is used, always in combination to limit resistance, in the treatment of tuberculosis, plague and certain other serious infections. Its principal adverse effects are ototoxicity (damage to hearing and balance) and nephrotoxicity, and the development of bacterial resistance is rapid when it is used alone. For these reasons it is now a reserve agent rather than a first-line drug, but its historical and pharmacological importance is undiminished. Streptomycin was the first of a class that now includes gentamicin, amikacin, kanamycin, and neomycin, all sharing the aminoglycoside structure, the action on the 30S ribosome, the bactericidal effect on aerobic Gram-negative bacilli, and the characteristic ototoxicity and nephrotoxicity; because they are not absorbed from the gut, they are given by injection for systemic infection. The isolation of streptomycin from a systematic search of soil actinomycetes also established the strategy by which most later antibiotics were discovered, namely the screening of soil microorganisms for antibacterial activity, and it is for this body of work that the field of antibiotic discovery from actinomycetes is chiefly remembered.

2.5.3 Cephalosporins

The cephalosporins originate from the fungus *Acremonium chrysogenum* (formerly *Cephalosporium acremonium*), isolated by Brotzu in 1945 from a sewage outfall in Sardinia. The active principle, **cephalosporin C**, was later isolated and characterised at Oxford, and from its nucleus, **7-aminocephalosporanic acid (7-ACA)**, the large family of semi-synthetic cephalosporins in present use is derived by chemical substitution. The cephalosporins are therefore produced in two stages: fermentation of *A. chrysogenum* to yield cephalosporin C, followed by chemical conversion to 7-ACA and substitution to give the individual drugs.

Cephalosporins are β-lactam antibiotics, structurally related to the penicillins and sharing their mode of action: they inhibit the synthesis of the bacterial cell wall by binding the enzymes (penicillin-binding proteins) that cross-link peptidoglycan, so that the wall is weakened and the cell undergoes osmotic lysis. They are bactericidal and, as a class, broad in spectrum. They are conventionally grouped into successive generations (first to fifth), later generations having, in general, greater activity against Gram-negative organisms and greater stability to the bacterial enzymes (β-lactamases) that destroy earlier β-lactams. Familiar examples by generation include cefalexin and cefazolin (first generation), cefuroxime (second), ceftriaxone and cefotaxime (third), cefepime (fourth), and ceftaroline (fifth), the progression showing the steady extension of Gram-negative activity. Their advantages over the early penicillins, namely greater stability to these enzymes and a broader spectrum, account for their extensive use in hospital practice. ### 2.5.4 Antibiotic resistance

The therapeutic value of any antibiotic is limited by the capacity of bacteria to become resistant to it, and an account of antibiotic production is incomplete without reference to resistance. Bacteria resist antibiotics by several mechanisms: enzymatic destruction or modification of the drug, of which the hydrolysis of β-lactams by β-lactamases is the most important example; alteration of the drug target, such as a modified penicillin-binding protein or a modified ribosome; reduced uptake or increased efflux of the drug from the cell; and the development of an alternative metabolic pathway that bypasses the inhibited step. The genes conferring resistance are frequently carried on plasmids and can spread between bacteria, so that resistance, once it arises, disseminates rapidly. The practical responses, namely the use of antibiotics in rational combination, the development of β-lactamase-stable agents and β-lactamase inhibitors, and the prudent restriction of antibiotic use, are themes that recur throughout pharmaceutical and clinical practice. The general comparison of the two prescribed antibiotics is given in Table 2.3.

Table 2.3: Comparison of streptomycin and the cephalosporins

Feature	Streptomycin	Cephalosporins
Producing organism	*Streptomyces griseus* (actinomycete)	*Acremonium chrysogenum* (fungus)
Chemical class	Aminoglycoside	β-lactam
Mode of action	Inhibits protein synthesis (30S ribosome)	Inhibits cell-wall synthesis
Action	Bactericidal	Bactericidal
Notable use	Tuberculosis (in combination)	Broad hospital use (generations 1–5)
Principal limitation	Ototoxicity, nephrotoxicity, resistance	β-lactamase susceptibility (earlier agents)

> **Quick recall.** An antibiotic is a microbial product that inhibits or kills other microorganisms. Streptomycin: from *Streptomyces griseus*; aminoglycoside; inhibits the 30S ribosome; first anti-tuberculosis antibiotic. Cephalosporins: from *Acremonium chrysogenum*; β-lactam; inhibit cell-wall synthesis; nucleus 7-ACA.

2.6 Summary

The study of a microorganism begins with the microscope. Magnification enlarges the image, but resolution, set by the wavelength of the illuminating radiation, determines the detail that can be seen; the compound light microscope resolves to about 0.2 μm and may view living cells, whereas the electron microscope resolves to about 0.2 nm but requires a dead specimen in vacuum. Because cells are nearly transparent, they are made visible by staining: simple staining with a single basic stain reveals morphology, Gram's staining divides bacteria into Gram-positive and Gram-negative by the structure of the wall, and acid-fast staining identifies the waxy-walled mycobacteria. Organisms alike in morphology and Gram reaction are separated by biochemical tests, of which the IMViC set distinguishes *Escherichia coli* (+ + − −) from the *Enterobacter–Klebsiella* group (− − + +).

The same microbiology, applied in reverse, yields antibiotics. An antibiotic is a microbial product that inhibits or kills other microorganisms, manufactured by fermentation and recovered by downstream processing. Streptomycin, from *Streptomyces griseus*, is an aminoglycoside that inhibits the 30S ribosome and was the first drug effective against tuberculosis; the cephalosporins, from *Acremonium chrysogenum*, are β-lactams that inhibit cell-wall synthesis and are built from

the 7-ACA nucleus. With the organisms now classified, observed and identified, and the dual role of the microorganism as both contaminant and producer established, the book turns in the next unit to the sources of microbial contamination and the means by which it is controlled in pharmaceutical manufacture.

2.7 Self-assessment

A. Multiple-choice questions

1. The resolving power of a microscope is determined chiefly by:

(a) the eyepiece magnification (b) the wavelength of the illuminating radiation (c) the length of the tube (d) the brightness of the source. Answer: (b).

2. In Gram's staining, the mordant is:

(a) crystal violet (b) safranin (c) Gram's iodine (d) acid-alcohol. Answer: (c).

3. A Gram-negative cell appears, after the complete Gram procedure:

(a) purple (b) pink/red (c) colourless (d) green. Answer: (b).

4. The acid-fast stain is used principally to identify:

(a) *Staphylococcus* (b) *Escherichia coli* (c) *Mycobacterium* (d) *Candida*. Answer: (c).

5. The IMViC pattern of *Escherichia coli* is:

(a) − − + + (b) + + − − (c) + − + − (d) − + − +. Answer: (b).

6. Streptomycin exerts its antibacterial action by:

(a) inhibiting cell-wall synthesis (b) inhibiting the 30S ribosomal subunit (c) disrupting the cell membrane (d) inhibiting folate synthesis. Answer: (b).

B. Short-answer questions (2 to 5 marks)

Q1. Distinguish between magnification and resolution.

Magnification is the degree to which the apparent size of an object is increased; resolution is the smallest distance between two points at which they can still be distinguished as two. Resolution, set by the wavelength of the illuminating radiation, fixes the real limit of useful magnification, since enlargement beyond the resolution gives only a larger blur.

Q2. List the steps of Gram's staining and state the colour of Gram-positive and Gram-negative cells at the end.

The steps are: crystal violet (primary stain); Gram's iodine (mordant); alcohol or acetone (decolouriser); safranin (counterstain). At the end the Gram-positive cell is purple, having retained the crystal violet–iodine complex in its thick peptidoglycan, and the Gram-negative cell is pink, having been decolourised and counterstained.

Q3. Why are mycobacteria stained by the acid-fast method rather than the Gram method?

The mycobacterial cell wall contains a high proportion of waxy mycolic acid that repels aqueous stains, so the cells take up ordinary stains poorly. The acid-fast method uses carbol fuchsin driven in by heat and then tests retention against acid-alcohol; mycobacteria retain the stain (acid-fast, red), distinguishing them from non-acid-fast organisms.

Q4. Name the four IMViC tests and give the result of each for *Escherichia coli*.

The tests are indole, methyl red, Voges–Proskauer, and citrate. For Escherichia coli *the results are: indole positive, methyl red positive, Voges–Proskauer negative, citrate negative (+ + − −).*

Q5. Define an antibiotic and state the mode of action of the cephalosporins.

An antibiotic is a substance produced by a microorganism that, in low concentration, inhibits or kills other microorganisms (the term now including semi-synthetic and synthetic analogues). Cephalosporins are β-lactam antibiotics that inhibit bacterial cell-wall synthesis by binding the penicillin-binding proteins that cross-link peptidoglycan, weakening the wall and causing osmotic lysis; they are bactericidal.

C. Long-answer questions (10 marks)

Q1. Describe Gram's staining: principle, procedure and the structural basis of the result.

A complete answer defines a differential stain, gives the four-step procedure with the role of each reagent, states the final colours, and explains the result in terms of cell-wall structure: the thick peptidoglycan of the Gram-positive cell retains the crystal violet–iodine complex on dehydration by the decolouriser, while the thin peptidoglycan and the dissolved lipid outer membrane of the Gram-negative cell do not, so the latter is counterstained pink. Credit is given for naming over-decolourisation as the principal source of error. (High-yield: Gram's staining is among the most frequently examined topics in the subject.)

Q2. Give an account of the production, mode of action, and therapeutic uses of streptomycin.

A complete answer states that streptomycin is an aminoglycoside obtained by submerged fermentation of Streptomyces griseus*, recovered and purified from the broth; explains that it acts by binding the 30S ribosomal subunit to cause misreading and arrest of protein synthesis (bactericidal); and gives its uses, principally tuberculosis in combination and certain Gram-negative infections, together with its limitations of ototoxicity, nephrotoxicity and rapid resistance.*

D. Structured note

Write a note on the electron microscope.

The electron microscope forms an image with a beam of electrons focused by electromagnetic lenses; the short wavelength of the electron beam gives a resolution of about 0.2 nm, a thousand-

fold finer than the light microscope, revealing viruses and internal cell structure. The transmission type passes electrons through a thin section to show internal detail; the scanning type scans the surface to give a three-dimensional view. The specimen must be examined dead and in vacuum, the chief limitation of the instrument.

References

1. Pelczar MJ, Chan ECS, Krieg NR. Microbiology: Concepts and Applications. New York: McGraw-Hill.
2. Prescott LM, Harley JP, Klein DA. Microbiology. New York: McGraw-Hill.
3. Denyer SP, Hodges NA, Gorman SP, editors. Hugo and Russell's Pharmaceutical Microbiology. 8th ed. Oxford: Wiley-Blackwell; 2011.
4. Schatz A, Bugie E, Waksman SA. Streptomycin, a substance exhibiting antibiotic activity against gram-positive and gram-negative bacteria. Proc Soc Exp Biol Med. 1944;55(1):66–69.

Chapter 3: Sources, Types and Control of Microbial Contamination

Learning outcomes

After studying this chapter the student will be able to:

- Define microbial contamination and classify contaminants by their source as intrinsic or extrinsic.
- State the types of microorganism that contaminate pharmaceutical products and the concept of **objectionable organism**s.
- Describe the control of microbial contamination during the manufacture of **non-**sterile and sterile dosage forms.
- Describe the control of each route of entry: atmosphere, water, raw materials, facility and personnel, equipment and packaging.
- Distinguish **terminal sterilization** from **aseptic processing**, and outline the GMP framework for contamination control.

These outcomes serve Course Outcome CO3, the evaluation of contamination sources and the application of GMP-based strategies for their control.

3.1 Introduction

Microbial contamination is the presence of unwanted microorganisms in a pharmaceutical material, product, or environment. It is the central hazard that pharmaceutical microbiology exists to control, because a contaminated medicine may be degraded in quality, spoiled in its physical form, or rendered a vehicle of infection. The governing principle of its control was stated in Chapter 1 and is the organising idea of this chapter: because the contaminant is invisible and cannot be removed by inspection of the finished product, contamination must be prevented at its source rather than detected after the event. A finished-product test examines only a small sample and can confirm cleanliness only at the level of that sample; assurance of quality comes from controlling the materials, the environment, and the process throughout manufacture. This chapter sets out where contaminants come from, what they are, and how each route of entry is controlled, and so provides the rationale for the clean-area design, sterilization and quality-control testing of the chapters that follow.

3.2 Sources and types of microbial contaminant

A contaminant is any microorganism present where it is not wanted. The sources of contamination are most usefully classified by whether the organism is carried in by the materials of the product or introduced from the surroundings during processing and use.

Intrinsic contamination is that present within the ingredients of the product before manufacture. Many pharmaceutical raw materials are natural products of plant, animal, or mineral origin, and carry their own microbial load: starches, gums, talc, sugars, gelatin and many crude drugs are characteristically contaminated. Water, the most widely used ingredient of all, is itself a habitat in which Gram-negative bacteria multiply, and is the most important single source of **intrinsic contamination**.

Extrinsic contamination is that introduced from the environment during manufacture, storage, or use. The air carries bacteria and fungal spores on dust and skin scales; personnel shed very large numbers of microorganisms from the skin, hair, respiratory tract, and clothing; equipment, surfaces and pipework harbour organisms, often as **biofilm**s; packaging carries its own surface load; and a multi-dose container is recontaminated by the patient at every use.

The organisms encountered as contaminants are a predictable group, dictated by the conditions of the pharmaceutical environment. The Gram-negative bacteria, in particular *Pseudomonas aeruginosa* and related species, are the most important, because they survive and multiply in water and dilute aqueous solutions, tolerate many preservatives, and are pathogenic when introduced into the eye or a wound. The spore-forming genera *Bacillus* and *Clostridium*, met in Chapter 1, are important because their spores survive drying and many processes that kill vegetative cells. Among the fungi, the moulds *Aspergillus* and *Penicillium* and the yeast *Candida* contaminate and spoil products of low water activity or acidic pH in which bacteria cannot grow. The principal sources and their characteristic organisms are summarised in Table 3.1.

Table 3.1: Principal sources of microbial contamination and their characteristic organisms

Source	Type	Characteristic organisms
Water	Intrinsic	Gram-negative bacteria, especially *Pseudomonas*
Raw materials (natural origin)	Intrinsic	Spore-formers (*Bacillus*), moulds, mixed flora
Air and atmosphere	Extrinsic	Fungal spores, *Bacillus*, micrococci on dust
Personnel	Extrinsic	Skin flora (*Staphylococcus*), respiratory organisms

Source	Type	Characteristic organisms
Equipment and surfaces	Extrinsic	Biofilm organisms, *Pseudomonas*
Packaging	Extrinsic	Environmental and surface flora

3.2.1 Objectionable organisms

Not every contaminant is of equal concern. The pharmacopoeias recognise the concept of the objectionable organism, an organism that must be absent from a particular product because of the hazard it presents by the route of administration, irrespective of the total count. For a non-sterile product the requirement is therefore twofold: the total viable count must lie within a defined limit, and specified organisms must be absent. The specified organisms commonly tested for, and the products from which each must be excluded, depend on the route of use; they include *Escherichia coli* as an indicator of faecal contamination, *Salmonella* species, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and bile-tolerant Gram-negative bacteria. An organism that would be tolerated in an oral product may be objectionable in a preparation for the eye or for application to broken skin, because the site of use determines the risk. The microbial limit tests by which these requirements are verified are the subject of Chapter 9.

3.2.2 Consequences of contamination

The consequences of contamination justify the effort of its control. A contaminated medicine may be reduced in potency, because microbial enzymes degrade the active ingredient; it may be spoiled in its physical form, so that an emulsion separates or a solution becomes turbid; it may develop toxic or pyrogenic substances, of which **bacterial endotoxin** is the most important; and, most seriously, it may transmit infection to the patient. The risk is greatest for products administered by routes that bypass the body's natural defences, namely the parenteral, ophthalmic and wound routes, and least for oral products taken by a patient with intact defences. The consequence therefore depends not only on the organism and the count but on the route of administration, and the standards applied to a product follow from that route, a principle that runs through the whole of the control described below.

> **Quick recall.** Intrinsic contamination is carried in by the ingredients (natural raw materials, water); **extrinsic contamination** is introduced from the surroundings (air, personnel, equipment, packaging). *Pseudomonas* indicates a water source, spore-formers a source that survived a kill step, moulds and yeasts a product too dry or acidic for bacteria. An objectionable organism must be absent from a given product whatever the total count.

3.3 Control during manufacture: the two product classes

The degree of control required is set by the nature of the product. A non-sterile product, such as a tablet, capsule, or oral liquid, is not required to be free of all organisms, but its microbial load, the **bioburden**, must be held within defined limits and the product must be free of objectionable organisms. A sterile product, such as an injection, an infusion, or an ophthalmic preparation, must contain no viable microorganisms whatever, because it is introduced by a route that bypasses the body's defences.

For a sterile product two strategies are available, and the distinction between them governs the design of the whole process. In terminal sterilization, the product is filled into its final container, sealed and then sterilized in that container, usually by moist heat, so that the final operation destroys any organism introduced during manufacture. This is the preferred method wherever the product can withstand it, because the sterilization is applied to the sealed final product and is therefore the most reliable. In aseptic processing, the product and its container are sterilized separately and are then brought together and filled under conditions clean enough that no organism is added, the method being used when the product cannot withstand terminal sterilization. Aseptic processing is inherently less secure than terminal sterilization, because there is no final sterilizing step to fall back on, and it therefore demands the most stringent control of environment and procedure, the subject of Chapter 5. The contrast is set out in Table 3.2.

Table 3.2: Terminal sterilization compared with aseptic processing

Feature	Terminal sterilization	Aseptic processing
Sequence	Fill and seal, then sterilize in final container	Sterilize components separately, then fill
Final sterilizing step	Present	Absent
Reliability	Higher	Lower; depends wholly on process control
Used when	Product withstands sterilization	Product is heat-labile
Environmental demand	High	Highest

The manufacture of a sterile product accordingly involves operations not required for a non-sterile product. The components, the containers, and the closures are sterilized by the methods of Chapter 7 before they are brought together. A heat-labile solution that cannot be terminally sterilized is rendered sterile by **filtration through a sterilising-grade membrane** of 0.22 micrometre pore size, which removes bacteria from the liquid, and is then filled aseptically into sterile containers in

a clean area of the highest grade. Each of these operations introduces an opportunity for contamination that the environmental control must close. The greater part of the difficulty of sterile manufacture lies in the aseptic operations that follow sterilization, for the reason given above: in aseptic processing there is no terminal step to correct an error, so the assurance of sterility rests wholly on the control of the environment, the equipment, and the operator.

Whichever class the product belongs to, contamination is controlled by addressing the routes of entry, considered next. These routes are the same for both classes; they differ only in the stringency with which control is applied.

3.4 Control of the routes of entry

The routes by which microorganisms enter a product during manufacture are conventionally treated as six: the atmosphere, water, raw materials, the facility and its personnel, the equipment, and the packaging. They are shown together in Figure 3.1, and each is considered in turn.

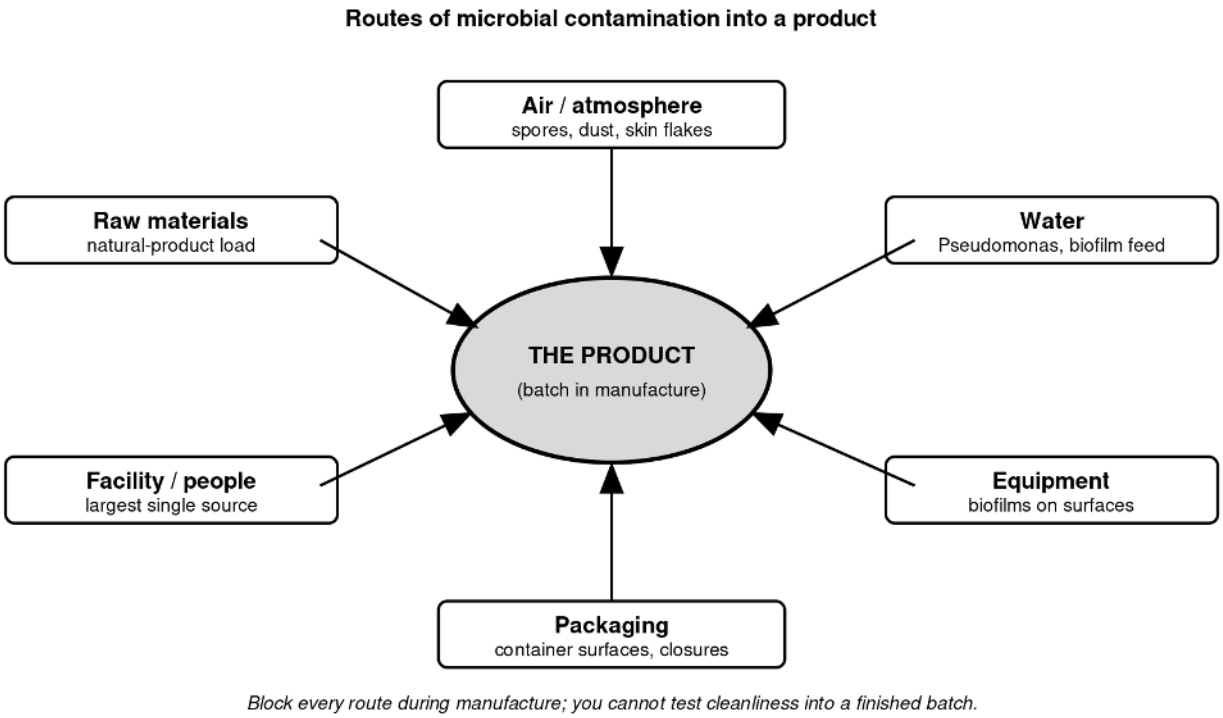


Figure 3.1 *Routes of microbial contamination into a product.*

3.4.1 Atmosphere (air)

Air is never sterile; it carries bacteria and fungal spores on suspended particles, and any product exposed to untreated air will acquire them. The control of air operates at two levels. For ordinary manufacture, the air supplied to a production area is filtered, the area is maintained at a positive pressure relative to its surroundings so that air flows outward through any gap, and the number of airborne particles is limited by controlling dust and movement. For sterile manufacture, essentially

all particles are removed by passing the supply air through a **high-efficiency particulate air (HEPA) filter**, and the critical working zone is bathed in a unidirectional flow of this filtered air. The engineering of clean air, the grading of clean areas, and the control of laminar flow are treated fully in Chapter 5. In outline, the supply air passes through an air-handling unit in which it is filtered in stages, a coarse pre-filter removing the larger particles and a fine or HEPA filter removing the remainder, before it is delivered to the room. The room is held at a positive pressure of the order of 10 to 15 pascals above the adjacent less-clean area, so that the direction of any leakage is always outward, and the air is changed many times an hour to dilute and remove the particles generated within. The combination of filtration, pressurisation and a high air-change rate keeps the airborne count within the limit set for the grade of the area.

3.4.2 Water

Water demands the closest attention of any ingredient, both because it is used in almost every product and because it supports the growth of the Gram-negative bacteria that are the most troublesome contaminants. A water system left static, or with dead legs of pipe in which water stagnates, will develop a heavy microbial population and a biofilm within a short time. Control rests on selecting the appropriate grade of water for the use and on maintaining the distribution system so that water cannot stagnate, by keeping it in constant circulation, holding it hot, or sanitising it regularly.

The pharmacopoeias define grades of water by the purity required and the use intended, and the Indian Pharmacopoeia is the governing compendium for an Indian manufacturer. **Purified Water** is prepared from potable water by distillation, reverse osmosis, ion exchange, or other suitable means and is used for non-sterile products and as the feed for higher grades. **Water for Injection (WFI)** is the highest routine grade, used in the preparation of parenteral products, and is distinguished by a strict limit on bacterial endotoxin, the heat-stable lipopolysaccharide of Gram-negative cell walls that causes fever when injected. The control of endotoxin is a separate requirement from the control of viable organisms, because endotoxin persists after the organism that produced it has been killed; a water may therefore be sterile and still pyrogenic. The grades of water are set out in Table 3.3.

Table 3.3: Common pharmacopoeial grades of water

Grade	Typical preparation	Endotoxin controlled?	Principal use
Potable water	Municipal treatment	No	Feed water only; not an ingredient
Purified Water	Distillation, reverse osmosis, ion exchange	No	Non-sterile products; feed for higher grades
Water for Injection	Distillation or suitable membrane process	Yes	Parenteral products

A pharmaceutical water system comprises a pre-treatment stage that conditions the incoming potable water by filtration, softening and removal of chlorine; a purification stage that produces the required grade by distillation or by reverse osmosis with associated steps; a storage vessel; and a distribution loop that returns water continuously to the store. The microbiological design of the system is directed entirely at preventing the growth and accumulation of organisms: the water is kept in constant circulation so that no part stands still, the loop is designed without dead legs in which water could stagnate, Water for Injection is commonly stored and circulated hot, at about 80 degrees Celsius, and the system is sanitised at intervals by heat or by a chemical agent. The microbial quality is verified by routine sampling of the water at the points of use, the count being compared against alert and action limits, and the endotoxin of Water for Injection is monitored in addition.

3.4.3 Raw materials

Raw materials carry the intrinsic contamination described in Section 3.2. They are controlled by purchasing to a defined microbial specification, by testing incoming consignments against microbial limits before acceptance, and, where a material carries a high load and the process permits, by a treatment to reduce the count before use. Materials of natural origin, such as talc, starches, gums and crude drugs, are the usual subjects of incoming microbial testing. The specification states a permitted total aerobic count, a permitted total yeast and mould count, and the absence of named objectionable organisms, the limits being stricter for a material destined for a sterile or an ophthalmic product than for one destined for an oral tablet. A consignment that exceeds the limit is rejected, and a material of consistently high load is either replaced by a cleaner source or subjected to a reducing treatment such as irradiation or, for a heat-stable material, dry heat, provided the treatment does not impair its pharmaceutical quality.

3.4.4 Facility and personnel

The building and the people who work in it are the largest single source of contamination in most operations, and are controlled together. The facility is designed so that operations of different cleanliness are separated, materials and personnel flow in one direction from less clean to more clean areas, surfaces are smooth, impervious and readily cleaned, and the air is treated as in Section 3.4.1. The personnel are controlled by gowning that covers the skin and hair, by hand disinfection, by training in conduct that minimises the shedding and dispersal of organisms, and by limiting the number of persons in the clean area. The importance of personnel control follows from the magnitude of the source: a single inadequately gowned operator can compromise an otherwise satisfactory facility. Gowning for a clean area is performed in a defined sequence in a changing room arranged as an airlock, the operator passing from the ordinary side to the clean side and donning, in order, a suit, hood, mask, goggles, gloves and dedicated footwear, with hand disinfection at the appropriate step; for aseptic work the garments are themselves sterile. Movement within the clean area is kept slow and deliberate, because rapid movement sheds and disperses particles, and the operator is trained never to pass a hand or an implement between a source of filtered air and an exposed product. Materials enter the clean area through a separate airlock or a pass-through hatch, are disinfected on entry, and follow a path that does not cross the path by which waste leaves, so that a clean article never travels over a contaminated one.

3.4.5 Equipment

Equipment transmits contamination when organisms survive on its surfaces between batches. The characteristic failure is the biofilm, a community of microorganisms adherent to a surface and embedded in a protective layer of secreted polymer, which resists cleaning and reinoculates each batch that passes. Control rests on cleaning validated to remove both chemical residue and microorganisms, on the design of equipment without dead legs, crevices and rough joints in which films can establish, and on the sanitisation or sterilization of equipment that contacts a sterile product. The capacity of bacteria to adhere and to secrete slime, noted in Chapter 1, is the property that underlies biofilm formation. Cleaning is increasingly performed by **cleaning-in-place (CIP)**, in which a fixed system circulates detergent and rinse solutions through the closed equipment without dismantling it, and sterilization by **sterilization-in-place (SIP)**, in which steam is admitted to the assembled system; both reduce the handling that would otherwise reintroduce contamination. The adequacy of cleaning is established by **cleaning validation**, which demonstrates, by testing for residue and for viable organisms after the defined cleaning cycle, that the procedure reproducibly returns the equipment to an acceptable state. Equipment that contacts a sterile product is, in addition, sterilized by the methods of Chapter 7.

3.4.6 Control of process gases and sanitisation of the area

Two further controls support those already described. Compressed air and process gases such as nitrogen, which are used to drive equipment and, in sterile manufacture, to overlay or purge a product, are themselves potential carriers of contamination and are therefore filtered, the gas that contacts a sterile product passing through a sterilising-grade filter. Separately, the surfaces of the clean area are kept under control by a documented **sanitisation programme**, in which floors, walls and work surfaces are cleaned and treated with a disinfectant on a defined schedule. Because no single disinfectant is active against every organism and resistant survivors may be selected by the repeated use of one agent, the programme commonly rotates two disinfectants of different type, with periodic use of a sporicidal agent; the selection and evaluation of disinfectants is the subject of Chapter 6.

3.4.7 Packaging

The container is at once the final barrier protecting the product and a route by which contamination may enter. Packaging materials carry a surface microbial load, so containers for sterile products are cleaned and sterilized before filling. The container has a second and continuing function, the protection of the product against the ingress of organisms and moisture throughout its shelf life and use; a well-designed closure maintains this protection, whereas a defective seal permits the recontamination of a multi-dose product at each opening, which is among the reasons such products carry a preservative, treated in Chapter 6. The choice of container contributes to microbial control in its own right: a single-dose container, opened once and discarded, removes the recontamination that besets a multi-dose container; a well-sealed glass or plastic container with an effective closure resists the ingress of organisms and moisture over the shelf life; and the integrity of the closure system of a sterile product is itself tested, since a container that does not maintain its seal cannot maintain sterility. Packaging is thus both the last barrier built during manufacture and the barrier on which the product depends for the whole of its life in storage and use.

> **Quick recall.** The six routes of entry are atmosphere, water, raw materials, facility and personnel, equipment and packaging. Personnel are usually the largest single source. Water and equipment fail through stagnation and biofilm. Sterile products require zero organisms; non-sterile products require the bioburden to be held within limits and objectionable organisms to be absent.

3.5 Good Manufacturing Practice and contamination control

The systematic control of contamination is codified in **Good Manufacturing Practice (GMP)**, the body of regulation that governs the conditions and procedures of pharmaceutical manufacture. In India the requirements of GMP are laid down in **Schedule M** of the Drugs and Cosmetics Rules, administered under the Drugs and Cosmetics Act 1940 by the Central Drugs Standard Control

Organisation (CDSCO) and the state licensing authorities. GMP addresses, among much else, the premises and their environmental control, the qualification of water and air systems, the cleaning and maintenance of equipment, the training and hygiene of personnel, the testing of materials, and the documentation that demonstrates control. The requirements of GMP are conventionally grouped under a small number of headings that together determine the quality of the product: the premises and environment, designed and controlled as described above; the equipment, qualified, maintained and cleaned to validated procedures; the materials, purchased to specification and tested before use; the personnel, trained, healthy and correctly gowned; and the procedures and documentation, written down and followed so that every batch is made in the same controlled way and the control can be demonstrated after the event. Documentation is itself a pillar of GMP and a contamination control in its own right. Every batch is made according to a written master formula and method, and the operations actually performed, the materials and equipment used, the environmental results obtained, and the tests carried out are recorded in a **batch manufacturing record** that is reviewed and authorised before the batch is released. The record makes the control demonstrable after the event, allows a contamination problem to be traced to its source, and ensures that each batch is made in the same validated way. Standard operating procedures govern cleaning, gowning, monitoring and every other repeated task, so that the control does not depend on the memory of an individual operator.

A further principle of GMP is the prevention of **cross-contamination**, the contamination of one product by another, which is controlled by dedicating facilities or equipment to particular products where necessary, by validated cleaning between products, and by air-handling arrangements that prevent the transfer of material from one area to another. The principle that unifies these requirements is the one with which the chapter began: that quality, and in particular freedom from contamination, is built into the product by the control of materials, environment and process, and is then confirmed, but not created, by testing of the finished product.

Confirmation that the controls are effective is obtained by **environmental monitoring**, the routine sampling of the air, surfaces, water and personnel of the manufacturing area. The air is sampled both passively, by exposing settle plates that collect organisms falling under gravity over a measured time, and actively, by drawing a measured volume of air onto the surface of a culture medium with an air sampler, the result being expressed as colony-forming units per cubic metre. Surfaces and the gloved hands of operators are sampled with contact plates and swabs. The non-viable particle count is measured in parallel with a particle counter, since it defines the grade of the area. The results of all these are trended against established alert and action limits, an alert limit signalling a drift that warrants investigation and an action limit signalling a result that requires a defined corrective response. Monitoring is most intensive during actual production, when personnel and activity load the environment most heavily, and it is at its most stringent in the critical zone

where a sterile product is exposed. The detailed methods of environmental monitoring are described in Chapter 10.

3.6 Summary

Microbial contamination, the presence of unwanted microorganisms, is controlled at its source because it cannot be removed by inspection of the finished product, and because its consequences, ranging from loss of potency through physical spoilage to the transmission of infection, are determined by the organism, the count, and above all the route by which the product is administered. Contaminants are classified by source as intrinsic, carried in by natural raw materials and water, or extrinsic, introduced from the air, personnel, equipment and packaging; the organisms of chief concern are the water-borne Gram-negative bacteria, the resistant spore-formers, and the moulds and yeasts of low-water and acidic products. The pharmacopoeias additionally require the absence of objectionable organisms determined by the route of use.

The stringency of control is set by the product class: a non-sterile product must hold its bioburden within limits and exclude objectionable organisms, whereas a sterile product must be free of all viable organisms, achieved by terminal sterilization where possible and by aseptic processing where not. Control is exercised over six routes of entry: the atmosphere, by filtration, positive pressure, and HEPA-filtered laminar air for sterile work; water, by selecting the correct pharmacopoeial grade and maintaining a circulating, sanitised system, with a separate limit on endotoxin for Water for Injection; raw materials, by specification and incoming testing; facility and personnel, by design, gowning and discipline, the personnel being the largest single source; equipment, by validated cleaning and biofilm-resistant design; and packaging, by cleaning, sterilization and protective closure. These controls are codified in Good Manufacturing Practice, in India through Schedule M, and confirmed by environmental monitoring. It should be appreciated that the controls operate together rather than in isolation, each closing one route of entry, so that the failure of any one may admit the contamination the others were designed to exclude; the assurance of a clean product therefore rests on the integrity of the whole system, maintained continuously and documented throughout. The next chapter examines what microorganisms do once they have gained entry, namely the spoilage of pharmaceuticals, and the constructive use of the same organisms in fermentation.

3.7 Self-assessment

A. Multiple-choice questions

1. Contamination present within the raw materials of a product before manufacture is termed:

(a) extrinsic (b) intrinsic (c) terminal (d) aseptic. Answer: (b).

2. The most important single source of intrinsic contamination in pharmaceutical manufacture is:

(a) packaging (b) personnel (c) water (d) the air. Answer: (c).

3. An organism that must be absent from a particular product irrespective of the total count is called:

(a) an indicator organism (b) an objectionable organism (c) a spore-former (d) a commensal. Answer: (b).

4. The bacterial endotoxin controlled in Water for Injection is:

(a) destroyed whenever the organism is killed (b) a heat-stable lipopolysaccharide of Gram-negative cell walls (c) a product of fungal growth (d) the same as a preservative. Answer: (b).

5. In aseptic processing, as distinct from terminal sterilization:

(a) the product is sterilized in its final sealed container (b) there is no final sterilizing step (c) no environmental control is needed (d) only non-sterile products are made. Answer: (b).

6. In India the requirements of GMP for pharmaceutical manufacture are laid down in:

(a) the Pharmacy Act 1948 (b) Schedule M of the Drugs and Cosmetics Rules (c) the Indian Pharmacopoeia monographs (d) the DPCO. Answer: (b).

7. A sterilising-grade membrane filter used to render a heat-labile solution sterile has a pore size of:

(a) 0.45 µm (b) 0.22 µm (c) 1.0 µm (d) 5.0 µm. Answer: (b).

8. Settle plates exposed in a clean area measure:

(a) the non-viable particle count (b) organisms falling under gravity onto a medium (c) the endotoxin level (d) the surface count only. Answer: (b).

9. An alert limit in environmental monitoring, as distinct from an action limit, signals:

(a) that the product must be rejected (b) a drift that warrants investigation before a limit is breached (c) that the area must be closed (d) the presence of an objectionable organism. Answer: (b).

10. Cross-contamination is the contamination of one product by:

(a) its own ingredients (b) another product (c) the patient (d) the pharmacopoeia. Answer: (b).

B. Short-answer questions (2 to 5 marks)

Q1. Distinguish intrinsic from extrinsic microbial contamination, with two examples of each.

Intrinsic contamination is carried in by the ingredients of the product, for example the natural microbial load of raw materials such as starch or talc, and the organisms living in process water. Extrinsic contamination is introduced from the surroundings during manufacture, storage, or use, for example airborne fungal spores, organisms shed by personnel, contamination from equipment surfaces, and recontamination of a multi-dose container by the patient.

Q2. State the concept of an objectionable organism and name four organisms commonly tested for in non-sterile products.

An objectionable organism is one that must be absent from a particular product because of the hazard it presents by the route of administration, irrespective of the total viable count; the organism that is acceptable in one product may be objectionable in another used by a more vulnerable route. Organisms commonly tested for include Escherichia coli, Salmonella species, Pseudomonas aeruginosa, and Staphylococcus aureus.

Q3. Why does Water for Injection carry a limit on bacterial endotoxin in addition to a limit on viable organisms?

Endotoxin is the heat-stable lipopolysaccharide of Gram-negative cell walls and causes fever when injected. It persists after the organism that produced it has been killed, so a water may be sterile yet still pyrogenic. The removal of viable organisms and the control of endotoxin are therefore separate requirements, and Water for Injection, being used in parenteral products, must satisfy both.

Q4. Distinguish terminal sterilization from aseptic processing.

In terminal sterilization the product is filled and sealed in its final container and then sterilized in that container, so that the final operation destroys any organism introduced during manufacture; it is preferred wherever the product can withstand it. In aseptic processing the product and container are sterilized separately and then combined under clean conditions, used for heat-labile products; there is no final sterilizing step, so the method depends entirely on process control and is inherently less secure.

Q5. Why are personnel regarded as the largest single source of contamination, and how are they controlled?

Personnel shed very large numbers of microorganisms from the skin, hair, respiratory tract, and clothing, so a single operator can introduce more organisms than any other route. They are controlled by gowning that covers the skin and hair, by hand disinfection, by training in conduct that minimises shedding and dispersal, and by limiting the number of persons in the clean area.

Q6. How is a pharmaceutical water system designed and controlled to limit microbial growth?

The system comprises pre-treatment, a purification stage (distillation or reverse osmosis), storage, and a continuously circulating distribution loop. Microbial growth is limited by keeping the water in constant circulation with no dead legs, by storing and circulating Water for Injection hot (about 80 degrees Celsius), and by periodic sanitisation. Quality is verified by routine sampling at the points of use against alert and action limits, with endotoxin monitored for Water for Injection.

Q7. What is environmental monitoring, and what does it sample?

Environmental monitoring is the routine sampling of the manufacturing environment to confirm that contamination controls are effective. It samples the air, both passively with settle plates and actively with air samplers (results in colony-forming units per cubic metre), surfaces and gloved hands with contact plates and swabs, the non-viable particle count with a particle counter, and the water system. Results are trended against alert and action limits, most intensively during production and most stringently in the critical zone.

C. Long-answer questions (10 marks)

Q1. Describe the sources of microbial contamination of pharmaceutical products and the control of each route of entry during manufacture.

A complete answer classifies sources as intrinsic and extrinsic with examples, names the characteristic contaminating organisms, and then treats each of the six routes of entry, air, water, raw materials, facility and personnel, equipment and packaging, giving the specific control for each (filtration and positive pressure and HEPA-filtered laminar air; water grades and a circulating sanitised system with endotoxin control; specification and incoming testing; one-directional flow, cleanable surfaces, and gowning; validated cleaning and biofilm-resistant design; cleaned and sterilized containers with protective closures). Credit is given for noting that personnel are the largest source and that quality is built in rather than tested in. (High-yield: contamination control is a core, frequently examined topic.)

Q2. Explain the control of microbial contamination in the manufacture of sterile and non-sterile dosage forms.

A complete answer distinguishes the requirements of the two classes (bioburden within limits and objectionable organisms absent for non-sterile; complete absence of viable organisms for sterile), explains terminal sterilization and aseptic processing with the reason terminal sterilization is preferred, and relates both to the control of the routes of entry and to the GMP framework, in India Schedule M, with environmental monitoring as the confirmatory check.

D. Structured note

Write a note on pharmacopoeial grades of water and their control.

Purified Water, prepared from potable water by distillation, reverse osmosis, or ion exchange, is used for non-sterile products and as feed for higher grades. Water for Injection, the highest routine grade, is used for parenterals and carries a strict endotoxin limit because endotoxin survives the death of the organism. Water systems are controlled by selecting the correct grade and by keeping the distribution system circulating, hot, or regularly sanitised to prevent stagnation and biofilm, with routine monitoring of the microbial count and, for Water for Injection, the endotoxin level.

Write a note on Good Manufacturing Practice in relation to contamination control.

Good Manufacturing Practice is the body of regulation governing the conditions and procedures of pharmaceutical manufacture, laid down in India in Schedule M of the Drugs and Cosmetics Rules and administered by the CDSCO and the state authorities. In relation to contamination it requires controlled premises and environment, qualified water and air systems, validated cleaning of equipment, trained and correctly gowned personnel, tested materials, the prevention of cross-contamination, and full documentation in the form of master formulae, batch records, and standard operating procedures. Its unifying principle is that freedom from contamination is built into the product by control of materials, environment and process, and confirmed, not created, by finished-product testing supported by environmental monitoring.

References

1. Denyer SP, Hodges NA, Gorman SP, editors. Hugo and Russell's Pharmaceutical Microbiology. 8th ed. Oxford: Wiley-Blackwell; 2011.
2. Hugo WB, Russell AD. Pharmaceutical Microbiology. Oxford: Blackwell Science.
3. Sandle T. Pharmaceutical Microbiology: Essentials for Quality Assurance and Quality Control. Cambridge: Woodhead Publishing/Elsevier; 2016.
4. Indian Pharmacopoeia. Ghaziabad: Indian Pharmacopoeia Commission. [latest edition]
5. The Drugs and Cosmetics Act 1940 and Rules 1945, Schedule M. Government of India. [latest amendment]

Chapter 4: Microbial Spoilage and Fermentation Technology

Learning outcomes

After studying this chapter the student will be able to:

- Define microbial spoilage and describe its manifestations in pharmaceutical dosage forms.
- Explain the factors that determine whether a product is spoiled, with particular reference to water activity.
- Define **fermentation** and classify its types.
- Describe the fermentation process and the construction and operation of a fermenter.
- State the principal pharmaceutical products obtained by fermentation.

These outcomes serve Course Outcomes CO3 and CO1, the understanding of spoilage and of the industrial application of microorganisms.

4.1 Introduction

Microbial spoilage is the deterioration of a pharmaceutical product caused by the growth and metabolic activity of microorganisms within it. Where the preceding chapter considered how organisms gain entry to a product, the present chapter considers what they do once established, and then turns the same biology to constructive use. The two halves of the chapter are connected by a single idea: the conditions that permit an unwanted organism to spoil a product are precisely those that are supplied deliberately to a wanted organism in fermentation. Spoilage is the uncontrolled growth of a contaminant in a finished medicine; fermentation is the controlled growth of a selected organism to manufacture a medicine. An understanding of the factors that govern microbial growth therefore serves both the prevention of spoilage and the design of a fermentation, and this chapter treats the two together. The first part defines spoilage, sets out its consequences and the factors that govern it, and shows how those factors are manipulated to preserve a product; the second part defines fermentation, classifies its types, describes the medium, the process, and the fermenter, and ends with the products obtained and the example of penicillin. The unity of the chapter lies in the recognition that growth, prevented in the one case and promoted in the other, obeys the same rules throughout.

4.2 Microbial spoilage of pharmaceuticals

Spoilage is more than a matter of an increased microbial count, and a product may be rendered unfit for use well before any visible change appears. The consequences of spoilage are of three kinds: **chemical**, **physical** and **infective**.

The chemical consequence is the degradation of the active ingredient or of an excipient by microbial enzymes, so that the product loses potency or accumulates a degradation product; many drugs, and excipients such as sugars, starches and surfactants, are susceptible to microbial attack. The physical consequence is the deterioration of the dosage form: an emulsion may be broken by organisms that attack the emulsifying agent, a suspension may cake, a cream may separate, and gas, odour, discolouration, or visible growth may appear. The infective consequence, the most serious, is that the spoiled product becomes a vehicle of infection, a danger of the greatest importance in products for the eye, for injection, and for application to broken skin. The manner of spoilage varies with the dosage form. In an aqueous solution or mixture, growth produces turbidity, sediment, gas and off-odours, and may split a **preservative** system that has been overwhelmed. In an emulsion or cream, attack on the emulsifying agent or the oil phase causes the phases to separate, the texture to change, and the appearance and smell to deteriorate. In a syrup, fermentation of the sugar produces gas and acid. In a tablet or powder, which is ordinarily too dry to support growth, an increase in moisture during faulty storage may permit moulds to develop on the surface. Recognising the form of spoilage characteristic of each dosage form is part of the routine assessment of a product's stability. The characteristic spoilage organisms of the principal dosage forms are summarised in Table 4.1. The clinical record provides the warning that gives these consequences their weight: aqueous products for the eye contaminated with *Pseudomonas aeruginosa* have caused serious, sometimes sight-destroying, eye infections, and it is for this reason that ophthalmic products are formulated, preserved and packaged with particular care. Spoilage is thus not only an economic loss, through the scrapping of an affected batch and the investigation and recall that follow, but a hazard to the patient.

Table 4.1: Common spoilage organisms by dosage form

Dosage form	Characteristic spoilage organisms	Basis
Aqueous solutions, eye-drops	*Pseudomonas* and other Gram-negative bacteria	High water activity, dilute preservative
Syrups and high-sugar liquids	Osmophilic yeasts, some moulds	High osmotic pressure excludes bacteria
Creams and emulsions	Gram-negative bacteria, moulds, *Candida*	Aqueous phase supports growth
Tablets and dry powders	Moulds, spore-forming bacteria (if moisture rises)	Low water activity limits growth

4.2.1 Factors affecting microbial spoilage

Whether a contaminated product is actually spoiled is determined by a small set of factors that together decide whether the contaminating organism can grow. These are shown in Figure 4.1, and the dominant factor is the availability of water.

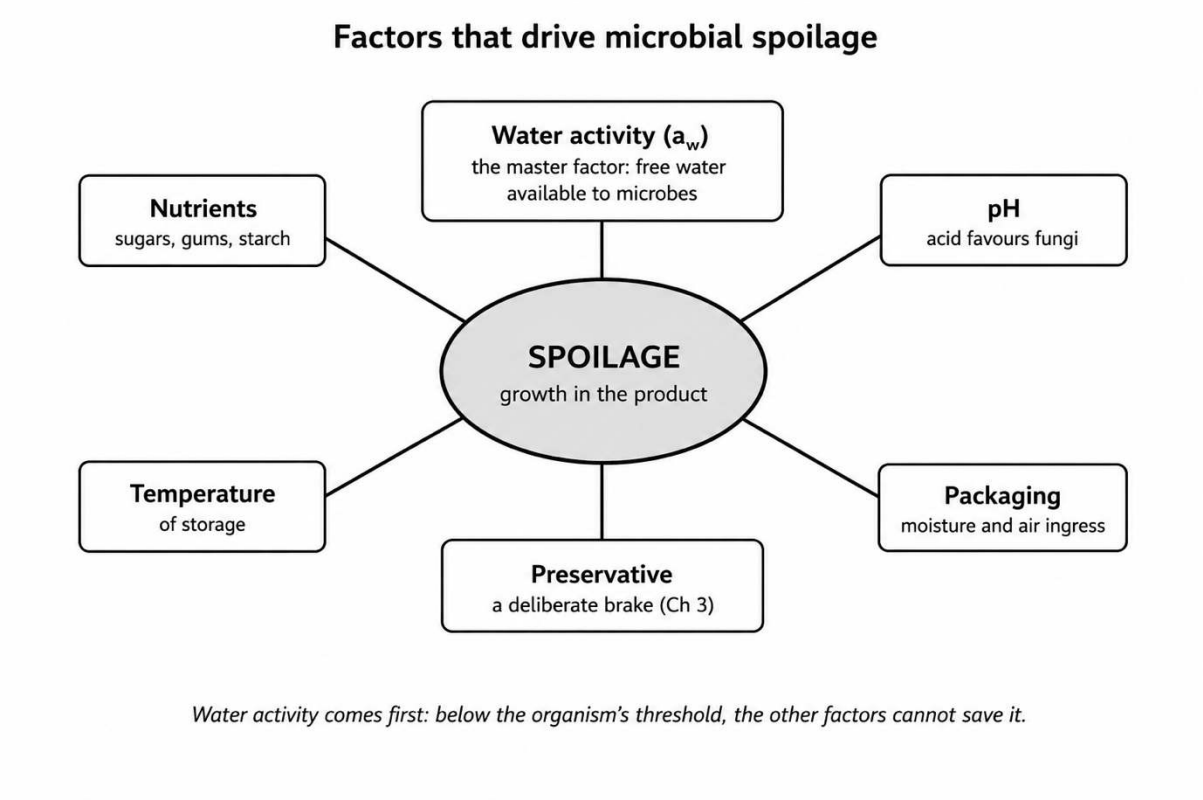


Figure 4.1 *Factors that drive microbial spoilage, with water activity central.*

Water activity (aw) is the measure of the water in a product that is available to microorganisms, expressed on a scale on which pure water has a value of 1.0. It is not the same as the water content, because solutes such as sugar and salt bind water and lower its availability without removing it. Water activity predicts spoilage better than any other single factor, because microbial growth requires a minimum level of available water that differs between groups of organisms. Most bacteria require a water activity above about 0.90, although the more tolerant *Staphylococcus aureus* grows down to about 0.86. Yeasts generally require above 0.88, but the osmophilic yeasts adapted to high sugar concentrations grow below 0.80. Moulds are the most tolerant of dryness: most grow down to between 0.80 and 0.70, and the xerophilic moulds to about 0.60, below which no microbial growth occurs. This ladder of tolerances explains a familiar pattern: a dilute aqueous product is spoiled by bacteria, a concentrated syrup resists bacteria but may be spoiled by osmophilic yeasts or moulds, and a dry tablet at low water activity resists almost all organisms.

The remaining factors modify the outcome. The nutrient content of the formulation supports growth, and many excipients are themselves nutrients. The pH determines which organisms can

grow: most bacteria require a near-neutral pH, whereas moulds and yeasts tolerate the acidity that excludes bacteria, so acidic products are characteristically spoiled by fungi. The temperature of storage accelerates or retards growth in the obvious way. The packaging and storage conditions determine the access of moisture and air over the life of the product. Finally, the preservative, where one is included, is a deliberate inhibitor of microbial growth, the subject of Chapter 6. The protection of a product against spoilage is therefore achieved by formulating it so that one or more of these factors denies the contaminant the conditions for growth.

> **Quick recall.** Spoilage requires available water above the organism's threshold. The water-activity ladder runs approximately: bacteria above 0.90 (*S. aureus* to 0.86), yeasts above 0.88 (osmophilic below 0.80), moulds 0.80 to 0.70 (xerophilic to 0.60); below 0.60 nothing grows. pH then selects bacteria (near neutral) or fungi (acid-tolerant). Nutrients, temperature, packaging and preservative modify the result.

4.2.2 Preservation against spoilage

The protection of a susceptible product against spoilage is achieved partly by formulation and partly by the addition of a preservative. By formulation, the product is made hostile to growth wherever the dosage form allows: the water activity is lowered by sugars or polyols, the pH is adjusted away from the range that favours bacteria, and the packaging is chosen to exclude moisture and to limit recontamination. Where these measures cannot by themselves prevent growth, particularly in aqueous multi-dose products that are repeatedly opened, a chemical preservative is included to inhibit the organisms that gain entry during use. The selection, the mode of action, and the evaluation of preservatives, including the preservative efficacy test by which a preservative system is shown to be adequate, are treated with the antiseptics and disinfectants in Chapter 6. The point to carry forward here is that preservation is the deliberate manipulation of the same factors that govern spoilage, applied so as to deny the contaminant the conditions for growth.

4.3 Fermentation

In the industrial sense used in pharmaceutical manufacture, fermentation is the large-scale cultivation of a selected microorganism under controlled conditions to obtain a useful product. The word has a narrower meaning in biochemistry, where it denotes the anaerobic generation of energy, but in pharmaceutical technology it denotes any controlled large-scale microbial culture, whether aerobic or anaerobic. Fermentation is the means by which antibiotics, vitamins, amino acids, organic acids, enzymes and recombinant proteins are manufactured, and it is the constructive counterpart of the spoilage considered above: the same control of nutrients, pH, temperature and aeration that must be denied to a contaminant is supplied to the production organism.

The product of a fermentation is classified by its relation to the growth of the organism. A **primary metabolite** is a product of the normal growth of the organism, formed during the logarithmic phase,

such as an amino acid or an organic acid. A **secondary metabolite** is a product not required for growth, formed chiefly after rapid growth has ceased, in or near the stationary phase; the antibiotics are the principal example. This distinction, met in Chapter 2, governs the management of the process, since the production of a secondary metabolite requires that growth be promoted first and product formation second. The two periods of an antibiotic fermentation are named accordingly: the **trophophase**, the early period of rapid growth in which little product is formed, and the **idiophase**, the later period in which growth has slowed and the secondary metabolite is formed most actively. The operator manages the fermentation so as to pass from one to the other at the right time, by the timing of nutrient feeding and the control of the conditions.

4.3.1 The fermentation medium

The medium supplies the producing organism with everything it needs to grow and to form the product, and its composition is a principal determinant of yield. It must provide a carbon and energy source, commonly a carbohydrate such as glucose, sucrose, or starch, or a cheaper industrial substrate; a nitrogen source, organic (such as corn-steep liquor, soya meal, or peptones) or inorganic (such as ammonium salts); the mineral elements phosphorus, sulphur, potassium, magnesium and trace metals; and, for fastidious organisms, specific growth factors. Two further additions are characteristic of industrial media. A **precursor** is a specific compound added to be incorporated directly into the product and so to raise its yield or direct its structure; the addition of phenylacetic acid to a penicillin fermentation to favour benzylpenicillin is the classic example. An antifoam agent is added to suppress the foam generated by aeration and agitation. Industrial media favour inexpensive, complex substrates such as corn-steep liquor precisely because the scale makes cost decisive.

4.3.1 Types of fermentation

Fermentations are classified on several independent bases, summarised in Table 4.2.

By the mode of operation, a fermentation is batch, fed-batch, or continuous. In a **batch fermentation** the vessel is charged with medium and inoculum, the culture is grown to completion, and the whole is then harvested; nothing is added or removed during the run except gases and agents for the control of pH and foam. It is simple to operate and to keep sterile, but the organism passes through every growth phase in one vessel and the yield is limited by the initial nutrients. In a **fed-batch fermentation** fresh nutrient is added during the run without the removal of broth, which allows the culture to be held in its most productive state for longer and avoids the inhibition that a single large charge of nutrient may cause; it is the mode most used for antibiotic production. In a **continuous fermentation** fresh medium is added and spent broth withdrawn at the same rate, holding the culture in a steady state of constant growth; it can be highly productive but runs for long periods, during which the risk of contamination accumulates.

By the physical arrangement, a fermentation is submerged or solid-state. In **submerged fermentation** the organism is suspended throughout a liquid medium; in **solid-state fermentation** it is grown on the surface of a moist solid. Submerged fermentation predominates in modern antibiotic manufacture because it is more readily controlled and scaled. By the requirement for oxygen, a fermentation is aerobic or anaerobic; the antibiotic-producing organisms are aerobic and require vigorous aeration.

The yield obtainable from a wild isolate is far below that required for economic manufacture, so the production strain is the product of prolonged **strain improvement**. By repeated cycles of mutation, induced by chemical mutagens or radiation, and selection of the highest-yielding survivors, and latterly by the methods of genetic engineering, the yield of penicillin has been raised several thousand-fold over the original isolate of *Penicillium*. Strain improvement, the optimisation of the medium, and the control of the fermentation conditions together determine the productivity of the process.

4.3.2 The fermentation process

A complete fermentation is more than the operation of the main vessel; it is a sequence of stages. It begins with **inoculum development**, the growth of the production organism in successive stages from a preserved stock culture, through shake flasks and seed vessels, to provide a large and active inoculum that will establish quickly in the production vessel and resist invasion by a contaminant. The medium and the empty vessel are sterilized, usually by steam, before the organism is introduced, because a single surviving contaminant can ruin a fermentation lasting several days. The organism is then grown in the production fermenter under controlled conditions while it forms the product. Throughout the run the culture must be protected against invasion by a foreign organism, for a contaminant may outgrow the producer, consume the substrate, spoil the product, or, in the worst case, destroy the producing strain. The defences are those of sterile manufacture applied to a vessel that must nonetheless be fed and aerated: the medium and vessel are sterilized before inoculation, the air is filtered through a sterilising-grade filter, all additions and withdrawals are made through sterile ports, and a positive pressure is maintained within the vessel so that any leak is outward. A large, vigorous inoculum is itself a defence, since a dense population of the producer establishes quickly and resists invasion. The longer the fermentation, the greater the cumulative risk, which is one reason that the continuous mode, productive though it is, is harder to sustain than the batch or fed-batch mode. The fermentation is followed by **downstream processing**, the recovery and purification of the product from the broth by such operations as filtration, solvent extraction, and chromatography; a secreted product is recovered from the liquid, whereas a product retained within the cells requires that the cells first be broken. Downstream processing is frequently more costly than the fermentation itself. Its stages follow a general sequence: the separation of the cells from the broth by filtration or centrifugation; the extraction of the product, from the liquid if it is secreted or from the disrupted cells if it is retained within them,

by solvent extraction, adsorption, or precipitation; the purification of the extracted product, often by chromatography, to remove related impurities; and the final isolation of the pure product, commonly by crystallisation and drying. The number and severity of the steps are kept to the minimum consistent with the required purity, since each step loses some product and adds to the cost. For a parenteral antibiotic the purity demanded is very high, and the later stages are correspondingly elaborate.

4.3.3 The fermenter

A **fermenter (bioreactor)** is the vessel in which a fermentation is conducted and its conditions are controlled. The commonest design in antibiotic manufacture is the **stirred-tank fermenter**, a closed, jacketed vessel that can be sterilized and that maintains the culture under controlled agitation, aeration, temperature and pH for the duration of the run. Its principal parts and their functions, shown in Figure 4.2, are as follows.

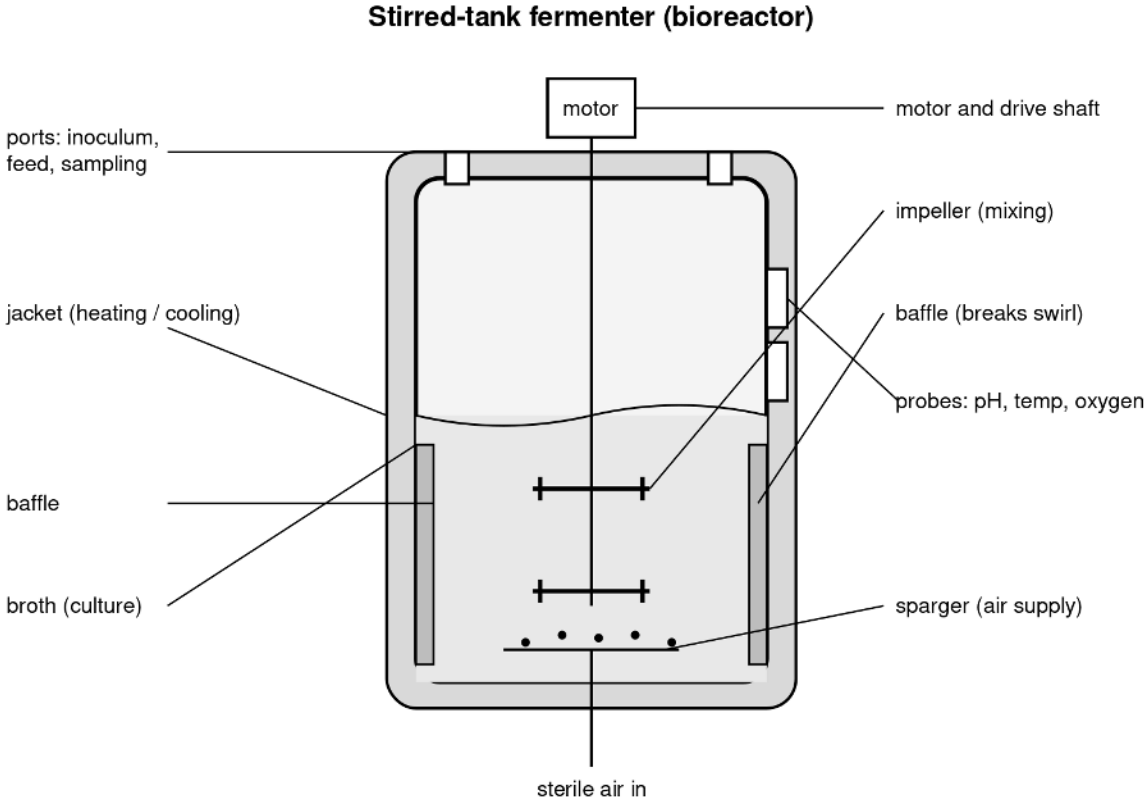


Figure 4.2 *A stirred-tank fermenter and the function of each part.*

The vessel is a sealed, usually stainless-steel tank built to withstand sterilization by steam under pressure, since the entire interior must be sterile at the outset and remain so.

The **impeller**, a set of stirring blades on a motor-driven shaft, agitates the culture so that cells, nutrients, dissolved oxygen, and heat are distributed evenly.

The **baffles**, vertical strips fixed to the wall, prevent the formation of a vortex and improve mixing and the transfer of oxygen.

The **sparger**, a perforated ring or plate at the base, introduces sterile air as fine bubbles to supply oxygen to the aerobic culture.

The jacket or internal coils carry heating or cooling fluid to maintain the temperature, since active growth generates heat.

The probes continuously measure temperature, pH and dissolved oxygen, and feed the controllers that regulate them.

The ports permit the sterile addition of inoculum, nutrient and pH-correcting agents, and the withdrawal of samples, without admitting contaminants.

The foam-control system, mechanical or chemical, suppresses the foam generated by aeration and agitation.

An alternative design, the **airlift fermenter**, dispenses with the impeller and mixes the culture by the rising stream of sparged air, which circulates the broth around an internal loop. Having no stirrer shaft entering the vessel, it has fewer moving parts and one fewer route for contamination, and it handles shear-sensitive cells gently, at the cost of less vigorous mixing. Whatever the design, the vessel is sterilized in place before each run and every entering air stream is filtered, so that the culture is protected by the same discipline that protects a sterile product.

The conditions within the fermenter are held within narrow limits because the yield is sensitive to each of them. The temperature is controlled through the jacket or coils to the optimum for the organism, since both growth and product formation fall away on either side of it. The pH is held at its set point by the automatic addition of acid or alkali, because metabolism continually tends to change it. The dissolved oxygen of an aerobic culture is maintained above the critical level by adjusting the rate of aeration and the speed of the impeller, oxygen being poorly soluble and rapidly consumed by a dense culture. Foam, which would otherwise carry cells into the exhaust and block the air filters, is suppressed mechanically or by an antifoam agent. Samples are withdrawn through sterile ports so that the cell mass, the substrate, and the product can be followed through the run.

Other designs of fermenter exist for particular purposes. The **tower (bubble-column) fermenter** is a tall vessel mixed by sparged air without mechanical agitation, related in principle to the airlift. Packed-bed and other immobilised-cell reactors retain the organism on a support while medium flows past, and are used where the catalyst is to be reused. The stirred-tank remains the standard for antibiotic manufacture because of the thorough mixing and the high rate of oxygen transfer it provides.

4.3.6 Scale-up

A process developed in a small laboratory fermenter cannot simply be transferred to a vessel of many thousands of litres, because the physical conditions change with size. As the volume increases, the ratio of surface to volume falls, so that the removal of heat and the transfer of oxygen become more difficult, and the time required to mix the contents lengthens. Scale-up is the

systematic enlargement of the process through intermediate (pilot) vessels, holding constant a chosen parameter such as the rate of oxygen transfer or the power input per unit volume, so that the yield obtained in the laboratory is reproduced at production scale. It is one of the central problems of industrial microbiology and a frequent reason that a promising laboratory result fails to translate into manufacture.

4.3.4 Products of fermentation

The range of pharmaceutical products obtained by fermentation is wide, and the principal ones are listed in Table 4.3. The antibiotics are obtained chiefly from the actinomycetes and the moulds; the largest-volume fermentation chemical, citric acid, is obtained almost entirely from *Aspergillus niger*; amino acids such as glutamic acid and lysine are obtained from *Corynebacterium glutamicum*; certain vitamins, including B12 and riboflavin, and many industrial enzymes are obtained from microbial cultures; and, through recombinant DNA technology, therapeutic proteins such as human insulin are obtained from engineered organisms. The unifying principle is that a living cell performs, cheaply and at scale, chemistry that would be difficult or impossible to perform by synthesis.

Table 4.3: Representative pharmaceutical products obtained by fermentation

Product	Class	Producing organism
Penicillin	Antibiotic	*Penicillium chrysogenum*
Streptomycin	Antibiotic	*Streptomyces griseus*
Citric acid	Organic acid	*Aspergillus niger*
Glutamic acid, lysine	Amino acids	*Corynebacterium glutamicum*
Vitamin B12, riboflavin	Vitamins	Bacteria and fungi
Human insulin	Recombinant protein	Engineered *Escherichia coli* or yeast

4.3.5 An example: the production of penicillin

The production of penicillin illustrates the process as a whole. A high-yielding strain of *Penicillium chrysogenum*, the result of decades of strain improvement, is grown from a preserved spore stock through laboratory and seed stages to provide the inoculum for a production fermenter of many thousands of litres. The sterilised medium is a complex one based on a carbohydrate and corn-steep liquor, with phenylacetic acid added as a precursor to favour benzylpenicillin and an antifoam to control foaming. The fermentation is aerobic and is conducted as a fed-batch, sterile air being

sparged continuously while the temperature, pH and dissolved oxygen are held at their set points; carbohydrate is fed during the run to sustain the idiophase in which the antibiotic is formed. After several days the broth is filtered to remove the mycelium, and the penicillin, which is secreted into the liquid, is recovered by solvent extraction at low pH, purified and crystallised as a salt. The whole sequence, from strain to sterile crystalline product, embodies the principles set out above and is the model on which the manufacture of later antibiotics was built.

> **Quick recall.** Fermentation is the controlled large-scale culture of a selected organism for a useful product. Primary metabolites form during growth; secondary metabolites (antibiotics) after it. Modes: batch, fed-batch (usual for antibiotics), continuous. The stirred-tank fermenter controls agitation (impeller, baffles), aeration (sparger), temperature (jacket), and pH and oxygen (probes), the whole kept sterile.

4.4 Summary

Microbial spoilage is the deterioration of a product by the growth of microorganisms within it, with chemical consequences (loss of potency, attack on excipients), physical consequences (breakdown of the dosage form, gas, odour, discolouration), and the most serious, infective, consequence that the product becomes a vehicle of infection. Whether spoilage occurs is decided chiefly by the water activity, the available water on a scale where pure water is 1.0, with bacteria requiring the most, yeasts less, and moulds least; pH then selects bacteria or fungi, and nutrients, temperature, packaging and any preservative modify the outcome.

The same control of growth conditions, applied constructively, is fermentation, the large-scale controlled culture of a selected organism to manufacture a product. The product may be a primary metabolite formed during growth or a secondary metabolite, such as an antibiotic, formed after it. Fermentations are operated batch, fed-batch, or continuous, and conducted as submerged or solid-state, aerobic or anaerobic cultures. The process comprises inoculum development, sterilization of medium and vessel, the controlled fermentation, and downstream recovery and purification. The stirred-tank fermenter controls agitation, aeration, temperature and pH while remaining sterile, and the airlift fermenter achieves mixing by aeration alone. The medium supplies the carbon, nitrogen, mineral and growth-factor needs of the producer, with precursors and antifoam as characteristic additions, and the production strain is the product of prolonged strain improvement that has raised yields manyfold. The conditions of temperature, pH and dissolved oxygen are held within narrow limits, the culture is protected against contamination throughout, and the process is enlarged from laboratory to plant by scale-up that preserves the critical physical conditions. By these means microorganisms manufacture antibiotics, vitamins, amino acids, organic acids, enzymes and recombinant proteins, the production of penicillin serving as the model for the manufacture of later antibiotics. The next unit turns to the deliberate construction and defence of the clean environment in which sterile products are made.

4.5 Self-assessment

A. Multiple-choice questions

1. The single factor that best predicts whether a product will be spoiled is:

(a) its colour (b) its water activity (c) its viscosity (d) its container size. Answer: (b).

2. Below a water activity of about 0.60:

(a) only bacteria grow (b) only yeasts grow (c) essentially no microbial growth occurs (d) moulds grow fastest. Answer: (c).

3. An antibiotic is, in relation to the growth of its producing organism, a:

(a) primary metabolite (b) secondary metabolite (c) structural protein (d) storage compound. Answer: (b).

4. The mode of fermentation in which fresh nutrient is added during the run without removal of broth is:

(a) batch (b) fed-batch (c) continuous (d) solid-state. Answer: (b).

5. In a stirred-tank fermenter, the part that supplies oxygen to the culture is the:

(a) impeller (b) baffle (c) sparger (d) jacket. Answer: (c).

6. Citric acid is manufactured chiefly by fermentation using:

(a) *Streptomyces griseus* (b) *Aspergillus niger* (c) *Penicillium chrysogenum* (d) *Corynebacterium glutamicum*. Answer: (b).

7. The early period of an antibiotic fermentation, characterised by rapid growth and little product, is the:

(a) idiophase (b) trophophase (c) stationary phase (d) death phase. Answer: (b).

8. A precursor is added to a fermentation medium in order to:

(a) suppress foam (b) be incorporated into the product and raise its yield (c) control the pH (d) sterilize the medium. Answer: (b).

9. The chief difficulty introduced by increasing the scale of a fermentation is:

(a) higher cost of medium only (b) impaired heat removal and oxygen transfer (c) loss of the inoculum (d) change of the organism. Answer: (b).

10. Penicillin is secreted into the broth and is therefore recovered chiefly by:

(a) disruption of the cells (b) solvent extraction from the filtered liquid (c) distillation (d) ion exchange of the mycelium. Answer: (b).

B. Short-answer questions (2 to 5 marks)

Q1. Define microbial spoilage and state its three kinds of consequence.

Microbial spoilage is the deterioration of a pharmaceutical product caused by the growth and metabolic activity of microorganisms within it. Its consequences are chemical (degradation of the active ingredient or excipients, with loss of potency), physical (breakdown of the dosage form, with gas, odour, discolouration, or visible growth), and infective (the product becomes a vehicle of infection, the most serious consequence, especially for ophthalmic, parenteral and topical products).

Q2. Explain water activity and its importance in spoilage.

Water activity is the measure of the water in a product available to microorganisms, on a scale where pure water is 1.0; it differs from water content because solutes bind water and lower its availability. It is the dominant factor in spoilage because each group of organisms requires a minimum available water: most bacteria above 0.90, yeasts above 0.88 (osmophilic below 0.80), and moulds down to about 0.60. Lowering the water activity below the threshold prevents growth and so protects the product.

Q3. Distinguish a primary from a secondary metabolite, with an example of each.

A primary metabolite is a product of the normal growth of the organism, formed during the logarithmic phase, for example an amino acid such as glutamic acid. A secondary metabolite is a product not required for growth, formed chiefly after rapid growth has ceased, for example an antibiotic such as penicillin. The distinction governs the management of the fermentation, since secondary-metabolite production requires growth to be promoted first.

Q4. Compare batch, fed-batch, and continuous fermentation.

In batch fermentation the vessel is charged once and harvested at the end, with nothing added or removed; it is simple and easily kept sterile but limited in yield. In fed-batch fermentation nutrient is added during the run without removing broth, prolonging the productive state; it is the usual mode for antibiotics. In continuous fermentation medium is added and broth withdrawn at the same rate to hold a steady state; it is productive but runs long, with an accumulating risk of contamination.

Q5. Name the principal parts of a stirred-tank fermenter and give the function of the impeller, the sparger, and the baffles.

The principal parts are the sterilizable vessel, the impeller on a driven shaft, the baffles, the sparger, the temperature jacket or coils, the probes for pH, temperature and dissolved oxygen, the ports for sterile addition and sampling, and the foam-control system. The impeller agitates the culture to distribute cells, nutrients, oxygen and heat; the sparger introduces sterile air as fine bubbles to supply oxygen; the baffles prevent vortex formation and improve mixing and oxygen transfer.

Q6. State the constituents of a fermentation medium and the function of a precursor and an antifoam.

A fermentation medium provides a carbon and energy source (a carbohydrate or cheaper substrate), a nitrogen source (organic such as corn-steep liquor, or inorganic such as ammonium salts), mineral elements, and growth factors for fastidious organisms. A precursor is a compound added to be incorporated directly into the product to raise its yield or direct its structure, for example phenylacetic acid in a penicillin fermentation. An antifoam agent is added to suppress the foam produced by aeration and agitation.

Q7. What is meant by scale-up, and why is it difficult?

Scale-up is the systematic enlargement of a fermentation from the laboratory through pilot vessels to production scale. It is difficult because the physical conditions change with size: as volume increases the surface-to-volume ratio falls, so heat removal and oxygen transfer become harder and mixing takes longer. Scale-up holds a chosen parameter constant, such as the oxygen-transfer rate or the power input per unit volume, so that the laboratory yield is reproduced at full scale.

Q8. How is a fermentation protected against contamination during the run?

The medium and the vessel are sterilized before inoculation, the air supplied for aeration is passed through a sterilising-grade filter, additions and samples are made through sterile ports, and the vessel is held under positive pressure so that leakage is outward. A large, active inoculum establishes the producer quickly and helps it resist invasion. The risk increases with the length of the run, which is one reason continuous fermentation is harder to sustain than batch or fed-batch.

Q9. Describe the forms that microbial spoilage takes in different dosage forms.

In aqueous solutions and mixtures spoilage produces turbidity, sediment, gas and off-odours; in emulsions and creams it attacks the emulsifier or oil phase, causing separation and changes in texture and smell; in syrups it ferments the sugar to gas and acid; and in tablets and powders, normally too dry to support growth, a rise in moisture during faulty storage may allow surface moulds to develop. The form of spoilage reflects the water activity and composition of the dosage form.

C. Long-answer questions (10 marks)

Q1. Discuss the factors affecting the microbial spoilage of pharmaceutical products.

A complete answer treats water activity as the dominant factor, with the approximate thresholds for bacteria, yeasts and moulds and the resulting pattern of spoilage in dilute, concentrated and dry products, and then treats nutrient content, pH (selecting bacteria or fungi), temperature of storage, packaging and storage conditions, and the preservative, explaining how formulation manipulates these factors to deny the contaminant the conditions for growth.

Q2. Describe the construction and operation of a stirred-tank fermenter, and outline the stages of a fermentation process.

A complete answer describes the labelled parts of the stirred-tank fermenter (vessel, impeller, baffles, sparger, jacket, probes, ports, foam control) with the function of each, and outlines the stages of the process: inoculum development, sterilization of medium and vessel, the controlled fermentation with management of growth then product formation, and downstream recovery and purification. Credit is given for noting the maintenance of sterility throughout and for distinguishing primary and secondary metabolite production. (High-yield: the fermenter and the fermentation process are frequently examined.)

Q3. Describe, with the example of penicillin, the stages of an industrial fermentation process.

A complete answer follows the process from a high-yielding strain of Penicillium chrysogenum through inoculum development to the production fermenter; describes the sterilised complex medium with a carbohydrate, corn-steep liquor, a phenylacetic-acid precursor, and an antifoam; explains the aerobic fed-batch operation with control of temperature, pH and dissolved oxygen and the management of trophophase and idiophase; and describes the downstream recovery by filtration of the mycelium, solvent extraction of the secreted penicillin, purification and crystallisation. Credit is given for the maintenance of sterility throughout and for the distinction between growth and production phases.

D. Structured note

Write a note on the products of fermentation of pharmaceutical importance.

Fermentation yields antibiotics (penicillin from Penicillium chrysogenum, streptomycin from Streptomyces griseus), the organic acid citric acid from Aspergillus niger, amino acids such as glutamic acid and lysine from Corynebacterium glutamicum, vitamins such as B12 and riboflavin, industrial enzymes, and, through recombinant DNA technology, therapeutic proteins such as human insulin. The common principle is that a microbial cell performs at scale chemistry that is difficult to achieve by synthesis.

Write a note on the modes of fermentation.

By mode of operation a fermentation is batch, fed-batch, or continuous. In the batch mode the vessel is charged once and harvested at the end, with only gases and control agents added during the run; it is simple and easily kept sterile but limited in yield. In the fed-batch mode fresh nutrient is added during the run without removal of broth, prolonging the productive phase; it is the mode most used for antibiotics. In the continuous mode medium is fed in and broth withdrawn at equal rates to maintain a steady state of constant growth; it gives a high output but runs for long periods with an accumulating risk of contamination. By physical arrangement a fermentation is submerged, the organism suspended throughout the liquid, or solid-state, the organism grown on a moist solid;

submerged culture predominates in antibiotic manufacture. By oxygen requirement it is aerobic or anaerobic, the antibiotic producers being aerobic and requiring vigorous aeration.

References

1. Waites MJ, Morgan NL, Rockey JS, Higton G. Industrial Microbiology: An Introduction. Oxford: Blackwell Science; 2001.
2. Denyer SP, Hodges NA, Gorman SP, editors. Hugo and Russell's Pharmaceutical Microbiology. 8th ed. Oxford: Wiley-Blackwell; 2011.
3. Pelczar MJ, Chan ECS, Krieg NR. Microbiology: Concepts and Applications. New York: McGraw-Hill.
4. Sandle T. Pharmaceutical Microbiology: Essentials for Quality Assurance and Quality Control. Cambridge: Woodhead Publishing/Elsevier; 2016.

Chapter 5: Aseptic Area Design, Laminar Flow, Clean-Area Classification and Biosafety

Learning outcomes

After studying this chapter the student will be able to:

- Describe the principles of design of an **aseptic area**, including the **cascade of cleanliness**, pressure differentials, and **airlock**s.
- Explain the principle, construction and use of laminar air **flow** equipment, and distinguish horizontal from vertical units.
- State the classification of clean areas by particle and microbial limits and match each grade to its use.
- Explain the four biological safety levels and the classes of **biological safety cabinet**.
- Summarise the methods of prevention of contamination in the manufacture of sterile products.

5.1 Introduction

An aseptic area is a controlled environment, designed and maintained so that the number of microorganisms and particles in it is held below defined limits, in which sterile products are prepared without the introduction of contamination. The requirement arises directly from the preceding unit: a sterile product that cannot be terminally sterilized must be made by aseptic processing, in which there is no final step to destroy a contaminant, so that the assurance of sterility rests entirely on the environment in which the product is exposed. The design of that environment is therefore not a matter of convenience but the principal safeguard of the product. This chapter describes how a clean environment is constructed and graded, how filtered air is delivered to the point at which the product is exposed, how clean areas are classified by the limits they must meet, and, where the hazard is reversed and the organism must be contained for the protection of the worker, how laboratories are graded by biological safety level. The chemical agents used to disinfect and preserve are treated separately in Chapter 6.

5.2 Design of the aseptic area

An aseptic area is not a single room but a series of connected spaces of increasing cleanliness, arranged so that the cleanest space, in which the product is exposed, is the most protected. The design rests on several principles that act together, and the failure of any one compromises the rest.

The first principle is the cascade of cleanliness. The area is arranged as a set of nested zones, from the general factory through rooms of intermediate cleanliness to the critical zone at the centre, so

that contamination is shed in stages and the critical zone is never directly open to a dirty space. A person or an article reaches the critical zone only by passing through the intervening zones and becomes cleaner at each step.

The second principle is the **pressure cascade**. The cleanest room is held at the highest air pressure, and the pressure falls step by step toward the exterior, the difference between adjacent rooms being of the order of 10 to 15 pascals. Because air flows from high pressure to low, any leakage or door opening drives air outward from the clean zone, so that dirtier air cannot enter. A reversal or loss of the pressure differential is among the first faults an inspector looks for, because it defeats the whole scheme. (Where a hazardous material must be contained, the pressure regime is deliberately inverted, as described in Section 5.5.)

The third principle is the airlock. People and materials enter the clean area through small intermediate rooms fitted with interlocked doors, so that the clean zone is never open directly to the exterior. Personnel change and gown in stages within the airlock; materials are disinfected or passed through a hatch. The airlock is the place at which the cascade is enforced in practice.

The fourth principle is the control of flow. The layout directs the movement of materials and personnel in one direction, from less clean to more clean and out again by a separate route, so that a clean article never crosses the path of a contaminated one and waste never travels back over clean material.

The fifth principle concerns **surfaces and construction**. Walls, floors and ceilings are smooth, hard, impervious and free of cracks and ledges, with the junctions coved (rounded) so that no corner can trap dirt or resist cleaning. Fittings are kept to a minimum and services are concealed, doors are flush and self-closing, and windows are sealed and non-opening. Sinks and drains are **excluded** from the cleaner grades, because they harbour Gram-negative bacteria. The changing rooms through which personnel enter are themselves designed as a graded series, the operator gowning progressively as described in Chapter 3 so that the level of cover matches the grade entered. The area is, in short, built to be cleaned and to be entered without bringing contamination in.

Underlying all of these is the heating, ventilation and air-conditioning (HVAC) system, which supplies filtered air at a controlled temperature and humidity, maintains the pressure cascade, and changes the air of each room many times an hour so that the particles and organisms released within are continuously diluted and **removed**. The number of air changes and the grade of filtration rise with the cleanliness required of the room. A general clean area may receive of the order of twenty air changes an hour, whereas a **Grade A** zone is supplied with a continuous flow that effects far more. The air-handling unit contains, in sequence, a coarse pre-filter that removes the larger particles, a finer secondary filter, and, for the cleaner grades, a terminal HEPA filter at the point of supply; it also conditions the temperature and humidity, both of which are controlled for the

comfort and the low shedding of gowned personnel and for the stability of the product. The delivery of filtered air to the critical zone itself is the function of laminar air flow, considered next.

> **Quick recall.** An aseptic area is a cascade of nested zones, cleanest at the centre. The pressure cascade (about 10 to 15 Pa between rooms) keeps clean air flowing outward. Airlocks enforce the step-down for people and materials, flow keeps clean and dirty routes apart, surfaces are smooth and coved, and the HVAC system filters the air and changes it many times an hour.

5.3 Laminar air flow equipment

Filtration and pressurisation reduce the general level of contamination in a room, but they cannot render any single point free of organisms, because room air is turbulent and an operator within it sheds organisms continuously. To protect the exact point at which the product is exposed, a different principle is used: the critical zone is bathed in a stream of filtered air that moves in one direction at a uniform velocity and sweeps airborne contamination away from the product before it can settle. This is **laminar air flow (LAF)**, also called unidirectional air flow.

The essential component of an LAF unit is the **high-efficiency particulate air (HEPA) filter**, which removes essentially all particles, including those carrying microorganisms, from the air passed through it. A HEPA filter is constructed of a pleated mat of fine glass fibres and removes at least 99.97 per cent of particles of 0.3 micrometre, the size most difficult to capture; it functions not as a simple sieve but by the interception, impaction and diffusion of particles onto the fibres. Because the integrity of the filter is essential to the protection it provides, each filter is tested on installation and periodically thereafter, by challenging it with an aerosol and scanning its face for leakage, so that a damaged filter or a faulty seal is detected. The filtered air is delivered across the work zone at a controlled, uniform velocity, conventionally of the order of 0.45 metre per second, fast enough to sweep contamination away but not so fast as to disturb the work. The filtered air leaves the face of the filter as a smooth, even stream in which all the air moves in parallel at the same speed, without the eddies of ordinary room air. Because the stream does not mix, it does not pick up and recirculate contamination but carries it steadily out of the zone. The contrast is with **turbulent (non-unidirectional) flow**, in which filtered air is merely supplied to a room and mixed with the room air to dilute the contamination; turbulent flow suffices for the supporting clean areas but not for the critical zone.

Laminar air flow is arranged in one of two directions, and the choice determines what is protected.

In a **horizontal laminar air flow** unit, the HEPA filter forms the back wall of the cabinet and the filtered air flows horizontally across the work surface toward the operator. The arrangement protects the product well, but the air, having passed over the product, flows toward the worker; a horizontal unit is therefore unsuitable when the material handled is hazardous to the operator.

In a **vertical laminar air flow** unit, the HEPA filter is in the ceiling of the cabinet and the filtered air flows downward over the work and is removed at the base or the front. Vertical flow is suitable for a wider range of work and is the basis of the biological safety cabinets described in Section 5.5, which can be arranged to protect the operator as well as the product.

The proper use of laminar flow requires an understanding of what it does and does not achieve. It does not sterilize; it supplies particle-free air to a defined zone and removes contamination from it. Its protection is lost wherever the unidirectional stream is interrupted: if a hand, an implement, or a piece of apparatus is placed between the filter and the exposed product, it casts a wake of disturbed air downstream in which contamination can reach the product. Correct technique therefore consists, above all, in never working in such a way that anything is interposed between the filter and the open product. The two arrangements are shown in Figure 5.1.

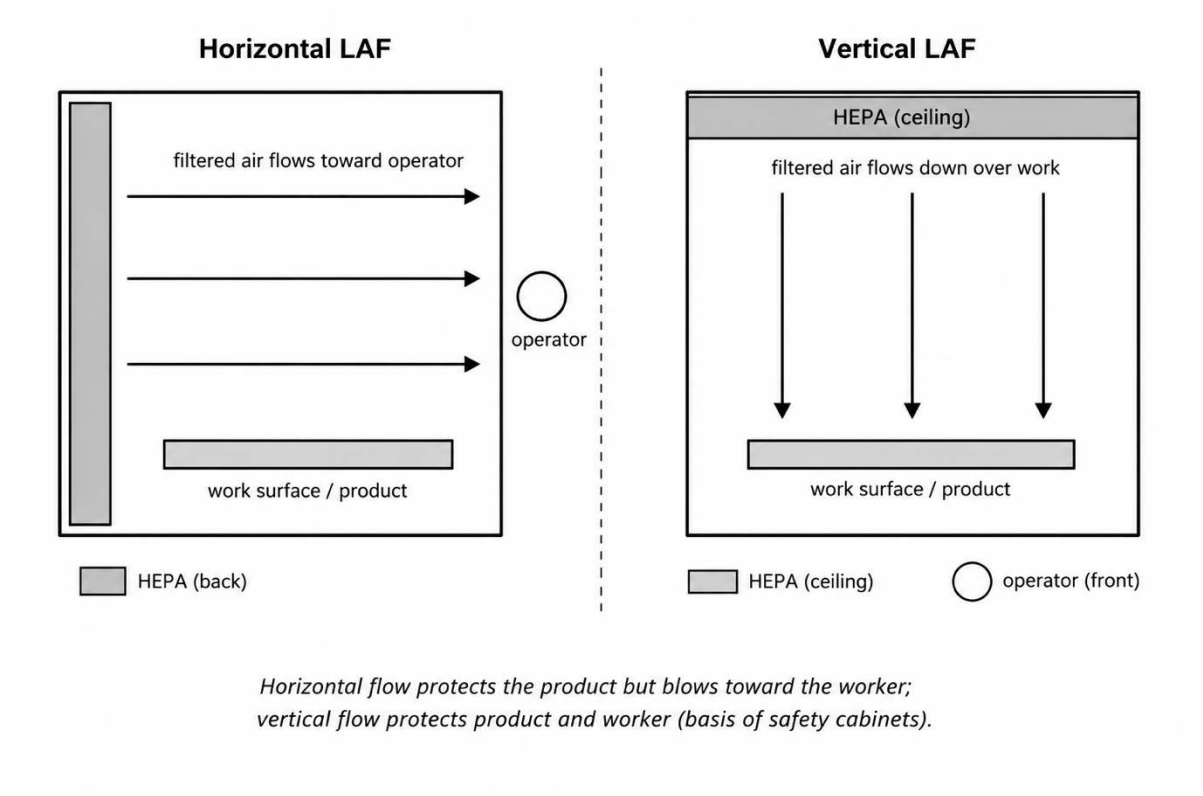


Figure 5.1 *Horizontal and vertical laminar air flow.*

Laminar air flow is provided in several physical forms. A laminar flow bench or cabinet provides a clean zone for a single operation on a bench; a laminar flow ceiling supplies an entire room or a filling line with downward unidirectional air. A further development encloses the critical zone altogether. A **restricted-access barrier system (RABS)** separates the operator from the critical zone by a physical barrier with glove ports, and an **isolator** encloses the critical zone completely in a sealed, decontaminated enclosure supplied with HEPA-filtered air and worked through gloves or fitted with automated handling. By removing the operator, the largest single source of

contamination, from the critical zone, these enclosed systems give a higher and more reliable assurance of sterility than an open clean area and are increasingly used for aseptic filling.

> **Quick recall.** Laminar (unidirectional) air flow bathes the critical zone in a uniform stream of HEPA-filtered air that sweeps contamination away from the product. Horizontal flow protects the product but blows toward the operator; vertical flow protects product and operator and underlies the biological safety cabinet. LAF does not sterilize, and its protection is lost if anything is placed between the filter and the product.

5.4 Classification of clean areas

To build and operate zones of defined cleanliness, the cleanliness must be specified and measured. It is specified by the concentration of airborne particles, because particles are the vehicles on which microorganisms travel and because they can be counted rapidly by an instrument. The international standard for airborne particle cleanliness is **ISO 14644**, which defines classes by the maximum permitted number of particles of a given size per cubic metre. For pharmaceutical manufacture the European Union GMP guide defines a parallel scheme of grades, Grade A, B, C and D, which, unlike the ISO classes alone, also sets limits on viable microorganisms and distinguishes the state of the room in which the measurement is made.

Two states are distinguished, and a grade is meaningful only when the state is stated. The **at-rest** state is that in which the installation is complete and operating but no personnel are present; the **in-operation** state is that in which the process is running and the normal number of personnel are present and working. The in-operation count is the higher, because personnel and activity generate particles.

The grades and their uses are as follows, and are summarised in Table 5.1.

Grade A is the critical zone, the local environment in which the product is exposed and filled. It corresponds to ISO Class 5 and is required to meet the limit of not more than 3,520 particles of 0.5 micrometre or larger per cubic metre in both the at-rest and the in-operation state, and it is supplied by laminar air flow. The viable count permitted in Grade A is very low, of the order of less than one colony-forming unit per cubic metre.

Grade B is the clean area that immediately surrounds and supports the Grade A zone in aseptic processing. It meets the Grade A particle limit at rest but is permitted a higher count in operation, because it is the staffed background to the critical zone rather than the critical zone itself.

Grade C and Grade D are clean areas of progressively lower cleanliness, used for the less critical stages of manufacture, such as the preparation of solutions before they are sterilized or filtered. They carry higher particle and microbial limits and form the supporting shell around the cleaner zones.

The microbial limits follow the same descending order and are verified by the environmental monitoring methods of Chapter 10, namely settle plates, active air sampling, and contact plates. The microbial limits set for the grades fall in the same order, and a representative scheme is given in Table 5.1; the figures are recommended action limits for the in-operation state and are verified by routine monitoring. The principle is constant: the nearer the air is to the exposed product, the cleaner it must be, in both particles and organisms.

Table 5.1: Representative microbial limits for the clean-area grades (in operation)

Grade	Air sample (CFU/m³)	Settle plate, 90 mm (CFU per 4 hours)	Contact plate, 55 mm (CFU per plate)
A	less than 1	less than 1	less than 1
B	10	5	5
C	100	50	25
D	200	100	50

A clean area is not classified once and then assumed to remain so; its compliance with these limits is confirmed continuously. On qualification the area is classified in the at-rest and in-operation states by particle counting, and in routine use the particle count and the viable count are **monitored** on a defined schedule, most intensively in and around the Grade A zone during filling. The viable count is determined by the methods described in Chapter 10, namely settle plates exposed for a measured time, active air samplers drawing a measured volume onto a medium, and contact plates and swabs for surfaces and gloved hands. The results are trended against alert and action limits, so that a drift is detected and corrected before a limit is breached. The monitoring of the clean area is thus the routine confirmation that the design described above is performing as intended. Before routine use, the area and its air-handling system are formally qualified, in the same manner as a sterilizer is validated (Chapter 8): the installation is shown to conform to the design, the system is shown to operate to specification across its range, and its performance is demonstrated by classifying the area and by mapping the airflow, the pressure differentials, and the recovery of the room after a disturbance. Only when this qualification is complete is the area approved for manufacture, and it is requalified periodically and after any significant change. The control of a clean area is therefore a continuing programme of qualification and monitoring, not a single act of construction. A change to the air-handling system, the introduction of new equipment, or a trend of adverse monitoring results all trigger a reassessment, because the controlled state, once achieved, must be actively maintained. In this respect the clean area resembles the validated sterilizer of Chapter 8: both are qualified before use, monitored in use, and requalified whenever a change might affect their performance.

The assignment of a grade to an operation follows from the risk of the operation, and differs between aseptic processing and terminal sterilization. In aseptic processing the critical operations, the filling of the sterilized product and the handling of sterilized components, are performed in a Grade A zone with a Grade B background, because any organism added here reaches the product directly and is not removed afterwards. In the manufacture of a product to be terminally sterilized, the same operations may be conducted in a lower grade, commonly Grade C, because the contamination introduced will be destroyed by the final sterilization; the grade required is set by the bioburden that the sterilization must then overcome. The preparation of solutions before sterilization or filtration is conducted in Grade C or D according to the risk. The grade is thus matched to the consequence of contamination at each step, which is the unifying logic of the scheme.

Table 5.2: Clean-area grades, with approximate particle limits and use

Grade	ISO equivalent	Max particles ≥0.5 µm per m³	Typical use
A	ISO 5 (at rest and in operation)	3,520	Critical zone: product exposure, filling, under LAF
B	ISO 5 at rest; ISO 7 in operation	3,520 (at rest)	Clean-room background to Grade A in aseptic work
C	ISO 7 at rest; ISO 8 in operation	352,000 (at rest)	Less critical stages, e.g. solution preparation
D	ISO 8 at rest	3,520,000 (at rest)	Least critical clean stages; supporting zone

5.5 Biological safety levels

In the clean area the object is to protect the product from the environment and the operator. The opposite requirement arises when the organism handled is itself a hazard to the worker and to the community, and must be contained. **Containment** is graded into four **biological safety levels (BSL)**, numbered 1 to 4, the level of containment being matched to the hazard presented by the organism. The principle is one of proportion: a harmless organism is handled on an open bench, whereas a lethal one is handled only within multiple barriers.

Biological safety level 1 (BSL-1) applies to organisms not known to cause disease in healthy adults, such as non-pathogenic strains of *Escherichia coli* used in teaching. Standard microbiological practice on an open bench, with hand-washing and the decontamination of waste, is sufficient.

Biological safety level 2 (BSL-2) applies to organisms of moderate hazard that cause human disease, usually through ingestion or through contact with the skin or mucous membranes, such as *Staphylococcus aureus*, *Salmonella* species, and the hepatitis B virus. Procedures likely to generate aerosols are carried out in a biological safety cabinet, access is restricted, and waste is decontaminated. Most pharmaceutical and hospital microbiology laboratories operate at this level.

Biological safety level 3 (BSL-3) applies to organisms that cause serious or lethal disease and may be transmitted by the airborne route, such as *Mycobacterium tuberculosis*. The laboratory is specially constructed, with controlled access and a directional airflow that draws air inward so that nothing escapes, and all work with the agent is performed in a safety cabinet. Containment now resides in the building as well as in the technique.

Biological safety level 4 (BSL-4) applies to the most dangerous organisms, those that cause life-threatening disease, are readily transmitted as aerosols, and for which no reliable vaccine or treatment exists, such as the Ebola and Marburg viruses. Work is conducted in a sealed positive-pressure suit or within a fully enclosed Class 3 cabinet line, in a dedicated and isolated facility with multiple barriers. This is the highest level of containment. The four levels are shown in Figure 5.2.

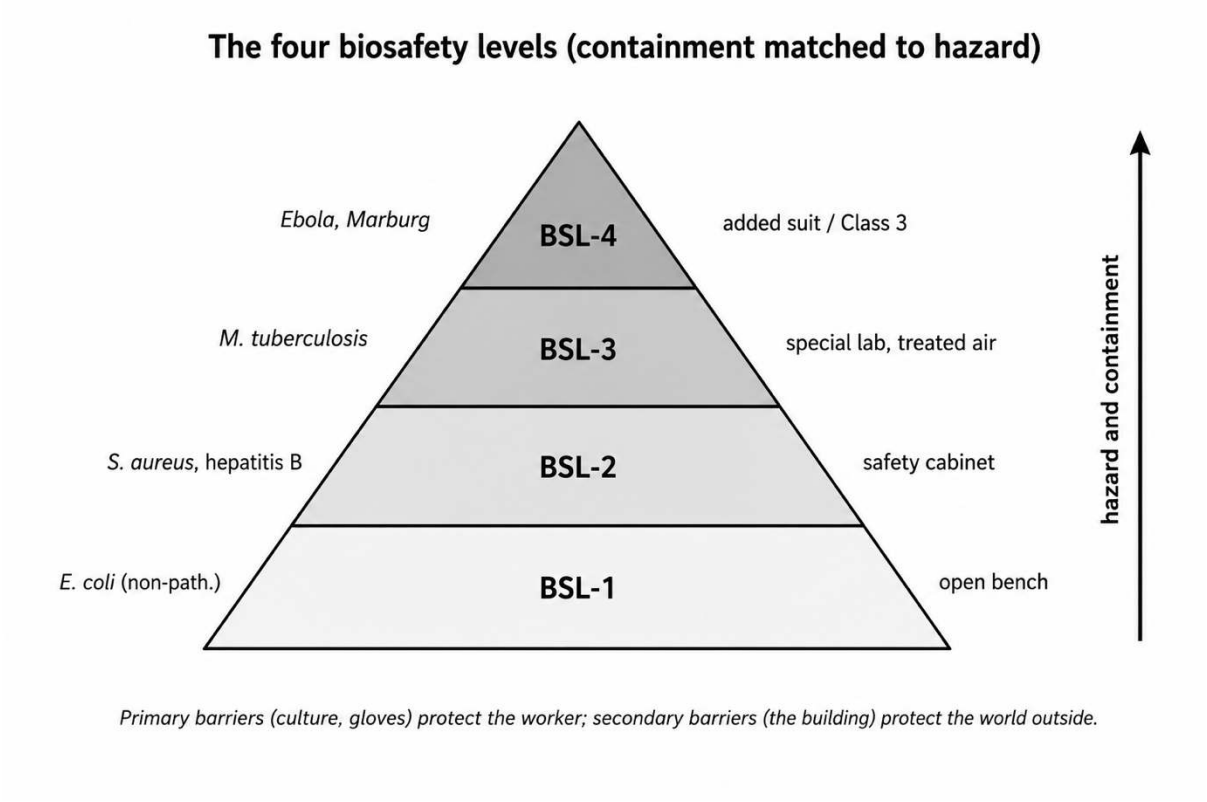


Figure 5.2 *The four biological safety levels, containment matched to hazard.*

Two concepts unify the scheme. A **primary barrier** protects the worker at the point of work and comprises the biological safety cabinet and personal protective equipment. A **secondary barrier** is the laboratory and its services, the directional airflow, airlocks and waste treatment, which protect the world outside. As the biosafety level rises, both barriers are strengthened together.

The biological safety cabinet is the principal primary barrier, and three classes are distinguished by what each protects. A Class 1 cabinet draws room air inward across the work and filters it before exhaust, so that it protects the worker and the environment but not the product, since the inflowing air is unfiltered. A Class 2 cabinet adds a downward curtain of HEPA-filtered air over the work, protecting the worker, the environment, and the product together, and is the type in general microbiological use. A Class 3 cabinet is a gas-tight enclosure held under negative pressure and worked through fixed gloves, and gives the highest containment for the most dangerous organisms. The level of containment is achieved not by the cabinet alone but by a set of practices and engineering controls that strengthen together as the level rises: standard microbiological technique and restricted access at the lower levels; the decontamination of all waste, the use of safety cabinets for aerosol-generating procedures, and medical surveillance of staff at the middle levels; and, at the highest levels, sealed and separately ventilated rooms maintained under negative pressure with respect to their surroundings, airlocks and showers for entry and exit, and the treatment of all exhaust air and effluent before release. The inward direction of airflow at BSL-3 and BSL-4, opposite to the outward flow of a product-protecting clean area, expresses the reversal of purpose: here the object is to keep the organism in, not to keep contamination out.

> **Quick recall.** Biological safety levels run 1 to 4, containment matched to hazard: BSL-1 harmless organisms, open bench; BSL-2 moderate hazard, safety cabinet for aerosols; BSL-3 airborne serious disease, special laboratory with inward airflow; BSL-4 lethal with no cure, sealed suit and dedicated facility. Primary barriers (cabinet, protective equipment) protect the worker; secondary barriers (the building) protect outside. Cabinet classes: 1 protects worker and environment, 2 also protects the product, 3 is a sealed glovebox.

5.5.1 Decontamination of the clean area and the design of filling operations

Two further points complete the account of the controlled environment. First, the fabric of the area is decontaminated not only by the routine disinfection of surfaces but periodically by the fumigation of the whole space with a gaseous sporicidal agent, such as vaporised hydrogen peroxide or, formerly, formaldehyde, which reaches surfaces that a cloth cannot; this supplements the daily sanitisation programme of Chapter 3. Second, the design of the filling operation itself reduces the exposure of the product. In **blow-fill-seal** technology the container is formed from molten plastic, filled and sealed in one continuous automated operation within a single machine, so that the product is exposed only momentarily and the operator is excluded altogether. The same principle, the

exclusion of the operator and the minimising of exposure, underlies the isolators described above and the trend of modern sterile manufacture generally.

5.6 Methods of prevention: a synthesis

The methods of preventing contamination in sterile manufacture, drawn together from this chapter and the last, form a layered defence in which each layer covers the failure of another. Organisms are excluded from the product by clean design, gowning and the pressure cascade; the organisms that nonetheless enter the air are removed by HEPA filtration and swept from the critical zone by laminar flow; surfaces and the product are protected by **disinfection and preservation**, the subject of Chapter 6; and the whole is monitored by environmental sampling so that a failure is detected before it reaches a product. No single measure is sufficient in isolation. The assurance of a sterile product made by aseptic processing rests on the integrity of the entire system, because, as emphasised throughout, there is no terminal step to correct an error once the product is sealed.

5.7 Summary

An aseptic area is a controlled environment in which sterile products are prepared without the introduction of contamination, and its design is the principal safeguard of a product made by aseptic processing. The design rests on a cascade of nested zones of increasing cleanliness, a pressure cascade that keeps clean air flowing outward, airlocks that step personnel and materials down in stages, controlled one-directional flow, smooth coved surfaces, and an HVAC system that filters the air and changes it many times an hour. Within this environment, laminar air flow delivers a unidirectional stream of HEPA-filtered air to the critical zone, horizontal flow protecting the product and vertical flow protecting product and operator; laminar flow does not sterilize and its protection is lost if anything is interposed between the filter and the product.

Clean areas are classified by airborne particle concentration under ISO 14644 and by the EU GMP grades A to D, measured both at rest and in operation, with Grade A (ISO Class 5) the critical filling zone and grades B, C and D the supporting shells, each with its own particle and microbial limits. Where the organism is a hazard to be contained rather than excluded, laboratories are graded into four biological safety levels, from the open bench of BSL-1 to the sealed facility of BSL-4, with primary barriers that protect the worker and secondary barriers that protect the community, and biological safety cabinets of three classes that serve as the chief primary barrier. Increasingly the critical zone is enclosed in an isolator or a restricted-access barrier system, or the product is filled by an automated technology such as blow-fill-seal, so that the operator, the largest single source of contamination, is removed from it altogether. The methods of prevention form a layered defence whose strength lies in the integrity of the whole, and it should be appreciated that the engineering described here protects the product only in combination with the procedural and chemical controls of the surrounding chapters, since a correctly graded room cannot compensate

for an undisinfected surface or an untrained operator. The next chapter treats the chemical agents, the disinfectants, antiseptics and preservatives, by which surfaces, skin and products are protected, completing the account of control by environment and by chemical means before the following unit turns to destruction by sterilization.

5.8 Self-assessment

A. Multiple-choice questions

1. The difference in air pressure maintained between adjacent rooms of a clean facility is of the order of:

(a) 1 to 2 Pa (b) 10 to 15 Pa (c) 100 to 150 Pa (d) 1000 Pa. Answer: (b).

2. Laminar air flow protects the product by:

(a) sterilizing the air (b) supplying a unidirectional stream of HEPA-filtered air that sweeps contamination away (c) heating the work zone (d) raising the humidity. Answer: (b).

3. A horizontal laminar air flow unit is unsuitable for hazardous material because:

(a) it cannot be sterilized (b) the air flows over the product toward the operator (c) it uses no HEPA filter (d) it has no work surface. Answer: (b).

4. The critical zone in which a sterile product is exposed and filled is:

(a) Grade A (b) Grade B (c) Grade C (d) Grade D. Answer: (a).

5. A grade of clean area is meaningful only when one states whether it was measured:

(a) by day or by night (b) at rest or in operation (c) hot or cold (d) wet or dry. Answer: (b).

6. Work with *Mycobacterium tuberculosis*, an airborne pathogen, requires a laboratory of biological safety level:

(a) 1 (b) 2 (c) 3 (d) 4. Answer: (c).

7. A Class 2 biological safety cabinet, unlike a Class 1, also protects:

(a) nothing additional (b) the product (c) only the environment (d) only the worker. Answer: (b).

8. A HEPA filter removes at least:

(a) 90% of particles of 5 μm (b) 99.97% of particles of 0.3 μm (c) 50% of all particles (d) all gases. Answer: (b).

9. An isolator improves the assurance of sterility chiefly by:

(a) heating the product (b) removing the operator from the critical zone (c) replacing the HEPA filter (d) lowering the pressure. Answer: (b).

10. Sinks and drains are excluded from the cleanest grades because they harbour:

(a) fungi only (b) Gram-negative bacteria (c) viruses (d) spores only. Answer: (b).

B. Short-answer questions (2 to 5 marks)

Q1. Explain the cascade of cleanliness and the pressure cascade in an aseptic area.

The cascade of cleanliness is the arrangement of the area as nested zones of increasing cleanliness, from the general factory to the critical zone at the centre, so that contamination is shed in stages and the critical zone is never open directly to a dirty space. The pressure cascade holds the cleanest room at the highest pressure, falling step by step outward by about 10 to 15 Pa between rooms, so that air always flows outward and dirtier air cannot enter. The two together protect the critical zone.

Q2. Distinguish horizontal from vertical laminar air flow, and state the use of each.

In horizontal LAF the HEPA filter is at the back and air flows horizontally across the work toward the operator; it protects the product but is unsuitable for operator-hazardous material because the air flows toward the worker. In vertical LAF the filter is in the ceiling and air flows downward over the work; it suits a wider range of work and is the basis of the biological safety cabinet, protecting product and operator.

Q3. State the particle standard for Grade A and explain why its surrounding room is Grade B.

Grade A corresponds to ISO Class 5 and must not exceed 3,520 particles of 0.5 µm or larger per cubic metre in both the at-rest and in-operation states, supplied by laminar flow. The surrounding room is Grade B because it is the staffed background supporting the critical zone, not the point of product exposure; personnel and activity raise its in-operation count, so it meets the Grade A particle limit only at rest.

Q4. Define the four biological safety levels with an example organism for each.

BSL-1: organisms not known to cause disease in healthy adults (non-pathogenic E. coli), open bench. BSL-2: moderate-hazard organisms (S. aureus, Salmonella, hepatitis B virus), safety cabinet for aerosols. BSL-3: serious airborne pathogens (M. tuberculosis), special laboratory with inward airflow. BSL-4: lethal organisms with no cure (Ebola, Marburg), sealed suit and dedicated facility.

Q5. Distinguish a primary from a secondary barrier in biological containment.

A primary barrier protects the worker at the point of work and comprises the biological safety cabinet and personal protective equipment. A secondary barrier is the laboratory and its services, the directional airflow, airlocks and waste treatment, which protect the community outside. As the biosafety level rises, both are strengthened.

Q6. What is an isolator, and what advantage does it offer in aseptic processing?

An isolator is a sealed, decontaminated enclosure that completely encloses the critical zone, supplied with HEPA-filtered air and worked through fixed gloves or fitted with automated

handling. Its advantage is that it removes the operator, the largest single source of contamination, from the critical zone, these systems give a higher and more reliable assurance of sterility than an open clean area. The restricted-access barrier system is a related arrangement using a physical barrier with glove ports.

Q7. How is the continuing compliance of a clean area with its grade confirmed?

On qualification the area is classified in the at-rest and in-operation states by particle counting. In routine use the particle count and the viable count are monitored on a schedule, most intensively in and around the Grade A zone during filling, the viable count being determined by settle plates, active air samplers, and contact plates. The results are trended against alert and action limits so that a drift is corrected before a limit is breached.

Q8. Why may a product intended for terminal sterilization be filled in a lower grade than one made aseptically?

In aseptic processing there is no final sterilizing step, so any organism introduced at filling reaches the product and remains; the critical operations therefore require Grade A in a Grade B background. A product to be terminally sterilized will have its contamination destroyed by the final sterilization, so the same operations may be performed in a lower grade, commonly Grade C, the grade being set by the bioburden the sterilization must overcome. The grade is matched to the consequence of contamination at each step.

C. Long-answer questions (10 marks)

Q1. Describe the design of an aseptic area for the manufacture of sterile products.

A complete answer treats the cascade of cleanliness, the pressure cascade with its magnitude and outward-flow logic, airlocks for personnel and materials, controlled one-directional flow, smooth coved cleanable surfaces, and the HVAC system providing filtered air, the pressure regime, and a high air-change rate, and relates the design to the delivery of laminar air flow to the critical zone. Credit is given for explaining that the design is the principal safeguard because aseptic processing has no terminal sterilizing step. (High-yield: aseptic-area design and clean-area classification are frequently examined.)

Q2. Classify clean areas and state the use of each grade; explain the at-rest and in-operation states.

A complete answer states that cleanliness is graded by airborne particle concentration under ISO 14644 and the EU GMP grades A to D, gives the Grade A limit (ISO 5; 3,520 particles ≥0.5 µm per m³) and the descending order of B, C and D with their uses, explains that at-rest measurement is made with the installation running but no personnel and in-operation with the process running and personnel present, and notes that the in-operation count is higher and that microbial limits follow the same order.

Q3. Explain the system of biological safety levels and the role of the biological safety cabinet.

A complete answer states that containment is graded into four biological safety levels matched to the hazard of the organism, describes BSL-1 to BSL-4 with an example organism and the practices and engineering controls of each, distinguishes primary barriers (cabinet and protective equipment, protecting the worker) from secondary barriers (the building and its airflow, which protect the community), and describes the three classes of biological safety cabinet by what each protects. Credit is given for noting that the airflow is directed inward at the higher levels, the reverse of the outward flow of a product-protecting clean area.

D. Structured note

Write a note on the biological safety cabinet.

The biological safety cabinet is the chief primary barrier in microbiological containment, and its class is chosen according to whether the product, as well as the worker and the environment, must be protected. A Class 1 cabinet draws unfiltered room air across the work and filters the exhaust, and protects the worker and environment but not the product. A Class 2 cabinet adds a downward curtain of HEPA-filtered air, which protects the worker, environment and product, and is in general use. A Class 3 cabinet is a sealed, gas-tight glovebox under negative pressure that gives the highest containment for the most dangerous organisms at BSL-4, the choice of class thus following directly from what must be protected.

References

1. European Commission. EudraLex Volume 4, Annex 1: Manufacture of Sterile Medicinal Products. Brussels: European Commission. [latest edition]
2. International Organization for Standardization. ISO 14644-1: Cleanrooms and associated controlled environments. Geneva: ISO. [latest edition]
3. Denyer SP, Hodges NA, Gorman SP, editors. Hugo and Russell's Pharmaceutical Microbiology. 8th ed. Oxford: Wiley-Blackwell; 2011.
4. Centers for Disease Control and Prevention and National Institutes of Health. Biosafety in Microbiological and Biomedical Laboratories. [latest edition]

Chapter 6: Disinfectants, Antiseptics, Preservatives and Their Evaluation

Learning outcomes

After studying this chapter the student will be able to:

- Define and distinguish a **disinfectant**, an **antiseptic**, and a **preservative**, and the terms describing antimicrobial action.
- Classify the chemical antimicrobial agents and state the mechanism of action of each class.
- Describe the preservatives used in pharmaceutical formulations.
- Describe the methods used to evaluate a disinfectant and a preservative.
- State the factors that affect the antimicrobial activity of a disinfectant.

These outcomes serve Course Outcome CO4, the selection of appropriate disinfectants, antiseptics and preservatives and the understanding of their evaluation.

6.1 Introduction and definitions

The chemical control of microorganisms is achieved by a group of agents distinguished not by their chemistry alone but by the place in which they are used. A disinfectant is a chemical agent applied to an inanimate object or surface to destroy or reduce the number of harmful microorganisms; because it does not contact living tissue, it may be relatively harsh. An antiseptic is a chemical agent applied to living tissue, such as the skin or a mucous membrane, to reduce the number of microorganisms; it must therefore be sufficiently free of toxicity and irritancy to be tolerated by the tissue. A preservative is a chemical agent incorporated within a product to prevent the growth of microorganisms in it during its shelf life and use. A single substance may serve in more than one of these roles at different **concentration**s, but the role is defined by the site of use: inanimate surface, living tissue, or the interior of a product.

A second set of terms describes the effect of the agent on the organism. An agent that kills microorganisms is described as **-cidal** (bactericidal, fungicidal, virucidal, sporicidal), whereas one that only prevents their multiplication without killing them is **-static** (bacteriostatic, fungistatic). The distinction is not absolute, since many agents are static at low concentration and cidal at higher concentration, but it is important in use: a static agent depends on the host defences or on subsequent removal to dispose of the inhibited organisms, whereas a cidal agent destroys them. A further term, **disinfection**, denotes the destruction of vegetative pathogens but not necessarily of spores, and is therefore a lesser process than **sterilization**, the destruction of all microorganisms including spores, which is the subject of the following unit.

> **Quick recall.** Disinfectant: inanimate surfaces. Antiseptic: living tissue. Preservative: within a product. The role is set by the site of use. A -cidal agent kills; a -static agent only inhibits. Disinfection destroys vegetative pathogens; sterilization destroys all organisms including spores.

6.2 Classification of chemical antimicrobial agents

The chemical antimicrobial agents are classified by chemical type, and within each type the members share a common mechanism of action. The principal classes are set out below and as a chart in Figure 6.1; their modes of action fall into a few categories, namely damage to the **cell membrane**, denaturation or modification of proteins, and reaction with nucleic acids.

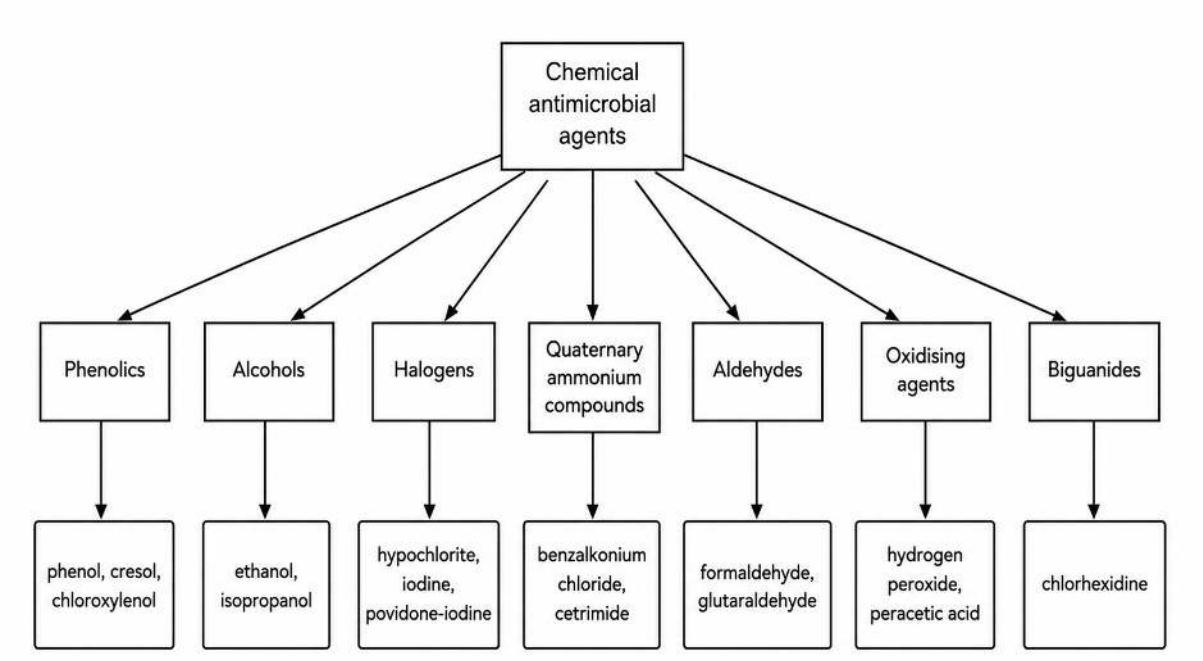


Figure 6.1 *Classification of the chemical antimicrobial agents.*

The **phenols and their derivatives** were among the first disinfectants and remain the reference against which others are measured. Phenol itself, and the substituted phenols (cresols, chlorocresol, chloroxylenol) and bisphenols, act by damaging the cell membrane and by denaturing proteins. They are used as surface disinfectants; chlorocresol and chloroxylenol are milder derivatives used in antiseptic and preservative roles. The phenolics retain useful activity in the presence of organic matter and are stable, which accounts for their long use in hospital disinfection, but they have an irritant odour and are not sporicidal.

The **alcohols**, chiefly ethanol and isopropanol, denature proteins and dissolve membrane lipids. They are most active in aqueous solution at about 60 to 90 per cent, because some water is required for their action, and are weaker when anhydrous. They act rapidly, evaporate without residue, and are widely used as skin antiseptics and surface disinfectants, but they do not kill spores and are

flammable, which limits the volume that may be used and stored. They are the basis of the alcohol-based hand rubs now standard for hand hygiene.

The **halogens** comprise the chlorine-releasing agents (such as hypochlorites and chloramines) and the iodine compounds (iodine solutions and the iodophores such as povidone-iodine). They are strong **oxidising agents** that act on a wide range of organisms; chlorine compounds are used to disinfect surfaces and water, and iodine compounds as skin antiseptics. The chlorine compounds act rapidly and cheaply over a wide spectrum, including viruses, but are corrosive and are readily inactivated by organic matter, so a higher concentration is used for soiled material; the iodophores combine the broad activity of iodine with reduced staining and irritation.

The **quaternary ammonium compounds (QACs)**, such as benzalkonium chloride and cetrimide, are cationic surface-active agents that disrupt the cell membrane. They are active against many bacteria and are mild enough for some antiseptic and preservative use, but they are relatively weak against many Gram-negative organisms, mycobacteria and spores, and their activity is reduced by hard water, soaps and organic matter, and they are inactivated by the anionic surfactants with which they form inactive complexes. They are nevertheless valued for their low odour, low toxicity, and detergent action, which combines cleaning with disinfection.

The **aldehydes**, formaldehyde and glutaraldehyde, act by alkylating and cross-linking proteins and nucleic acids. They are among the few chemical agents that destroy spores, and are used to disinfect heat-sensitive instruments such as endoscopes and, as fumigants, to disinfect enclosed spaces. Glutaraldehyde is less irritant and more stable in use than formaldehyde, but both are toxic and require care in handling and adequate ventilation.

The oxidising agents, hydrogen peroxide and peracetic acid, act by oxidising essential cell components and have the advantage of decomposing to harmless products, which makes them useful where residue is a concern. Both are sporicidal at appropriate concentrations, peracetic acid being effective even in the presence of organic matter, and they are used to sterilize heat-sensitive items and, as vapour, to fumigate enclosed spaces.

The **biguanides**, notably **chlorhexidine**, damage the cell membrane and are important skin antiseptics, active chiefly against vegetative bacteria. Chlorhexidine binds to the skin and so gives a persistent effect that outlasts its application, a property that makes it valuable for surgical hand antisepsis; it is, however, inactivated by soaps and by anionic materials and has little action on spores. The heavy-metal compounds, such as the organomercurials and silver compounds, and certain dyes, such as the acridines and crystal violet, complete the list of agents the student should recognise; their use is now limited but they retain niche applications. Two further groups complete the account. The acids and esters, such as benzoic acid and the parabens, and the gaseous agents, such as ethylene oxide and formaldehyde, are antimicrobial agents of importance; the gaseous agents are powerful sterilants used for heat-sensitive materials and are treated as methods of

sterilization in Chapter 7, while the acids and esters are important chiefly as preservatives, considered in the next section. The classes, examples and modes of action are summarised in Table 6.1.

6.2.1 Mechanism of action of antimicrobial agents

Although the chemical classes are diverse, their lethal or inhibitory action reduces to a small number of attacks upon the cell, and the same target may be reached by agents of different chemistry. The first is damage to the cell membrane: surface-active agents such as the quaternary ammonium compounds and the biguanides, and the lipid-solvent action of the phenols and alcohols, disorganise the membrane so that the cell loses its selective permeability and its contents leak out. The second is the **denaturation of proteins**: the alcohols, phenols and acids coagulate or unfold the cell proteins, and the aldehydes and the heavy metals react with the functional groups of enzymes and inactivate them. The third is **reaction with nucleic acids and other reactive damage**: the alkylating aldehydes cross-link nucleic acids, the oxidising agents and the halogens oxidise a wide range of cell constituents indiscriminately, and certain dyes interfere with nucleic acid function. An agent that acts on a single specific target tends to be inhibitory and selective, whereas an agent that oxidises or alkylates many constituents at once tends to be broadly and irreversibly lethal, which is why the oxidising agents and aldehydes are among the few that destroy spores.

6.2.2 The ideal disinfectant

No single agent is ideal, but the properties of an ideal disinfectant define the criteria by which a real one is chosen. It would have a wide spectrum, acting on bacteria, their spores, fungi and viruses; it would act rapidly even in the presence of organic matter; it would be stable on storage and in use; it would be non-corrosive to the surfaces and non-toxic and non-irritant to the persons who use it; it would penetrate into crevices and be active over a wide range of **pH** and **temperature**; it would not be inactivated by hard water, soaps, or plastics; and it would be cheap, with an acceptable odour. Because these requirements conflict, the choice of a disinfectant for a given purpose is a compromise among them, guided by the organisms to be killed, the surface to be treated, and the conditions of use.

Table 6.1: Principal classes of chemical antimicrobial agents

Class	Examples	Mode of action	Notes
Phenolics	Phenol, cresol, chloroxylenol	Membrane damage, protein denaturation	Reference standard; surface disinfection
Alcohols	Ethanol, isopropanol	Protein denaturation, lipid solvent	Best at 60–90%; no sporicidal action

Class	Examples	Mode of action	Notes
Halogens	Hypochlorite, iodine, povidone-iodine	Oxidation	Surfaces, water, skin
Quaternary ammonium compounds	Benzalkonium chloride, cetrimide	Membrane disruption (cationic surfactant)	Weak vs Gram-negatives and spores; hard water reduces activity
Aldehydes	Formaldehyde, glutaraldehyde	Alkylation and cross-linking	Sporicidal; heat-sensitive instruments
Oxidising agents	Hydrogen peroxide, peracetic acid	Oxidation of cell components	Decompose to harmless products
Biguanides	Chlorhexidine	Membrane damage	Skin antiseptic

6.3 Preservatives

A preservative is the antimicrobial agent incorporated within a product, and it has the most demanding role of the three, since it must inhibit the organisms introduced during manufacture and repeated use, over the whole life of the product, without harming the patient and without reacting with the formulation. An ideal preservative would be active against a wide range of organisms at low concentration, soluble and stable in the product, non-toxic and non-irritant at the concentration used, compatible with the other ingredients and the container, and free of colour, odour and taste. No single agent satisfies all these requirements, and combinations are often used.

The preservatives in common pharmaceutical use include the parabens (the methyl and propyl esters of p-hydroxybenzoic acid), which are widely used in oral and topical products and are often combined to broaden the spectrum; benzoic acid and its salts, effective only in acidic products because the active species is the un-ionised acid; benzalkonium chloride, a quaternary ammonium compound used to preserve ophthalmic and nasal products; chlorocresol and chlorbutol, used in injections and other products; phenylmercuric salts, used at very low concentration in some ophthalmic products; and sorbic acid, used in some preparations. Each of the common agents has a characteristic field of use. The parabens are active against fungi and Gram-positive bacteria and somewhat less so against Gram-negative organisms, so a combination of methyl and propyl esters is used to broaden the cover; they are stable over a wide pH range and are the most widely used preservatives in oral and topical products. Benzoic acid and the benzoates are active only at acid pH, where the un-ionised acid predominates, and are therefore confined to acidic preparations. Benzalkonium chloride is the standard preservative of ophthalmic and nasal products, used at low

concentration, but is incompatible with anionic substances. Chlorocresol and chlorbutol are used in injections and in some topical products, and the phenylmercuric salts, despite their mercury content, retain a limited use in certain ophthalmic products at very low concentration. The choice among them is governed by the nature of the product, its pH, its route of administration, and the compatibility of the preservative with the formulation, and no preservative is selected without regard to the formulation as a whole.

The effectiveness of a preservative system is not assumed but demonstrated by the **preservative efficacy test** (the antimicrobial effectiveness test of the pharmacopoeias), in which the product is deliberately inoculated with specified test organisms and the decline in their number is followed over a defined period; the system passes if the count falls by the required amount within the required time. In the test the product is inoculated with a defined level of each of a panel of test organisms, typically including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Candida albicans*, and *Aspergillus brasiliensis*, the inoculated product is stored at a controlled temperature, and the viable count of each organism is determined at intervals over the test period. The preservative system passes if the count of each organism falls by the prescribed amount within the prescribed time and shows no subsequent increase. The test thus confirms that the preservative actually protects the product under conditions resembling use, and it is required for multi-dose aqueous products and other preparations at risk of microbial growth during use.

A preservative may be compromised in the formulation in ways the formulator must anticipate. It may be inactivated by another ingredient, as the quaternary ammonium compounds are inactivated by anionic surfactants; it may partition into the oil phase of an emulsion or be adsorbed onto a suspended solid or the container, so that the concentration in the aqueous phase, where the organisms grow, falls below the effective level; and its activity depends on the pH, as for benzoic acid, which is active only in the un-ionised form. The selection of a preservative is therefore inseparable from the design of the whole formulation.

6.4 Antiseptics and their use on the skin

The antiseptics, the agents applied to living tissue, deserve separate notice because the constraint of tissue tolerance limits the choice. The skin carries a resident flora, which lives in the deeper layers and is difficult to remove, and a transient flora, acquired from the environment and removed comparatively easily; antisepsis aims to remove the transient flora and to reduce the resident flora. The principal skin antiseptics are the alcohols (ethanol and isopropanol at 60 to 90 per cent), which act rapidly but transiently and are often combined with a longer-acting agent; chlorhexidine, a biguanide that binds to the skin and gives a persistent effect, widely used for hand antisepsis and pre-operative skin preparation; the iodophores, such as povidone-iodine, which release iodine slowly and are well tolerated; and, for specific purposes, chloroxylenol and the quaternary ammonium antiseptics. The use of an antiseptic before an injection, the surgical scrubbing of the

hands, and the preparation of the operation site are routine applications in which the agent must combine adequate activity with tolerance by the tissue. The same agents at higher concentration may serve as disinfectants, the difference lying, as always, in the site of use.

6.5 Evaluation of disinfectants

A claim made for a disinfectant must be substantiated by test, and several methods exist, each measuring a different aspect of performance and each closer than the last to the conditions of actual use.

6.5.1 The phenol coefficient

The oldest and most frequently examined method compares a disinfectant with phenol, the historical standard, under defined conditions, and expresses the result as the phenol coefficient. In the **Rideal-Walker test**, dilutions of the disinfectant and of phenol are each challenged with a standard culture of *Salmonella typhi*, and after fixed contact times the cultures are subcultured to determine the highest dilution of each that kills the organism in the set time. The phenol coefficient is the ratio of the killing dilution of the disinfectant to the killing dilution of phenol; a value greater than one indicates an agent more active than phenol, a value less than one an agent less active. The principle is shown in Figure 6.2.

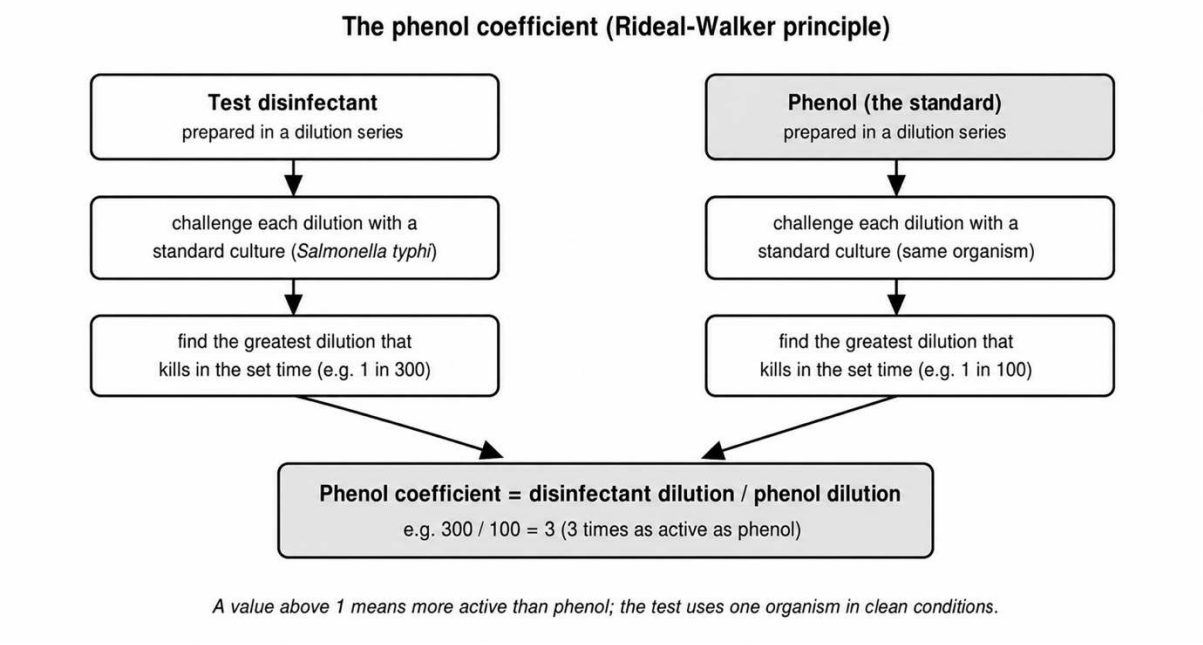


Figure 6.2 *The phenol coefficient (Rideal-Walker principle).*

> Phenol coefficient = (highest dilution of disinfectant killing in the set time) ÷ (highest dilution of phenol killing in the same time)

A worked illustration makes the calculation concrete: if the disinfectant kills the test organism at a dilution of 1 in 300 while phenol kills only at 1 in 100 under the same conditions, the phenol

coefficient is 300 ÷ 100 = 3, that is, the disinfectant is three times as active as phenol on a dilution basis. The **Chick-Martin test** is a related method that determines the phenol coefficient in the presence of an organic load (originally dried yeast, later faeces or another standard soil), and so gives a value closer to the performance of the agent on a soiled surface, which is the condition of most practical disinfection. The two tests differ in that the Rideal-Walker test is conducted under clean conditions and tends to give the higher coefficient, whereas the Chick-Martin test, by including organic matter, gives the lower and more realistic value. The phenol coefficient is simple and historically important, but it is limited: it is determined on a single organism under clean conditions and measures only killing under those conditions, and it does not predict performance on a soiled surface against a mixed population.

6.5.2 Determination of intrinsic activity: MIC and MBC

The phenol coefficient compares one agent with another, but the intrinsic activity of an antimicrobial agent against a given organism is expressed by two further measurements. The **minimum inhibitory concentration (MIC)** is the lowest concentration of the agent that prevents visible growth of the organism after a defined incubation. It is determined by a broth dilution method, in which the agent is prepared in a series of two-fold dilutions, each tube is inoculated with the organism and incubated, and the lowest concentration showing no turbidity is the MIC; it measures inhibition and so expresses static activity. The **minimum bactericidal concentration (MBC)** is the lowest concentration that kills the organism, determined by subculturing the clear tubes of the MIC series onto agar free of the agent and finding the lowest concentration from which no growth is recovered, a reduction of the population by 99.9 per cent being the usual criterion; it measures killing and so expresses cidal activity. When the MBC is close to the MIC the agent is bactericidal at concentrations near those that inhibit; when the MBC greatly exceeds the MIC the agent is essentially bacteriostatic at usable concentrations. These measurements are applied to antiseptics, preservatives and antibiotics alike, and reappear in the microbial assay of Chapter 9.

6.5.3 Selection of a disinfectant

The result of these tests guides the selection of a disinfectant for a purpose, which is a matter of matching the agent to the organisms, the surface, and the conditions. An agent of wide spectrum and sporicidal activity, such as a chlorine compound or a peracetic acid solution, is chosen where spores must be destroyed; a milder agent suffices where only vegetative bacteria are at issue. The agent must be compatible with the surface, since some corrode metals or damage plastics and rubber, and it must retain activity under the conditions of use, which excludes the quaternary ammonium compounds where the water is hard or anionic detergents are present. In practice a sanitisation programme rotates two agents of different class, with periodic use of a sporicide, both to cover the gaps in the spectrum of any one agent and to prevent the selection of resistant survivors, as noted in Chapter 3.

A special case is the disinfection of an enclosed space by a gaseous agent, or **fumigation**, used to decontaminate a clean area, a safety cabinet, or an isolator. Formaldehyde gas, generated by heating paraformaldehyde or by evaporating formalin, was long used for this purpose, but its toxicity and the persistence of its residue have led to its replacement in many settings by **vaporised hydrogen peroxide**, which is sporicidal and decomposes to water and oxygen and leaves no residue. The space is sealed, the agent is introduced and held for a defined period, and the space is then ventilated before re-entry. Fumigation reaches surfaces that cannot be wiped and is therefore the means of achieving a sporicidal treatment of a whole room.

6.5.4 Tests closer to use

Several tests bring the evaluation closer to practice. The **use-dilution test** challenges dried films of standard organisms on small carriers with the dilution of disinfectant recommended for use, and so verifies that the in-use concentration actually kills. The **in-use test** samples disinfectant taken from containers actually in service in the hospital or the plant, to detect a working solution that has been diluted, exhausted, or itself contaminated. The **capacity (Kelsey-Sykes) test** measures the capacity of a disinfectant to retain its activity when repeatedly challenged with successive additions of organisms in the presence of organic matter, simulating the repeated reuse of a disinfectant on a soiled surface. The capacity test in particular reflects the realities of hospital practice, where a single container of disinfectant is used repeatedly to disinfect successive soiled articles until its activity is exhausted; the test reports the dilution at which the disinfectant can still be relied upon to deal with a defined succession of challenges. Each of these tests trades the simplicity of the phenol coefficient for a closer correspondence to the way the disinfectant is actually used, and the choice of test depends on the question to be answered: the phenol coefficient for a comparison of intrinsic activity, the use-dilution and in-use tests for the verification of a working concentration, and the capacity test for the behaviour of a disinfectant in repeated practical use.

6.6 Factors affecting the antimicrobial activity of disinfectants

The activity of a disinfectant in use is not a fixed property of the chemical but depends on the conditions, and a disinfectant that passes a laboratory test may fail in practice if the conditions are unfavourable. The principal factors are the following.

The concentration of the agent is the first determinant. Activity in general increases with concentration, and many agents pass from static to cidal as the concentration rises; for most agents the rate of kill rises steeply with concentration, the relationship being described by the concentration exponent, which measures how sharply the activity falls on dilution. The important exception is the alcohols, which are most active at 60 to 90 per cent and weaker when anhydrous, because some water is needed to denature the cell proteins, so that for them more is not always

better. The practical consequence is that a disinfectant must be made up to the recommended concentration exactly, since a small dilution may cause a large loss of activity.

The **time of contact** is the second: killing is not instantaneous but proceeds with time, so the agent must remain in contact with the surface, wet, for a defined minimum period, and removing it too soon leaves the disinfection incomplete.

The temperature influences activity, which generally increases with temperature because the chemical reactions and the disruption of the cell proceed faster when warm.

The pH affects activity in two ways: it may alter the agent itself, since some agents are active only in the un-ionised form whose proportion depends on pH, and it affects the charge on the microbial surface.

Organic matter is among the most important practical factors. Blood, pus, food and other organic soil may react with and neutralise the agent, or coat the organisms and shield them from it; this is the reason a surface is cleaned before it is disinfected, and the reason the Chick-Martin and capacity tests include an organic load. Agents differ greatly in their susceptibility to organic matter: the chlorine compounds and the quaternary ammonium compounds are much reduced by it, whereas the phenolics and glutaraldehyde retain more of their activity, a difference that influences the choice of agent for a soiled environment.

The number and type of organism both matter. A larger initial population takes longer to kill, because killing is logarithmic, and organisms differ greatly in resistance: bacterial spores are the most resistant, followed by mycobacteria and some Gram-negative bacteria, with ordinary vegetative bacteria the most readily killed. An agent adequate against vegetative cells may have no effect on spores.

Finally, the formulation and the water used modify activity: surfactants and other additives affect the wetting of the surface and the availability of the agent, and hard water inactivates certain agents, notably the quaternary ammonium compounds, by reaction with their active cation.

> **Quick recall.** Disinfectant activity rises with concentration (except alcohols, optimal at 60–90%), contact time, and temperature, and varies with pH. It is reduced by organic matter, by a heavy or resistant microbial load (spores most resistant), by hard water for the QACs, and by poor wetting. The rule in practice is to clean before disinfecting.

6.7 Summary

The chemical control of microorganisms is exercised by disinfectants on inanimate surfaces, antiseptics on living tissue, and preservatives within products, the role of an agent being set by its site of use; agents are further described as cidal, which kill, or static, which only inhibit. The agents are classified by chemical type, the phenolics, alcohols, halogens, quaternary ammonium

compounds, aldehydes, oxidising agents, and biguanides being the principal classes, with modes of action that reduce to damage of the membrane, denaturation of proteins, or reaction with nucleic acids; the aldehydes and the oxidising agents are notable for sporicidal activity. Preservatives, of which the parabens, benzoic acid, benzalkonium chloride, and chlorocresol are common examples, have the most demanding role and their adequacy is demonstrated by the preservative efficacy test.

A disinfectant is evaluated by the phenol coefficient (the Rideal-Walker test, and the Chick-Martin test in the presence of organic matter), which compares its killing dilution with that of phenol, and by tests closer to use, namely the use-dilution, in-use, and capacity (Kelsey-Sykes) tests. Its activity in practice depends on the concentration, the contact time, the temperature, the pH, the presence of organic matter, the number and type of organism, and the formulation and water hardness, the practical consequence being that a surface is cleaned before it is disinfected. The selection of an agent for a given purpose is, in every case, a compromise among spectrum, speed, stability, tolerance, and cost, guided by the organisms to be controlled and the conditions of use. The next unit turns from the inhibition and killing of vegetative organisms by chemical agents to the complete destruction of all organisms, including spores, by sterilization.

6.8 Self-assessment

A. Multiple-choice questions

1. A chemical agent applied to living tissue to reduce microorganisms is a/an:

(a) disinfectant (b) antiseptic (c) preservative (d) sterilant. Answer: (b).

2. An agent that only prevents the multiplication of bacteria without killing them is described as:

(a) bactericidal (b) bacteriostatic (c) sporicidal (d) virucidal. Answer: (b).

3. The alcohols are most active as disinfectants at a concentration of about:

(a) 10–20% (b) 40–50% (c) 60–90% (d) 100% (anhydrous). Answer: (c).

4. Which class of agents is notable for sporicidal activity?

(a) alcohols (b) quaternary ammonium compounds (c) aldehydes (d) acridine dyes. Answer: (c).

5. The phenol coefficient compares a disinfectant with:

(a) ethanol (b) phenol (c) chlorine (d) glutaraldehyde. Answer: (b).

6. The capacity of a disinfectant to remain active on repeated challenge with organisms in the presence of organic matter is measured by the:

(a) Rideal-Walker test (b) use-dilution test (c) Kelsey-Sykes test (d) phenol coefficient alone. Answer: (c).

7. The activity of quaternary ammonium compounds is particularly reduced by:

(a) warm temperature (b) hard water (c) low microbial load (d) long contact time. Answer: (b).

8. The minimum inhibitory concentration (MIC) of an agent is the lowest concentration that:

(a) kills the organism (b) prevents visible growth of the organism (c) dissolves the agent (d) neutralises organic matter. Answer: (b).

9. Benzoic acid is effective as a preservative only in:

(a) alkaline products (b) acidic products (c) anhydrous products (d) oily products. Answer: (b).

10. Chlorhexidine, used for skin antisepsis, belongs to the class of:

(a) aldehydes (b) biguanides (c) halogens (d) alcohols. Answer: (b).

11. The Chick-Martin test differs from the Rideal-Walker test in that it is conducted:

(a) at higher temperature (b) in the presence of an organic load (c) without a test organism (d) with phenol only. Answer: (b).

12. Fumigation of a clean area is now commonly performed with:

(a) ethanol vapour (b) vaporised hydrogen peroxide (c) chlorhexidine (d) a quaternary ammonium compound. Answer: (b).

13. The steep dependence of a disinfectant's activity on its concentration is expressed by its:

(a) phenol coefficient (b) concentration exponent (c) partition coefficient (d) MIC. Answer: (b).

14. The persistent action of chlorhexidine on the skin is due to its:

(a) volatility (b) binding to the skin (c) oxidising power (d) acidity. Answer: (b).

B. Short-answer questions (2 to 5 marks)

Q1. Define disinfectant, antiseptic and preservative, with an example of each.

A disinfectant is applied to inanimate surfaces to destroy or reduce harmful organisms, for example hypochlorite on a floor. An antiseptic is applied to living tissue to reduce organisms without harming it, for example povidone-iodine or alcohol on skin. A preservative is incorporated within a product to prevent microbial growth during its life, for example benzalkonium chloride in eye-drops or a paraben in a syrup. The role is defined by the site of use.

Q2. Distinguish -cidal from -static agents.

A -cidal agent kills microorganisms (bactericidal, fungicidal, sporicidal), whereas a -static agent only prevents their multiplication without killing them (bacteriostatic, fungistatic). Many agents are static at low concentration and cidal at higher concentration. A static agent relies on the host defences or subsequent removal to dispose of the inhibited organisms, while a cidal agent destroys them.

Q3. Describe the phenol coefficient and how it is calculated.

The phenol coefficient compares a disinfectant with phenol under defined conditions (the Rideal-Walker test). Dilutions of the disinfectant and of phenol are each challenged with a standard culture of Salmonella typhi, and the highest dilution of each that kills the organism in the set time is found. The phenol coefficient is the ratio of the killing dilution of the disinfectant to that of phenol; a value above one means the disinfectant is more active than phenol. For example, killing at 1 in 300 against phenol at 1 in 100 gives a coefficient of 3.

Q4. List the qualities of an ideal preservative.

An ideal preservative is active against a wide range of organisms at low concentration; soluble and stable in the product; non-toxic and non-irritant at the concentration used; compatible with the other ingredients and the container; and free of colour, odour and taste. No single agent meets all these requirements, so combinations are often used.

Q4a. Why is the role of an agent defined by its site of use rather than by its chemistry?

The same chemical may serve as a disinfectant on a surface, an antiseptic on the skin, or a preservative within a product, the concentration and formulation being adjusted to the site; for example, a phenolic is a harsh surface disinfectant but a dilute chlorinated phenol is a skin antiseptic. What distinguishes the three is therefore not the chemistry but where the agent is applied and the constraints that the site imposes, principally the requirement of tolerance by living tissue and of compatibility within a product.

Q5. State four factors that affect the antimicrobial activity of a disinfectant.

Any four of: concentration (activity rises with concentration, except alcohols, optimal at 60 to 90%); contact time (killing proceeds with time, so a minimum wet contact is needed); temperature (activity generally rises with temperature); pH (alters the agent and the cell surface); organic matter (neutralises or shields the organisms, so surfaces are cleaned first); the number and type of organism (spores most resistant); and the formulation and water hardness (hard water inactivates QACs).

Q6. Distinguish the minimum inhibitory concentration from the minimum bactericidal concentration, and state how each is determined.

The MIC is the lowest concentration of an agent that prevents visible growth of the organism, determined by a broth dilution series and read as the lowest clear tube; it measures inhibition (static activity). The MBC is the lowest concentration that kills the organism, determined by subculturing the clear tubes onto agar free of the agent and finding the lowest concentration from which no growth is recovered (a 99.9 per cent reduction); it measures killing (cidal activity). When the MBC is close to the MIC the agent is bactericidal; when the MBC greatly exceeds the MIC it is essentially bacteriostatic at usable concentrations.

Q7. What is the preservative efficacy test and why is it required?

The preservative efficacy test demonstrates that the preservative system of a product actually controls microorganisms under conditions resembling use. The product is inoculated with a panel of test organisms (such as S. aureus, P. aeruginosa, E. coli, C. albicans, and A. brasiliensis), stored, and the viable count of each followed over the test period; the system passes if the count falls by the prescribed amount within the prescribed time without subsequent increase. It is required for multi-dose aqueous products and other preparations at risk of microbial growth during use, because the adequacy of a preservative cannot be assumed from its concentration alone.

C. Long-answer questions (10 marks)

Q1. Classify the chemical antimicrobial agents and give the mode of action and an example of each class.

A complete answer treats the phenolics (membrane damage and protein denaturation; phenol, cresol), alcohols (protein denaturation and lipid solvent; ethanol at 60 to 90%), halogens (oxidation; hypochlorite, iodine), quaternary ammonium compounds (membrane disruption; benzalkonium chloride), aldehydes (alkylation and cross-linking, sporicidal; glutaraldehyde), oxidising agents (oxidation; hydrogen peroxide), and biguanides (membrane damage; chlorhexidine), and notes that the modes reduce to membrane damage, protein denaturation, and reaction with nucleic acids. (High-yield: the classification and evaluation of disinfectants are frequently examined.)

Q2. Describe the evaluation of a disinfectant and the factors that affect its activity in use.

A complete answer describes the phenol coefficient (Rideal-Walker, with the ratio of killing dilutions; Chick-Martin in the presence of organic matter) and the use-dilution, in-use, and capacity (Kelsey-Sykes) tests, noting the limitation of the phenol coefficient, and then discusses concentration, contact time, temperature, pH, organic matter, microbial load and type, and formulation and water hardness, concluding with the rule that a surface is cleaned before it is disinfected.

Q3. Define disinfectant, antiseptic and preservative; classify the chemical antimicrobial agents; and outline how a disinfectant is evaluated.

A complete answer defines the three agents by site of use (inanimate surface, living tissue, within a product), classifies the agents (phenolics, alcohols, halogens, quaternary ammonium compounds, aldehydes, oxidising agents, biguanides, with acids and gaseous agents) giving the mode of action and an example of each, and outlines the evaluation by the phenol coefficient (Rideal-Walker and Chick-Martin), the determination of MIC and MBC, and the use-dilution, in-use, and capacity tests and ends with the factors that affect activity in use.

D. Structured note

Write a note on preservatives and the evaluation of a preservative system.

A preservative is incorporated within a product to prevent microbial growth over its life; the common agents are the parabens, benzoic acid (in acidic products), benzalkonium chloride (ophthalmic and nasal), chlorocresol and chlorbutol, and sorbic acid. The choice depends on the product, its pH, its route, and compatibility. The adequacy of a preservative system is demonstrated by the preservative efficacy test, in which the product is inoculated with specified organisms and the decline in their number is followed against the required criteria over a defined period.

Q8. Explain why a disinfectant must be made up exactly to its recommended concentration, and why a surface is cleaned before it is disinfected.

Activity increases with concentration, and for most agents the rate of kill falls steeply on dilution (a high concentration exponent), so a solution weaker than recommended may have greatly reduced activity and fail; an excessive concentration wastes agent and may damage the surface or harm the user. A surface is cleaned before disinfection because organic matter (blood, pus, soil) neutralises many agents and shields the organisms, so that disinfection of a dirty surface is partly wasted on the soil and may leave viable organisms.

Write a note on the antiseptics used on the skin.

Antisepsis of the skin aims to remove the easily removed transient flora and to reduce the resident flora of the deeper layers. The principal agents are the alcohols (ethanol and isopropanol at 60 to 90 per cent), rapid but transient and often combined with a persistent agent; chlorhexidine, a biguanide that binds to the skin and acts persistently, used for hand antisepsis and pre-operative skin preparation; and the iodophores such as povidone-iodine, which release iodine slowly and are well tolerated. The agents must combine adequate activity with tolerance by the tissue, and serve as disinfectants at higher concentration, the difference lying in the site of use.

References

1. Hugo WB, Russell AD. Pharmaceutical Microbiology. Oxford: Blackwell Science.
2. Denyer SP, Hodges NA, Gorman SP, editors. Hugo and Russell's Pharmaceutical Microbiology. 8th ed. Oxford: Wiley-Blackwell; 2011.
3. Sandle T. Pharmaceutical Microbiology: Essentials for Quality Assurance and Quality Control. Cambridge: Woodhead Publishing/Elsevier; 2016.
4. Indian Pharmacopoeia. Ghaziabad: Indian Pharmacopoeia Commission. [latest edition]

Chapter 7: Methods of Sterilization and Advances

Learning outcomes

After studying this chapter the student will be able to:

- Define sterilization and classify its methods.
- Describe the physical methods of sterilization by moist heat and dry heat, with their conditions and uses.
- Describe sterilization by radiation, by gaseous agents, by liquid chemical agents, and by filtration.
- State the advances in sterilization technology.
- Select an appropriate method of sterilization for a given product.

These outcomes serve Course Outcome CO5, the comparison of sterilization methods and the assessment of their suitability.

7.1 Introduction

Sterilization is the process by which all viable microorganisms, including bacterial spores, are destroyed or removed from an article. It is to be distinguished from disinfection, which destroys vegetative pathogens but not necessarily spores; sterilization is the absolute process, and an article is either sterile or it is not. Because the most resistant organism is the bacterial spore, every sterilization process is designed to destroy the spore, and an agent or condition that kills vegetative cells but spares spores does not sterilize. The requirement for sterility arises for products administered by routes that bypass the body's defences, the parenteral and ophthalmic products above all, and for the instruments and materials used in their manufacture and administration.

A second principle, developed fully in Chapter 8, governs the whole subject and should be held in mind throughout this chapter: because microbial death is exponential, the destruction of microorganisms can never be shown to be absolute, only to reduce the probability of a survivor to an acceptably low level. Sterilization is therefore defined in practice by the conditions known to achieve that probability, the conditions described in this chapter, and the assurance of sterility is treated quantitatively in the next. The methods of sterilization are classified as physical, radiation, gaseous, chemical and mechanical, and are shown together in Figure 7.1.

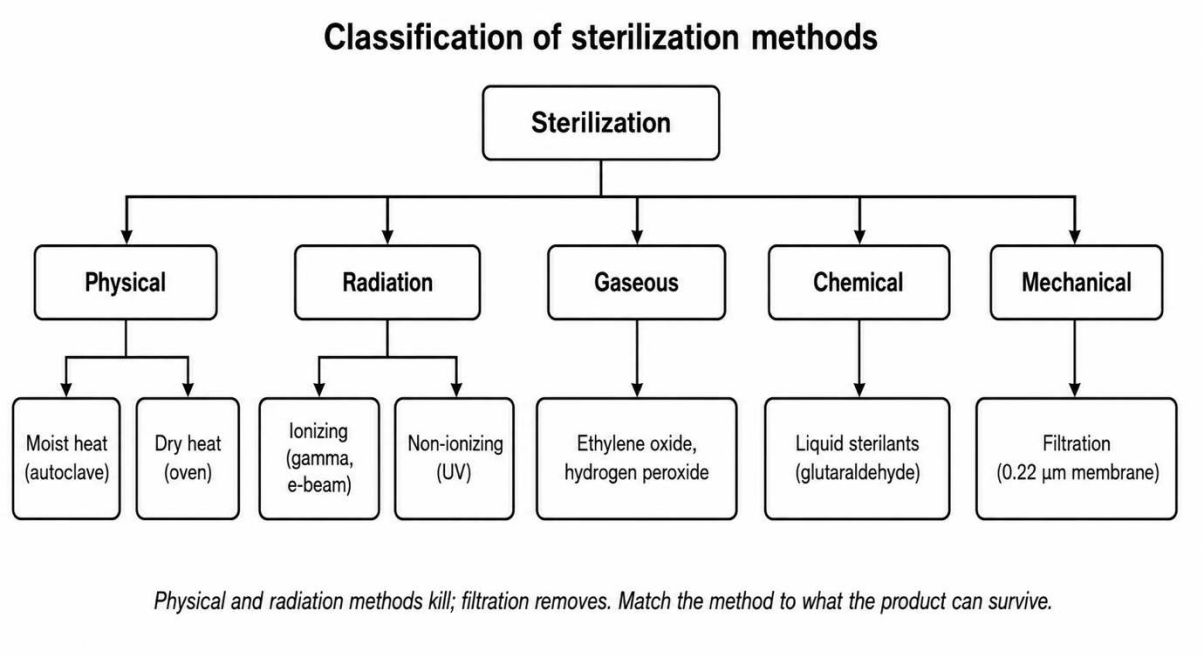


Figure 7.1 *Classification of the methods of sterilization.*

7.2 Physical methods: sterilization by heat

Heat is the oldest, the most reliable, and the most widely used sterilizing agent, and it is the method of choice wherever the product can withstand it. It acts in two distinct forms, moist heat and dry heat, which differ in mechanism, in the conditions required, and in their applications.

7.2.1 Moist-heat sterilization

Moist-heat sterilization is conducted with saturated steam under pressure in an **autoclave**. It destroys microorganisms by the coagulation and denaturation of their proteins, an action in which water participates directly, so that moist heat kills at a far lower temperature and in a far shorter time than dry heat. The essential requirement is contact between saturated steam and every surface of the load: the steam must be saturated (in equilibrium with water) and the air must be removed from the chamber and the load, because trapped air both lowers the temperature attained and prevents the steam from reaching the surfaces.

The principle of the autoclave is that water boils at a temperature determined by the pressure, so that raising the pressure raises the temperature of the steam. At a gauge pressure of about 15 pounds per square inch (approximately 1 bar) above atmospheric, the steam reaches 121 degrees Celsius, the standard sterilizing temperature. The reference cycle is 121 degrees Celsius for 15 minutes, the holding time being measured after the whole load has reached the temperature; a higher-temperature, shorter cycle of 134 degrees Celsius for 3 minutes is used for instruments and porous loads. The temperature and pressure of a typical cycle are shown against time in Figure 7.2, the cycle comprising the removal of air, the rise to temperature, the holding period, and the cooling.

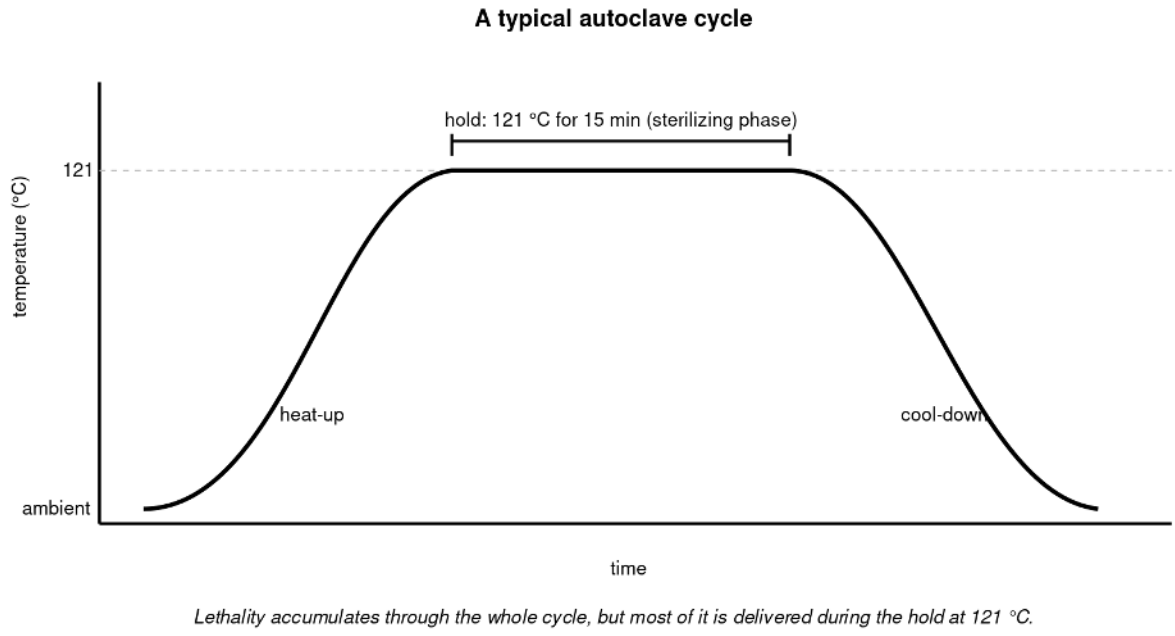


Figure 7.2 *A typical autoclave cycle of temperature against time.*

The removal of air defines the two principal designs of autoclave. In the **downward-displacement (gravity) autoclave**, steam admitted at the top displaces the denser air downward and out through a drain at the base; it is simple but may leave air trapped in a wrapped or porous load. In the **pre-vacuum (high-vacuum) autoclave**, the air is removed by a pump before steam is admitted, so that steam penetrates even the centre of a porous load immediately; it is preferred for dressings and wrapped instruments. The conduct of the cycle differs between a load of sealed fluids and a porous load. A load of bottled or ampouled fluids is heated through its own mass and is cooled slowly and under controlled pressure at the end, to prevent the containers from bursting as the internal pressure falls; the lethality delivered to such a load is conveniently expressed as its F_0, the equivalent time at 121 degrees Celsius, treated in Chapter 8. A porous load, such as dressings or wrapped instruments, requires the active removal of air so that steam can penetrate, and is dried under vacuum at the end. The performance of the autoclave is confirmed for each cycle by recording the temperature, pressure and time, and is validated by mapping the temperature throughout the load with thermocouples and by placing **biological indicators** at the points expected to be coolest and least accessible, the centre of a large fluid container or the interior of a porous pack. A leak test of the chamber and a steam-penetration test complete the routine checks. The detail of this validation belongs to Chapter 8; the point here is that the autoclave, for all its reliability, is not trusted blindly but is shown by measurement to deliver the intended conditions to every part of the load. Two related but lesser heat processes should be distinguished from true sterilization: sterilization by **boiling** at 100 degrees Celsius, which kills vegetative organisms but not spores and so is a disinfection rather than a sterilization; and **tyndallization** (intermittent sterilization), in which a material is heated to 100 degrees Celsius on each of three successive days, the intervening

incubation allowing surviving spores to germinate into heat-sensitive vegetative cells that the next heating destroys, a method of historical interest for heat-sensitive media. Moist-heat sterilization proper is used for aqueous solutions, dressings, surgical instruments, culture media, and any heat- and moisture-stable article, and is the first method to be considered for any product. The reference conditions are collected in Table 7.1.

Table 7.1: Reference conditions for heat sterilization

Method	Condition	Acts by
Moist heat (autoclave)	121 °C for 15 min (about 15 psi above atmospheric)	Coagulation and denaturation of protein
Moist heat (porous loads, instruments)	134 °C for 3 min	Coagulation and denaturation of protein
Dry heat (**hot-air oven**)	160 °C for 2 h, or 170 °C for 1 h, or 180 °C for 30 min	Oxidation
Dry heat (**depyrogenation**)	about 250 °C	Destroys endotoxin as well as organisms

7.2.2 Dry-heat sterilization

Dry-heat sterilization is conducted with hot air in a hot-air oven. It destroys microorganisms by oxidation rather than by the coagulation of protein, and because dry air transfers heat far less efficiently than steam, it requires higher temperatures and longer times. The standard conditions are 160 degrees Celsius for 2 hours, 170 degrees Celsius for 1 hour, or 180 degrees Celsius for 30 minutes, the choice being a trade between temperature and time. Dry heat is used for articles that steam would damage or fail to penetrate: glassware, metal instruments, fixed oils, fats and dry powders. It has the further important property that, at higher temperatures of the order of 250 degrees Celsius, it destroys bacterial endotoxin as well as organisms, a process called depyrogenation that is applied to the glass containers of parenteral products. The hot-air oven is constructed with a means of circulating the hot air, by a fan, so that the temperature is uniform throughout the chamber and the load, since dry air, a poor conductor, would otherwise leave cold regions. Related applications of dry heat at higher intensity include **flaming**, the brief passage of an article such as a scalpel or the neck of a culture bottle through a flame; **red heat**, the heating of an inoculating loop to redness in a flame, which sterilizes it immediately; and **incineration**, the complete burning of contaminated waste and disposable articles. These are methods of the laboratory and of waste disposal rather than of pharmaceutical manufacture, but they share the principle of destruction by dry heat.

> **Quick recall.** Sterilization destroys all organisms including spores; disinfection does not. Moist heat (autoclave, 121 °C/15 min, saturated steam, air removed) coagulates protein and is preferred wherever the product allows. Dry heat (oven, 160–180 °C) acts by oxidation, suits glass, metal, oils and powders, and at about 250 °C depyrogenates. Air removal and saturated steam are the keys to the autoclave.

7.3 Sterilization by radiation

Radiation sterilizes without heat and is therefore valuable for materials that heat would damage. It is of two kinds, ionizing and non-ionizing, which differ greatly in penetrating power and in use.

Ionizing radiation carries enough energy to eject electrons and break chemical bonds, and it destroys microorganisms chiefly by damaging their nucleic acid, directly and through the reactive species it generates in water. The forms used are gamma rays, emitted by a radioactive source of cobalt-60; accelerated electrons (an electron beam) from a machine; and X-rays. Ionizing radiation penetrates deeply and acts at ordinary temperature, so it sterilizes articles in their final sealed packaging, and it is the standard method for single-use plastic medical devices such as syringes, catheters, surgical gloves, and sutures, and for some heat-sensitive materials. The sterilizing dose is commonly of the order of 25 kilogray, the exact dose being set by the bioburden and its resistance. Its limitations are the cost of the facility and the radioactive source, and the degradation of some plastics and some drugs by the radiation. The sterilizing dose is established for a given product by relating the dose to the bioburden and its radiation resistance, and the process is validated and routinely controlled by dosimetry, the measurement of the absorbed dose, supported where appropriate by biological indicators. Because the dose can be measured directly and precisely, a radiation process lends itself to control by the dose delivered, in a manner analogous to the parametric release of a heat process described in Chapter 8.

Non-ionizing radiation, principally ultraviolet light of about 254 nanometres, has too little energy to ionize but enough to damage nucleic acid by causing adjacent bases to bond together. Its great limitation is its very poor penetrating power: it is absorbed at surfaces and does not pass through most solids or through turbid liquids. Ultraviolet radiation is therefore used not to sterilize sealed products but to reduce the microbial population of air, of exposed surfaces, and of water, for example in the air of an aseptic area or a safety cabinet and in water-treatment systems. It is properly regarded as a means of disinfection of surfaces and air rather than of sterilization, because its action is confined to what the radiation directly reaches; an organism shadowed by a particle or lying beneath the surface is not affected. Its advantages are that it adds nothing to the material treated and leaves no residue.

> **Quick recall.** Ionizing radiation (gamma from cobalt-60, electron beam, X-rays) damages nucleic acid, penetrates deeply, acts cold, and sterilizes pre-packed heat-sensitive devices at

> about 25 kGy. Non-ionizing radiation (UV at 254 nm) damages nucleic acid but barely penetrates, so it disinfects air, surfaces and water rather than sealed products.

7.4 Sterilization by gaseous agents

Some articles can withstand neither heat nor radiation, and for these a sterilizing gas, which acts at low temperature, is used. **Ethylene oxide** is the principal gaseous sterilant. It is a small, highly reactive gas that destroys microorganisms by alkylating the proteins and nucleic acids of the cell, and it penetrates packaging and the complex interior of devices well. It sterilizes heat-sensitive medical devices, plastics and electronic components that no other method can treat, and it is widely used by manufacturers of single-use devices and by hospital sterile-services departments for instruments that cannot be autoclaved. Its penetration of packaging means that the device is sterilized in the pack in which it will be stored and used, so that it remains sterile until the pack is opened. Its disadvantages are serious and define its use: ethylene oxide is toxic, flammable and carcinogenic, the cycle is long and must be conducted under controlled temperature and humidity, and the sterilized article must be aerated afterward, for an extended period, to remove absorbed gas before it is safe to use. The ethylene oxide cycle is conducted under controlled conditions of gas concentration, temperature (commonly 30 to 60 degrees Celsius), and relative humidity, the humidity being essential because the gas acts only on hydrated organisms; the cycle comprises conditioning, gas exposure, and a prolonged aeration to remove residual gas, and is validated with the biological indicator *Bacillus atrophaeus*. Other gaseous agents include **low-temperature steam with formaldehyde**, in which steam at about 73 degrees Celsius carries formaldehyde to sterilize heat-sensitive instruments, and **vaporised hydrogen peroxide**, the last increasingly preferred because it acts at low temperature and leaves only water and oxygen behind.

7.5 Sterilization by liquid chemical agents

Where a heat-sensitive instrument or surface must be sterilized by a liquid, the strong chemical agents described in Chapter 6 are used, applied for a contact time long enough to destroy spores. **Glutaraldehyde** and **peracetic acid** are the common agents, used to process delicate instruments such as endoscopes that cannot be autoclaved. Sterilization by a liquid chemical agent is distinguished from high-level disinfection only by the contact time and conditions, since, given long enough, these agents achieve sterility; it is, however, harder to validate than heat and leaves a residue that must be rinsed away with sterile water. For this reason liquid chemical sterilization is reserved for articles that cannot be treated by a more reliable method. The article must first be cleaned of organic matter, which would otherwise neutralise the agent and shield the organisms, then fully immersed for the validated contact time, then rinsed with sterile water and handled aseptically thereafter, since any subsequent contact with a non-sterile surface or rinse would

recontaminate it. The difficulty of validating and controlling these manual steps is the chief reason the method is regarded as a last resort.

7.6 Mechanical method: sterilization by filtration

Filtration sterilizes not by killing the organisms but by removing them. A heat-sensitive liquid or a gas is passed through a **membrane filter** whose pores are too small to allow the passage of microorganisms, which are retained on the filter while the fluid passes through. The **sterilizing-grade membrane** has a pore size of 0.22 micrometre (commonly written 0.2 micrometre), small enough to retain bacteria. Filtration is the method of choice for heat-sensitive solutions, such as many injectable drug solutions, serum and culture media, and, with a **hydrophobic membrane**, for sterilizing the air supplied to a fermenter or an autoclave, and for venting sterile containers.

Two limitations must be understood. First, an ordinary sterilizing-grade filter retains bacteria but does not reliably remove viruses or mycoplasma, which may be smaller than its pores; a separate, finer process is required where these must be excluded. Second, the sterility of the product depends entirely on the integrity of the filter, so the filter must be tested for integrity, by a method such as the bubble-point test, after use, and the filtration must be validated by challenging the filter with a high concentration of a small test organism, *Brevundimonas diminuta*, to demonstrate retention. Filters are of several constructions. The membrane filter, a thin polymer film with pores of closely controlled size, is the standard sterilizing filter and retains organisms chiefly at its surface. **Depth filters**, such as sintered glass and the older asbestos-free fibrous pads, retain organisms throughout a thick matrix by adsorption and entrapment and are used to clarify before a final membrane stage. A hydrophobic membrane is used to sterilize gases and to vent containers, since it allows gas but not water or organisms to pass, and the **high-efficiency particulate air (HEPA) filter** of Chapter 5 is the air-filtration counterpart of the liquid membrane. Filtration delivers a sterile filtrate that must then be filled aseptically into sterile containers, since the process does not sterilize the container.

> **Quick recall.** Gaseous sterilization (ethylene oxide; alkylation) treats heat-sensitive devices in their packaging but is toxic and needs long aeration. Liquid chemical sterilants (glutaraldehyde, peracetic acid) treat delicate instruments but are hard to validate. Filtration through a 0.22 µm membrane removes bacteria from heat-sensitive fluids and air, but does not retain viruses, requires an integrity test, and must be followed by aseptic filling.

7.6a Evaluation and indicators of sterilization

The efficiency of a sterilization process is not assumed but demonstrated, and the means of doing so are introduced here and treated quantitatively in Chapter 8. A process is monitored by physical means (the recording of temperature, pressure, time, or absorbed dose), by **chemical indicators** that change colour on exposure to the sterilizing condition (such as autoclave tape and, for steam

penetration, the Bowie-Dick test), and by biological indicators, standardised preparations of resistant spores placed in the load and incubated after the cycle, no growth confirming that the process destroyed even this resistant challenge. The biological indicator is the most direct evidence, because it tests the ability of the process to kill rather than merely the attainment of a condition; the indicator organism is chosen to be more resistant than any expected contaminant and is matched to the method. The validation of the process by these means, and the sequence of installation, operational and performance qualification, are described in Chapter 8.

7.7 Advances in sterilization technology

The classical methods have been supplemented by newer technologies that aim to sterilize heat-sensitive materials more quickly, more safely, or with less toxic residue than ethylene oxide. Vaporised hydrogen peroxide and **gas (low-temperature) plasma**, in which hydrogen peroxide vapour is energised into a reactive plasma, sterilize heat-sensitive instruments at low temperature and leave only water and oxygen, and so avoid the toxic residue and the long aeration of ethylene oxide. **Electron-beam and X-ray** systems provide the benefits of ionizing radiation without a radioactive source; the electron beam is very rapid though less penetrating than gamma radiation. Pulsed light and other surface technologies are used in specialised settings. Each advance addresses a limitation of the classical methods: gas plasma and vaporised hydrogen peroxide replace the toxic residue and long aeration of ethylene oxide; the electron beam and the X-ray remove the need for a stored radioactive source; and the newer methods generally act faster or at lower temperature. The common purpose of these advances is to reach the same assurance of sterility with less damage to the product and less hazard to the operator and the environment, and their adoption is governed, like that of any sterilization process, by validation against the same standards of assurance described in Chapter 8.

7.7a Sterilization of pharmaceutical products and articles

The application of these methods to the principal pharmaceutical articles may be summarised, since the syllabus expects the student to match a product to its method. Aqueous injections and infusions that are heat-stable are sterilized by moist heat in the final container (terminal sterilization at 121 degrees Celsius for 15 minutes); a heat-labile injection is sterilized by filtration through a 0.22 micrometre membrane and filled aseptically. The glass containers and metal components of parenterals are sterilized, and the glass depyrogenated, by dry heat. Ophthalmic solutions are treated like small-volume parenterals, by autoclaving or by filtration with aseptic filling. Surgical dressings and gowns, which are porous, are sterilized by moist heat in a pre-vacuum autoclave, or, if pre-packed and single-use, by ionizing radiation. Oils, fats and dry powders, which steam cannot penetrate and would not tolerate, are sterilized by dry heat. Single-use plastic devices, such as syringes and catheters, are sterilized in their packaging by ionizing radiation or, where radiation

would damage them, by ethylene oxide. Delicate heat-sensitive instruments such as endoscopes are processed by a liquid chemical agent or by a low-temperature method such as gas plasma. The match of product to method follows in every case from what the product can withstand, the principle formalised in the selection ladder below.

7.8 Selection of a method of sterilization

The choice of method follows from a single question: what can the product withstand? The selection proceeds down a ladder from the most reliable method to the least. If the product can withstand moist heat, it is sterilized in the autoclave, the cheapest, fastest and most easily validated method. If it can withstand heat but not moisture, dry heat is used. If it cannot withstand heat but can be pre-packed and irradiated, radiation is used. If it is a heat-sensitive solution, it is sterilized by filtration. Only when none of these is possible, as for a complex heat-sensitive device, is a gaseous or a liquid chemical agent used, these being slower and harder to validate. The order is not arbitrary but runs from the most certain method to the least, so that a product is given the most robust process it can bear. The methods are compared in Table 7.2.

Table 7.2: Comparison of the methods of sterilization

Method	Agent and mechanism	Typical conditions	Suited to	Principal limitation
Moist heat	Saturated steam; protein coagulation	121 °C, 15 min	Aqueous products, dressings, instruments	Product must tolerate heat and moisture
Dry heat	Hot air; oxidation	160–180 °C, 0.5–2 h	Glass, metal, oils, powders	High temperature, long time
Ionizing radiation	Gamma, e-beam, X-ray; nucleic-acid damage	about 25 kGy	Pre-packed heat-sensitive devices	Cost; degrades some plastics and drugs
Gaseous	Ethylene oxide; alkylation	Low temperature, long cycle	Heat-sensitive devices and plastics	Toxic; long aeration required
Liquid chemical	Glutaraldehyde, peracetic acid	Long contact time	Delicate instruments (endoscopes)	Hard to validate; residue

Method	Agent and mechanism	Typical conditions	Suited to	Principal limitation
Filtration	0.22 µm membrane; removal	Single pass	Heat-sensitive solutions and air	Does not retain viruses; needs integrity test

7.8a Sterilization in the pharmacopoeia

The Indian Pharmacopoeia, in common with the British and United States Pharmacopoeias, prescribes the recognised methods of sterilization and the conditions for each, and requires that the method chosen be validated and that its effectiveness be demonstrated. The compendia recognise sterilization by saturated steam, by dry heat, by ionizing radiation, by gas, and by filtration, and set out reference conditions for each, such as 121 degrees Celsius for 15 minutes for steam and the dry-heat conditions tabulated above. They require, further, that a product be sterilized by the most reliable method it can withstand, that terminal sterilization be preferred to aseptic processing wherever possible, and that the sterility of the product be confirmed by the sterility test of Chapter 10. The pharmacopoeia thus codifies both the methods described in this chapter and the assurance and testing described in the chapters that follow.

7.9 Summary

Sterilization is the destruction or removal of all viable microorganisms, including spores, and is the absolute process that disinfection is not; because the spore is the most resistant organism, every method is designed to destroy it. The methods are physical, radiation, gaseous, chemical and mechanical. Moist heat, saturated steam under pressure in an autoclave at 121 degrees Celsius for 15 minutes, coagulates protein and is the preferred method wherever the product allows, requiring the removal of air and saturated steam in contact with every surface; dry heat in a hot-air oven at 160 to 180 degrees Celsius acts by oxidation, suits glass, metal, oils and powders, and at about 250 degrees Celsius depyrogenates. Ionizing radiation (gamma, electron beam, X-ray) damages nucleic acid, penetrates deeply, and sterilizes pre-packed heat-sensitive devices at about 25 kilogray, whereas non-ionizing ultraviolet radiation, of poor penetration, disinfects air, surfaces and water.

For materials that withstand neither heat nor radiation, ethylene oxide gas sterilizes by alkylation but is toxic and requires long aeration; liquid chemical agents such as glutaraldehyde and peracetic acid sterilize delicate instruments but are hard to validate; and filtration through a 0.22 micrometre membrane removes bacteria from heat-sensitive fluids and air, though it does not retain viruses, requires an integrity test, and must be followed by aseptic filling. Newer technologies, vaporised hydrogen peroxide, gas plasma, and electron-beam and X-ray systems, sterilize heat-sensitive

materials with less hazard and residue. In their application to pharmaceutical products, heat-stable injections are autoclaved in the final container, heat-labile solutions are filtered and filled aseptically, glass and metal are sterilized and depyrogenated by dry heat, porous dressings are autoclaved or irradiated, oils and powders are treated by dry heat, and plastic devices by radiation or ethylene oxide. The method is selected by descending a ladder from the most reliable, moist heat, to the least, according to what the product can withstand, and the chosen method is in every case validated and its effectiveness demonstrated. The next chapter treats the quantitative basis of these processes, the kinetics of microbial death, the sterility assurance level, and the validation by which a sterilization process is shown to be effective.

7.10 Self-assessment

A. Multiple-choice questions

1. Sterilization differs from disinfection in that sterilization destroys:

(a) only vegetative bacteria (b) all microorganisms including spores (c) only fungi (d) only viruses. Answer: (b).

2. The standard reference cycle for moist-heat sterilization is:

(a) 100 °C for 30 min (b) 121 °C for 15 min (c) 160 °C for 2 h (d) 134 °C for 30 min. Answer: (b).

3. Moist heat sterilizes by:

(a) oxidation (b) coagulation and denaturation of protein (c) alkylation (d) radiation damage. Answer: (b).

4. Dry-heat sterilization is the method of choice for:

(a) aqueous injections (b) glassware, oils and powders (c) rubber gloves (d) culture media. Answer: (b).

5. The pore size of a sterilizing-grade membrane filter is:

(a) 0.45 µm (b) 0.22 µm (c) 1.0 µm (d) 5.0 µm. Answer: (b).

6. Ethylene oxide sterilizes by:

(a) coagulating protein (b) alkylating proteins and nucleic acids (c) oxidation by free radicals (d) filtration. Answer: (b).

7. Ultraviolet radiation is used to disinfect air and surfaces rather than to sterilize sealed products because it:

(a) is too expensive (b) has very poor penetrating power (c) generates heat (d) leaves a residue. Answer: (b).

8. Tyndallization (intermittent sterilization) destroys spores by:

(a) a single prolonged heating (b) repeated heating on successive days, with germination between heatings (c) dry heat (d) radiation. Answer: (b).

9. Sterilization by boiling at 100 °C is properly regarded as:

(a) sterilization, since all organisms die (b) a disinfection, since spores survive (c) depyrogenation (d) filtration. Answer: (b).

10. The humidity of an ethylene oxide cycle is important because the gas acts only on:

(a) dry organisms (b) hydrated organisms (c) spores alone (d) viruses alone. Answer: (b).

11. A hydrophobic membrane filter is used to:

(a) sterilize oils (b) sterilize gases and vent containers (c) depyrogenate glass (d) clarify syrups. Answer: (b).

B. Short-answer questions (2 to 5 marks)

Q1. State the principle of the autoclave and the standard sterilizing conditions.

The autoclave sterilizes with saturated steam under pressure. Because the temperature of steam rises with pressure, raising the gauge pressure to about 15 psi above atmospheric raises the steam temperature to 121 degrees Celsius. The standard cycle is 121 degrees Celsius for 15 minutes (with 134 degrees Celsius for 3 minutes for porous loads), measured after the load reaches temperature, with the air removed and saturated steam in contact with every surface.

Q2. Distinguish moist-heat from dry-heat sterilization.

Moist heat, saturated steam under pressure, kills by coagulating and denaturing protein, with water taking part, and so acts at a lower temperature and shorter time (121 degrees Celsius, 15 minutes); it suits aqueous products, dressings and instruments. Dry heat, hot air, kills by oxidation and, transferring heat poorly, needs higher temperatures and longer times (160 to 180 degrees Celsius); it suits glass, metal, oils and powders, and at about 250 degrees Celsius depyrogenates.

Q3. Describe sterilization by ionizing radiation and state its uses.

Ionizing radiation, chiefly gamma rays from cobalt-60, and also electron beams and X-rays, destroys microorganisms by damaging their nucleic acid. It penetrates deeply and acts at ordinary temperature, so it sterilizes articles in their final packaging; the usual dose is about 25 kilogray. It is the standard method for single-use plastic medical devices such as syringes, gloves and sutures. Its limitations are the cost of the facility and the degradation of some plastics and drugs.

Q4. Why is filtration followed by aseptic filling, and what are its limitations?

Filtration removes organisms from a fluid by retaining them on a 0.22 micrometre membrane, but it does not sterilize the container, so the sterile filtrate must be filled aseptically into a sterile container. Its limitations are that it does not reliably retain viruses or mycoplasma, which may be

smaller than the pores, and that the sterility depends on the integrity of the filter, which must therefore be integrity-tested after use and validated by a bacterial challenge.

Q5. State the advantages and disadvantages of ethylene oxide sterilization.

Ethylene oxide sterilizes heat-sensitive devices, plastics and electronics at low temperature, penetrating packaging and complex shapes by alkylating proteins and nucleic acids. Its disadvantages are that it is toxic, flammable and carcinogenic, the cycle is long and requires controlled temperature and humidity, and the article must be aerated for an extended period afterward to remove absorbed gas.

Q6. By what method would you sterilize each of the following, and why: a heat-stable aqueous injection; a heat-labile injection; a glass container for a parenteral; a single-use plastic syringe?

A heat-stable aqueous injection is sterilized by moist heat in its final container (terminal sterilization), the most reliable method, since it tolerates heat and moisture. A heat-labile injection cannot be heated and is sterilized by filtration through a 0.22 micrometre membrane, then filled aseptically. A glass container is sterilized and depyrogenated by dry heat, which suits glass and destroys endotoxin. A single-use plastic syringe, heat-sensitive and pre-packed, is sterilized by ionizing radiation, which penetrates the packaging and acts at ordinary temperature, or by ethylene oxide where radiation would damage it.

Q7. Why must air be removed from the chamber and load of an autoclave?

Trapped air lowers the temperature attained at a given pressure, because a mixture of air and steam is cooler than saturated steam at the same pressure, and it physically prevents steam from reaching the surfaces it occupies, leaving cold, non-sterilized regions. Air is removed by downward displacement in a gravity autoclave or, more effectively for porous loads, by a pump in a pre-vacuum autoclave; the adequacy of air removal in a porous-load autoclave is checked by the Bowie-Dick test.

Q8. What is depyrogenation, and how is it achieved?

Depyrogenation is the destruction or removal of pyrogens, chiefly bacterial endotoxin, which causes fever when injected and is not destroyed by ordinary sterilization because it is heat-stable. It is achieved for glass and metal by dry heat at a high temperature, of the order of 250 degrees Celsius, which destroys the endotoxin as well as any organisms; it is applied to the glass containers of parenteral products.

C. Long-answer questions (10 marks)

Q1. Classify the methods of sterilization and describe sterilization by moist heat and dry heat in detail.

A complete answer classifies the methods as physical (moist and dry heat), radiation, gaseous, chemical and mechanical; describes the autoclave (principle of pressure raising the steam temperature, the 121 degrees Celsius/15 minutes cycle, the need for air removal and saturated steam, the gravity and pre-vacuum designs, and the uses); and describes the hot-air oven (oxidation, the 160 to 180 degrees Celsius conditions, the uses, and depyrogenation at about 250 degrees Celsius). (High-yield: heat sterilization is among the most frequently examined topics.)

Q2. Compare the methods of sterilization and explain how a method is selected for a product.

A complete answer compares moist heat, dry heat, radiation, gaseous, liquid chemical, and filtration by mechanism, conditions, suitable products, and limitations (as in Table 7.2), and explains the selection ladder: moist heat first where the product allows, then dry heat, then radiation for pre-packed heat-sensitive devices, then filtration for heat-sensitive solutions, and a gaseous or liquid chemical agent only when no more reliable method is possible.

Q3. Describe sterilization by filtration, including the types of filter and the limitations of the method.

A complete answer states that filtration sterilizes by removing organisms on a sterilising-grade membrane of 0.22 micrometre pore size, describes the membrane filter (surface retention), depth filters such as sintered glass (retention through a matrix), the hydrophobic membrane for gases and venting, and the HEPA filter for air, and gives the limitations: viruses and mycoplasma are not reliably retained, the filter must be integrity-tested after use and validated by a bacterial challenge with Brevundimonas diminuta, and the sterile filtrate must be filled aseptically because the container is not sterilized.

Q4. Describe sterilization by gaseous agents and by liquid chemical agents, and state when each is used.

A complete answer describes ethylene oxide (alkylation; controlled concentration, temperature and humidity; conditioning, exposure and aeration; uses for heat-sensitive packaged devices; toxicity and the need for aeration), mentions low-temperature steam-formaldehyde and vaporised hydrogen peroxide, and describes liquid chemical sterilization with glutaraldehyde or peracetic acid (full immersion for a long contact time, after cleaning, with rinsing in sterile water and aseptic handling). It states that these are used only for articles that cannot withstand heat, radiation, or filtration, being slower and harder to validate.

D. Structured note

Write a note on the advances in sterilization technology.

Newer technologies sterilize heat-sensitive materials with less hazard than ethylene oxide. Vaporised hydrogen peroxide and gas (low-temperature) plasma sterilize at low temperature and leave only water and oxygen; electron-beam and X-ray systems give the benefits of ionizing

radiation without a radioactive source, the electron beam being rapid but less penetrating; and pulsed light and related methods serve specialised surface applications. The aim is to achieve the required assurance of sterility with less damage to the product and less risk to the operator and the environment.

Write a note on ethylene oxide sterilization.

Ethylene oxide is a reactive gas that sterilizes by alkylating the proteins and nucleic acids of microorganisms. It acts at low temperature and penetrates packaging and complex devices, so it sterilizes heat-sensitive medical devices, plastics and electronics. The cycle is conducted under controlled gas concentration, temperature and humidity (the humidity being essential, since the gas acts only on hydrated organisms) and comprises conditioning, exposure and a prolonged aeration to remove residual gas. Its disadvantages are that the gas is toxic, flammable and carcinogenic, the cycle is long, and the aeration is essential before the article is safe to use; the process is validated with Bacillus atrophaeus spores.

References

1. Denyer SP, Hodges NA, Gorman SP, editors. Hugo and Russell's Pharmaceutical Microbiology. 8th ed. Oxford: Wiley-Blackwell; 2011.
2. Hugo WB, Russell AD. Pharmaceutical Microbiology. Oxford: Blackwell Science.
3. British Pharmacopoeia. Appendix on methods of sterilisation. London: The Stationery Office. [latest edition]
4. Indian Pharmacopoeia. Ghaziabad: Indian Pharmacopoeia Commission. [latest edition]

Chapter 8: Sterility Assurance, Death Kinetics, Validation and Activity Testing

Learning outcomes

After studying this chapter the student will be able to:

- Explain the kinetics of microbial death and define and calculate the **D-value**, the **z-value**, and the **F-value**.
- Explain the sterility assurance level, the **overkill approach**, and the role of **bioburden**.
- Describe the modelling of microbial death and the deviations from log-linear kinetics.
- Describe the validation of a sterilization process and the use of sterility indicators.
- Describe the determination of bacteriostatic and bactericidal activity.

These outcomes serve Course Outcome CO5, the assessment of the effectiveness and validation of sterilization.

8.1 Introduction

The previous chapter described how sterilization is carried out; the present chapter explains the quantitative basis on which a process is shown to be effective. The basis is a single fact about the way microorganisms die: their death is exponential. A sterilizing process destroys not a fixed number of organisms in each interval but a fixed proportion of those remaining, so that the survivor count falls by a constant factor in each equal period and approaches zero without ever reaching it. From this fact two consequences follow that organise the whole of sterility assurance. First, sterility cannot be expressed as the certain absence of organisms, but only as a probability that an organism survives, which is made acceptably small. Second, that probability can be calculated, from the resistance of the organism and the conditions of the process, which is the purpose of the parameters defined in this chapter. The chapter develops the kinetics of microbial death, the sterility assurance level that follows from it, the validation by which a process is shown to deliver that level, and, finally, the laboratory tests by which the bacteriostatic and bactericidal activity of an agent is measured. The treatment is quantitative throughout, because the assurance of sterility is, uniquely among the topics of this book, a matter of calculation: the resistance of the organism and the lethality of the process together fix the probability of a survivor, and the validation confirms that the calculated lethality is in fact delivered to every part of the load. The student should therefore master both the arithmetic of the D-, z-, and F-values and the reasoning that connects them to the sterility assurance level and to the design and proof of a real process.

8.2 The kinetics of microbial death

When a population of organisms is exposed to a lethal agent at constant intensity and the number of survivors is counted with time, the number falls exponentially. A plot of the logarithm of the number of survivors against time is therefore a straight line descending with a constant slope, the **survivor curve** (Figure 8.1). The exponential character means that each equal interval of treatment destroys the same fraction of the survivors, so the same time is required to reduce the population from a million to a hundred thousand as from a hundred to ten. Three linked quantities describe this behaviour and constitute the quantitative language of sterilization.

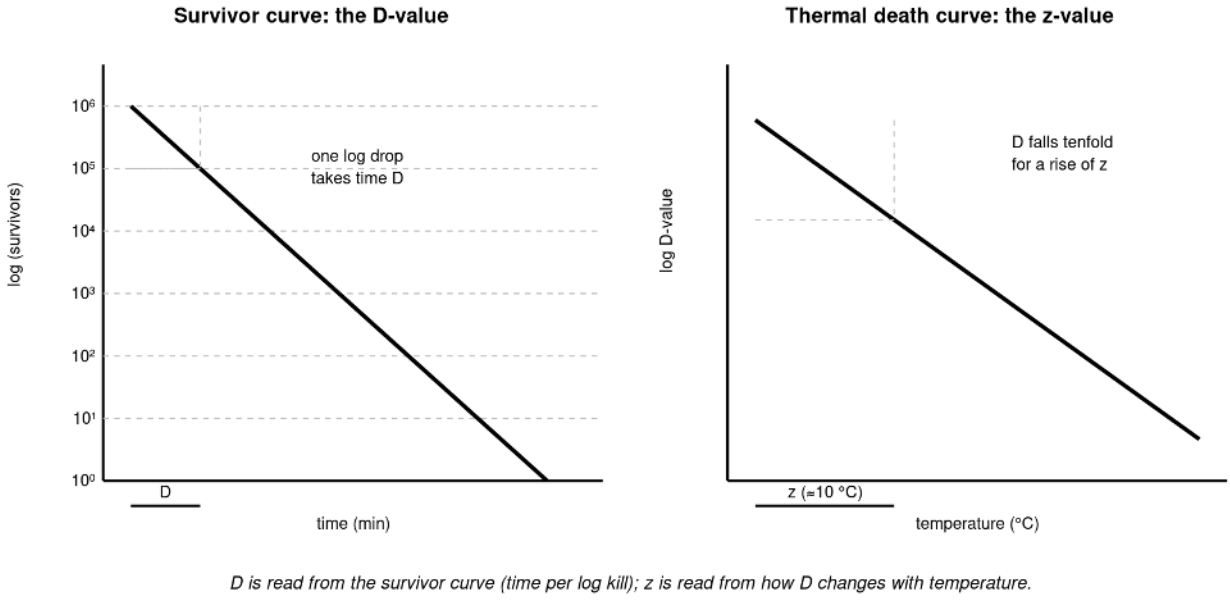


Figure 8.1 *The survivor curve (D-value) and the thermal death curve (z-value).*

8.2.1 The decimal reduction time (D-value)

The decimal reduction time, or D-value, is the time required, at a stated temperature, to destroy 90 per cent of the population, that is, to reduce it by one logarithmic cycle (one factor of ten). The survivor relationship follows from the exponential law:

> $N = N_0 \times 10^{(-t/D)}$

where N_0 is the initial number, N the number after time t, and D the decimal reduction time at the temperature concerned. The number of logarithmic reductions achieved in time t is t/D, and the time to achieve a chosen number of reductions is that number multiplied by D.

> Worked example 8.1. A spore population of 1×10^6 per unit has a D-value of 2 minutes at 121 degrees Celsius. How many survive after 12 minutes at 121 degrees Celsius?
> Number of logarithmic reductions = t/D = 12 ÷ 2 = 6. Therefore $N = N_0 \times 10^{(-6)} = 10^6 \times 10^{(-6)} = 1$ survivor per unit. Six D-values reduce a million spores to one; the count falls tenfold every 2 minutes, never by a fixed number.

The D-value depends on the organism, on the lethal agent, and on the temperature, and must always be quoted with the temperature, written for example D_{121}. A resistant spore has a long D-value, a vegetative cell a short one.

8.2.2 The z-value

The D-value falls as the temperature rises, since killing is faster at higher temperature. The z-value measures this dependence: it is the increase in temperature, in degrees Celsius, required to reduce the D-value to one tenth of its value. A small z-value denotes an organism whose killing is very sensitive to temperature; for saturated steam against bacterial spores the z-value is close to 10 degrees Celsius, the figure used throughout moist-heat calculations. The dependence of the D-value on temperature is shown graphically by the **thermal death time** curve, a plot of the logarithm of the D-value against temperature, which is a straight line whose slope gives the z-value: the temperature interval over which the line falls by one logarithmic cycle is, by definition, the z-value. The two curves, the survivor curve (log survivors against time at one temperature) and the **thermal death time curve** (log D against temperature), together describe the resistance of an organism completely, the first yielding the D-value and the second the z-value. The D-value at any temperature follows from a reference value by:

> $D_T = D_{ref} \times 10^{((T_{ref} - T) / z)}$
>
> Worked example 8.2. A spore has D = 2 minutes at 121 degrees Celsius and a z-value of 10 degrees Celsius. Find its D-value at 111 degrees Celsius and at 131 degrees Celsius.
> At 111 degrees Celsius (10 degrees cooler): $D = 2 \times 10^{((121 - 111)/10)} = 2 \times 10^{1} = 20$ minutes. At 131 degrees Celsius (10 degrees hotter): $D = 2 \times 10^{((121 - 131)/10)} = 2 \times 10^{(-1)} = 0.2$ minutes. A fall of one z-value multiplies the killing time tenfold; a rise of one z-value divides it by ten, which is why a modest increase in temperature greatly shortens the process.

8.2.3 The F-value and F_0

A real cycle does not hold a single temperature throughout; the load heats, holds and cools, and is being killed at every moment, faster when hot and slower when cool. To add the killing delivered at different temperatures into one figure, a common reference is used. The F-value is the lethality of a whole process expressed as the equivalent number of minutes at a reference temperature for an organism of a stated z-value. For moist-heat sterilization the reference temperature is 121 degrees Celsius and the reference z-value is 10 degrees Celsius, and the F-value is then written F_0. One minute of real time at a temperature T is worth a lethal rate L of equivalent minutes at 121 degrees Celsius:

> $L = 10^{((T - 121) / z)}$

> Worked example 8.3. With z = 10 degrees Celsius, find the lethal rate at 111, 121 and 125 degrees Celsius.
> At 111 degrees Celsius: L = 10^((111 − 121)/10) = 10^(−1) = 0.1, so a minute here is worth only 0.1 minute of F_0. At 121 degrees Celsius: L = 10^0 = 1.0, as it must be. At 125 degrees Celsius: L = 10^((125 − 121)/10) = 10^(0.4) = 2.51, so a minute here is worth 2.51 minutes of F_0. The total F_0 is the sum of L over every minute of the cycle. The pharmacopoeias commonly require an F_0 of at least 8 minutes, and many cycles are designed for 12 minutes or more to provide a margin.

The three quantities combine into a single statement of the killing achieved: the F_0 delivered, divided by the organism's D_{121}, gives the number of logarithmic reductions, which is the quantity required by the next section. The same idea is expressed by the older terms of thermal death. The thermal death time is the time required to kill a stated population at a stated temperature, and the **thermal death point** is the lowest temperature that kills the population in a stated time; both are particular points on the survivor relationship, now subsumed in the more general D-, z-, and F-value treatment. It follows from the definitions that the F-value required to achieve a given reduction is F = D × (number of logarithmic reductions), so that a process delivering an F_0 of, for example, 12 minutes against an organism of D_{121} = 1.5 minutes achieves eight logarithmic reductions; this simple product links the resistance of the organism, the lethality of the process, and the reduction achieved.

8.2.4 Deviations from log-linear kinetics

The log-linear model is the working assumption and holds well for most spore populations, but real survivor curves sometimes depart from the straight line, and two departures should be recognised. A **shoulder** is an initial period in which the curve is flatter than the straight portion, before the logarithmic decline begins; it arises where the population must be heated through or where clumped cells protect one another. A **tail** is a flattening at the end of the curve, where a small, unusually resistant fraction persists after the bulk has been destroyed. The tail is the more important for safety, since it represents the hardest organisms to kill, and the overkill approach described below is designed in part to drive even such a resistant fraction below the target. The modelling of microbial death is, in essence, the use of the log-linear survivor curve to predict the outcome of a process: from the initial population, the resistance of the organism (its D-value), and the lethality delivered (the F-value), the surviving population, and hence the probability of a survivor, is calculated. The model is the foundation of the rational design of a sterilization cycle, in which the conditions are chosen to deliver the F-value that reduces the expected bioburden to the required sterility assurance level, rather than being fixed by tradition alone. Where shoulders or tails make the real curve depart from the straight line, the model is applied conservatively, the resistant tail taken as the governing case. The practical value of the model is twofold. It allows a cycle to be designed rationally, by

choosing conditions that deliver the F-value required to reduce the expected bioburden to the target SAL, and it allows the assurance achieved by an existing cycle to be calculated and demonstrated. The model also makes plain why sterility is a probability and not a certainty: extrapolation of the straight line never reaches zero survivors but only ever-smaller fractional probabilities, so that the statement of sterility is always a statement of the chance of a survivor, made acceptably small.

> **Quick recall.** Microbial death is exponential, so the survivor curve is a straight line on a logarithmic plot. D-value: time for one log (90%) reduction at a stated temperature. z-value: temperature rise for a tenfold change in D (about 10 °C for steam). F_0: lethality as equivalent minutes at 121 °C (z = 10 °C). Log reductions = t/D = F_0/D_{121}. Shoulders and tails are deviations from the straight line.

8.2.5 Factors affecting the heat resistance of microorganisms

The D-value of an organism is not a fixed constant but depends on conditions, and the same factors that affect heat resistance affect the design of the process. The species and form of the organism is paramount: spores are far more resistant than vegetative cells, and species differ widely, so the reference organisms used to challenge a process are chosen for their high resistance. The water content of the environment is decisive, which is why moist heat, acting on hydrated organisms, kills at a much lower temperature than dry heat. The pH of the medium influences resistance, organisms generally being most resistant near neutrality. The composition of the medium matters, since fats, sugars and proteins protect organisms against heat, so that an organism in an oily or sugary product has a higher apparent D-value than the same organism in water. The initial population does not change the D-value but does change the time to reach a given survivor level, since more reductions are needed from a larger start. These factors explain why the D-value must always be quoted with its conditions and why a cycle validated for one product cannot be assumed to suffice for another.

8.3 Sterility assurance and the sterility assurance level

Because death is exponential, no finite process reduces the survivor count to zero, and sterility is therefore defined as a probability. The **sterility assurance level (SAL)** is the probability that a single sterilized item contains a viable microorganism after the process. The accepted standard for products sterilized in their final container is an SAL of 10^{-6}, that is, a probability of not more than one non-sterile item in a million.

The log-death model converts this requirement into a definite target, because the passage from a starting population to an SAL of 10^{-6} is a fixed number of logarithmic reductions.

> Worked example 8.4. A product carries a bioburden of 1×10^6 resistant spores per unit. How many logarithmic reductions are needed to reach an SAL of 10^{-6}, and how long must the cycle hold if D_{121} = 2 minutes?

> Logarithmic reductions = $\log(N_0) - \log(SAL) = \log(10^6) - \log(10^{-6}) = 6 - (-6) = 12$. Time = $12 \times D = 12 \times 2 = 24$ minutes at 121 degrees Celsius. The probability of a survivor has not reached zero; it has reached one in a million, which is the operative definition of sterile for this product.

This twelve-log target is the basis of the overkill approach, the usual design for heat-stable products. Rather than rely on a precise knowledge of the bioburden, the cycle is designed to deliver at least a twelve-logarithm reduction of a resistant reference spore, which guarantees an SAL of 10^{-6} even for a heavily contaminated and highly resistant load. It is deliberately more severe than the minimum, and is chosen because heat-stable products can withstand it and because it is robust against variation in the bioburden. Where a product cannot withstand the overkill cycle, a **bioburden-based approach** is used: the actual number and resistance of the organisms on the product are determined, and a milder cycle is designed to achieve the SAL of 10^{-6} for that real load. The relation of bioburden, logarithmic reduction, and SAL is shown in Figure 8.2.

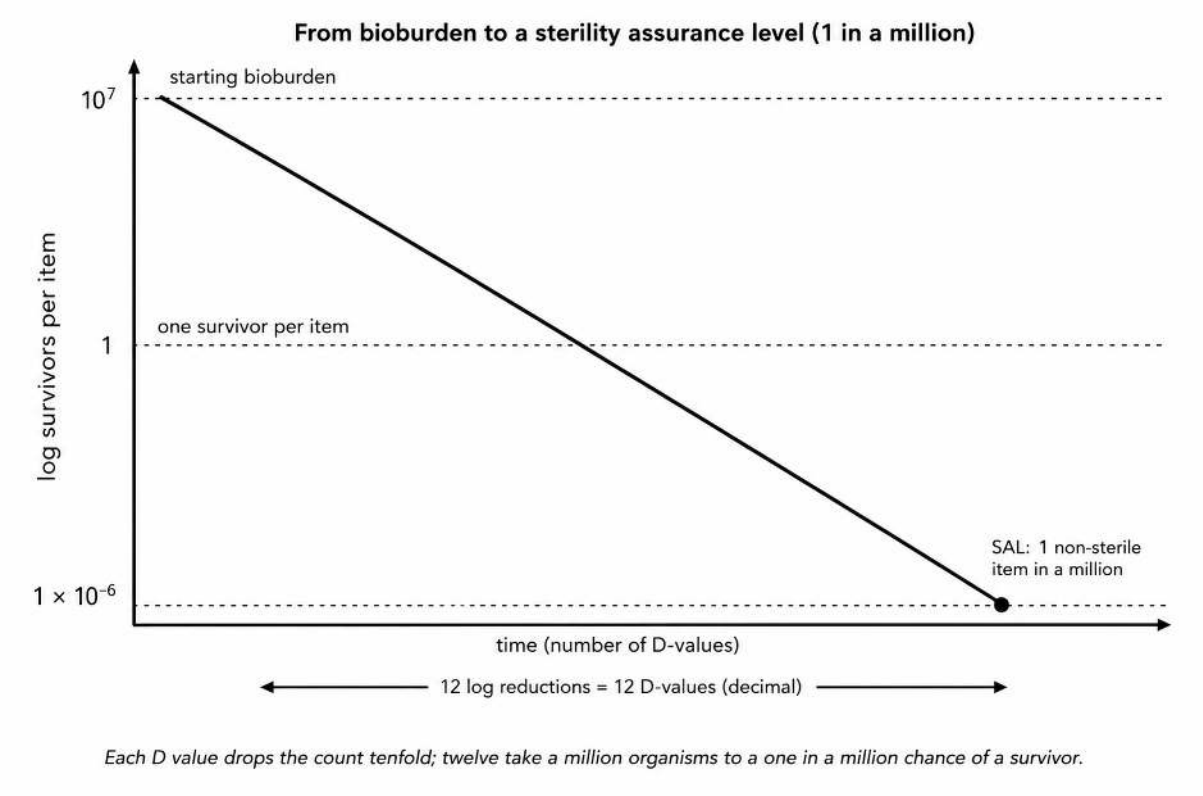


Figure 8.2 *From a starting bioburden to a sterility assurance level.*

The overkill cycle is often validated by the **half-cycle method**, in which the process is shown to destroy a large population of a resistant biological indicator (typically 10^6 spores) in half the routine exposure time; if half the cycle gives complete kill of 10^6 spores, the full cycle delivers at least a further six logarithmic reductions, so that the total reduction is at least twelve logarithms and an SAL of 10^{-6} is assured even for a heavily contaminated load. The number of logarithmic reductions of the indicator achieved by a process is termed the **spore log reduction**, and the overkill

requirement is expressed as a spore log reduction of at least twelve. The attraction of the overkill approach is that it does not depend on a precise and continuing knowledge of the natural bioburden, only on the demonstrated destruction of a far more resistant and more numerous challenge.

8.3.1 Bioburden determination

Both approaches require the bioburden, the number of viable organisms on or in the product before sterilization, to be known. It is determined by the viable-count methods of Chapter 1, the product or its rinse being plated and the colonies counted as colony-forming units, with separate counts of bacteria and fungi where required. The bioburden sets the starting figure N_0 for the calculation, guides the design of the cycle, and provides an early warning, through a rising count, that the contamination control of the earlier chapters has weakened. The bioburden is thus the single number at which the clean-area work and the sterilization work meet. Its determination requires that any antimicrobial substance in the product, such as a preservative, be neutralised or removed before the count, since otherwise the count would be falsely low; the product is therefore commonly recovered by membrane filtration, the membrane being washed free of the inhibitory substance and then placed on a recovery medium, or by direct plating with a neutraliser. The bioburden is monitored as a trend, an unexpected rise prompting investigation of the upstream controls before it can compromise the sterilization.

8.4 Validation and evaluation of sterilization

Because the survivor that the process must destroy is invisible, the success of a sterilization cannot be judged by inspecting the load; the process must be validated, and its routine performance monitored, by independent evidence of three kinds.

Physical measurement records what the load actually experienced. Thermocouples placed at the coldest and most inaccessible points of the load record the temperature throughout the cycle, the pressure and time are logged, and the F_0 delivered is calculated from the temperature record by the lethal-rate summation of Section 8.2. This confirms that the process delivered the intended lethality.

Chemical indicators give a rapid visible confirmation that the conditions were met. They are substances that change colour on exposure to the sterilizing condition, such as autoclave tape, and the Bowie-Dick test that confirms the removal of air and the penetration of steam in a porous-load autoclave. A chemical indicator distinguishes a treated item from an untreated one, but it does not by itself prove sterility. Chemical indicators range from simple process indicators, which merely show that an item has been exposed to a process (such as the colour change of autoclave tape), to more elaborate indicators that integrate temperature and time and approach the response of a biological indicator. They are valued for the immediate result they give at the point of use, in contrast to the biological indicator, whose result must await incubation.

Biological indicators provide the most direct evidence, since they test the one thing that matters, the ability of the process to kill. A biological indicator is a standardised preparation of a large number of spores of an organism chosen to be more resistant to the process than any expected contaminant; it is placed at the most difficult position in the load, exposed to the cycle, and then incubated. If no growth results, the process has destroyed even this resistant challenge. The reference organisms are matched to the method: *Geobacillus stearothermophilus* spores for moist heat, *Bacillus atrophaeus* spores for dry heat and for ethylene oxide, and other resistant spores for radiation and other processes (Table 8.1).

Table 8.1: Biological indicator organisms by sterilization method

Method	Biological indicator (resistant spore)
Moist heat (steam)	*Geobacillus stearothermophilus*
Dry heat	*Bacillus atrophaeus*
Ethylene oxide gas	*Bacillus atrophaeus*
Ionizing radiation	*Bacillus pumilus* (historically), or dose set by bioburden

The evaluation of the efficiency of a sterilization process draws these strands together. A process is efficient when it reliably delivers, to every part of the load, a lethality sufficient to achieve the required sterility assurance level for the expected bioburden, and this is judged by the physical lethality delivered (the F_0, calculated from the temperature record), the response of **chemical indicators**, and, decisively, the destruction of the biological indicator. A biological indicator that grows after the cycle is direct evidence of an inefficient process, whereas its destruction, together with a satisfactory physical record, is evidence of an efficient one. The biological indicator has the limitation that its result is delayed by the incubation it requires, so that it cannot release a batch in real time; this is among the reasons that a well-understood physical process such as steam sterilization is controlled and released by its validated physical parameters.

Validation assembles this evidence in a formal sequence. **Installation qualification (IQ)** documents that the sterilizer is correctly installed, with its services, instruments and calibration in place. **Operational qualification (OQ)** demonstrates that the empty machine operates to specification across its working range. **Performance qualification (PQ)** demonstrates that the loaded machine delivers the required lethality at every point, using temperature mapping and **biological indicators** at the coldest and most inaccessible positions. Only after all three is the cycle approved for routine use. The autoclave is, in addition, subjected to routine engineering checks: a leak-rate (vacuum) test confirms that the chamber holds its vacuum, and, for a porous-load sterilizer, the Bowie-Dick test confirms on each working day that air is removed and steam

penetrates evenly. The cycle is requalified periodically and after any change to the load, the machine, or the process, since a change that is not revalidated may invalidate the assurance the original validation gave. The rigour of this validation is what permits **parametric release** of well-understood processes such as steam sterilization, in which a batch is released on the validated physical record of the cycle rather than on a separate sterility test of the finished product, a direct expression of the principle that sterility is assured by controlling and proving the process, not by testing the product.

> **Quick recall.** A sterilization process is validated by physical measurement (thermocouples, calculated F_0), chemical indicators (colour change; Bowie-Dick), and biological indicators (resistant spores: *G. stearothermophilus* for steam, *B. atrophaeus* for dry heat and ethylene oxide). Validation follows IQ, OQ and PQ, and a validated process may be released parametrically.

8.5 Determination of bacteriostatic and bactericidal activity

A question related to but distinct from the performance of a sterilization process is the measurement of the action of an antimicrobial agent on an organism, whether the agent inhibits growth or kills. Two laboratory measurements, introduced with the evaluation of disinfectants in Chapter 6, express this.

The **minimum inhibitory concentration (MIC)** is the lowest concentration of an agent that prevents the visible growth of the organism after a defined incubation. It is determined by a broth dilution method: the agent is prepared in a series of two-fold dilutions, each tube is inoculated with the organism and incubated, and the lowest concentration showing no turbidity is the MIC. Because it measures the prevention of growth, the MIC expresses bacteriostatic activity.

The **minimum bactericidal concentration (MBC)** is the lowest concentration that kills the organism. It is determined by subculturing the clear tubes of the MIC series onto agar free of the agent and finding the lowest concentration from which no growth is recovered, a reduction of the population by 99.9 per cent being the usual criterion. Because it measures killing, the MBC expresses bactericidal activity. The relation of the two is informative: when the MBC is close to the MIC the agent is bactericidal at concentrations near those that inhibit, whereas when the MBC much exceeds the MIC the agent is essentially bacteriostatic at usable concentrations. A static agent depends on the host defences to clear the inhibited organisms, while a cidal agent destroys them. The determination of these activities follows a defined procedure: serial two-fold dilutions of the agent are prepared in broth, each is inoculated with a standardised culture, and after incubation the MIC is read as the lowest clear tube and the MBC by subculture, as described. A control without the agent confirms the viability of the inoculum, and, where the agent itself might be carried over and inhibit growth on subculture, a neutraliser is included so that the result reflects killing and not

mere carry-over of the agent. The same care over the neutralisation of an antimicrobial substance is essential in the sterility test of Chapter 10, where a residual preservative or antibiotic would otherwise prevent the growth of any organism present and produce a falsely negative result. The determination of bacteriostatic and bactericidal activity is thus both a measurement of an agent in its own right and a necessary preliminary to the valid testing of a preserved product.

8.6 Summary

The assurance of sterility is the quantitative heart of pharmaceutical microbiology. Microbial death is exponential, so the survivor curve is a straight line on a logarithmic plot and sterility can be expressed only as a probability. Three quantities describe the kinetics: the D-value, the time for a tenfold reduction at a stated temperature; the z-value, the temperature rise that reduces the D-value tenfold, about 10 degrees Celsius for steam; and the F_0, the lethality of a moist-heat cycle as equivalent minutes at 121 degrees Celsius, summed from the lethal rate at each moment. The number of logarithmic reductions delivered is t/D, or equivalently F_0/D_{121}, and the time to reach a target is the number of reductions multiplied by the D-value; deviations from the straight line take the form of shoulders and tails.

Sterility is specified as a sterility assurance level, conventionally 10^{-6} for terminally sterilized products, reached for heat-stable products by an overkill cycle delivering at least a twelve-logarithm reduction of a resistant spore, and for fragile products by a measured bioburden-based cycle; the bioburden, determined by viable count, is the starting figure and the link to contamination control. Because the surviving organism is invisible, the process is validated, not the product inspected: by physical measurement of the delivered F_0, by chemical indicators, and most directly by biological indicators of resistant spores, in the formal sequence of installation, operational and performance qualification, after which a process may be released parametrically. The efficiency of a process is judged by the physical lethality delivered, the response of chemical indicators, and the destruction of the biological indicator, and the heat resistance of an organism, expressed in its D-value, depends on its species and form, the water content, the pH, and the protective constituents of the medium. Finally, the action of an antimicrobial agent on an organism is quantified by the minimum inhibitory concentration, which measures inhibition, and the minimum bactericidal concentration, which measures killing, the neutralisation of any residual agent being essential both here and in the sterility test. Because that demonstration can never, by the very nature of exponential death, be a proof of absolute sterility, the next unit turns to the demonstration, by test, that the product is in fact of the required microbiological quality, and to the inherent limitations of such testing.

8.7 Self-assessment

A. Multiple-choice questions

1. The decimal reduction time (D-value) is the time required to:

(a) kill all organisms (b) reduce the population by 90 per cent (one log) (c) double the population (d) reduce the population by half. Answer: (b).

2. The z-value is the temperature change that alters the D-value by a factor of:

(a) 2 (b) 5 (c) 10 (d) 100. Answer: (c).

3. The reference temperature for the F_0 value is:

(a) 100 °C (b) 115 °C (c) 121 °C (d) 134 °C. Answer: (c).

4. The accepted sterility assurance level for terminally sterilized products is:

(a) 10^{-3} (b) 10^{-6} (c) 10^{-12} (d) zero. Answer: (b).

5. The biological indicator for moist-heat sterilization is:

(a) *Bacillus atrophaeus* (b) *Escherichia coli* (c) *Geobacillus stearothermophilus* (d) *Clostridium tetani*. Answer: (c).

6. The minimum bactericidal concentration is determined by:

(a) reading the lowest clear broth tube (b) subculturing the clear tubes onto agar free of the agent (c) a zone of inhibition (d) a particle count. Answer: (b).

7. The stages of validation of a sterilizer, in order, are:

(a) PQ, OQ, IQ (b) IQ, OQ, PQ (c) OQ, IQ, PQ (d) IQ, PQ, OQ. Answer: (b).

8. The half-cycle method validates an overkill process by showing complete kill of 10^6 resistant spores in:

(a) twice the routine time (b) half the routine time (c) the routine time (d) one tenth of the routine time. Answer: (b).

9. Heat resistance of an organism is increased by the presence in the medium of:

(a) water (b) fats, sugars and proteins (c) acid (d) salt only. Answer: (b).

10. The chief limitation of the biological indicator as a routine release test is that:

(a) it is inaccurate (b) its result is delayed by incubation (c) it is not specific (d) it cannot be placed in the load. Answer: (b).

11. The number of logarithmic reductions of a biological indicator achieved by a process is termed the:

(a) F-value (b) spore log reduction (c) z-value (d) bioburden. Answer: (b).

B. Short-answer questions (2 to 5 marks)

Q1. Define the D-value, the z-value, and the F_0 value, with the units of each.

The D-value is the time, in minutes, at a stated temperature, to reduce the population by 90 per cent (one log). The z-value is the rise in temperature, in degrees Celsius, that reduces the D-value to one tenth, about 10 degrees Celsius for steam. The F_0 value is the lethality of a moist-heat cycle expressed as the equivalent number of minutes at 121 degrees Celsius for an organism of z = 10 degrees Celsius.

Q2. Explain why a sterilized product cannot be claimed to contain no organisms, and how the SAL resolves this.

Microbial death is exponential, so each interval destroys a fixed fraction of the survivors and the count approaches zero without reaching it; no finite process gives certainty of no survivors. The sterility assurance level resolves this by defining sterility as an acceptably low probability of a survivor, an SAL of 10^{-6} meaning not more than one non-sterile item in a million.

Q3. Distinguish the overkill approach from the bioburden-based approach.

The overkill approach designs the cycle to deliver at least a twelve-logarithm reduction of a resistant reference spore, guaranteeing an SAL of 10^{-6} regardless of the actual bioburden; it suits heat-stable products and is robust. The bioburden-based approach measures the actual number and resistance of the organisms on the product and designs a milder cycle to achieve the same SAL for that real load; it is used for products that cannot withstand the overkill cycle and demands close control of the bioburden.

Q4. Describe the use of biological indicators in the validation of sterilization.

A biological indicator is a standardised preparation of a large number of spores of an organism more resistant to the process than any expected contaminant. It is placed at the most difficult position in the load, exposed to the cycle, and incubated; no growth indicates that the process destroyed even this resistant challenge. The organism is matched to the method: Geobacillus stearothermophilus for moist heat, Bacillus atrophaeus for dry heat and ethylene oxide.

Q5. Distinguish the minimum inhibitory concentration from the minimum bactericidal concentration.

The MIC is the lowest concentration that prevents visible growth, found from a broth dilution series as the lowest clear tube; it measures inhibition (static activity). The MBC is the lowest concentration that kills, found by subculturing the clear tubes onto agar free of agent and identifying the lowest concentration giving no growth (a 99.9 per cent reduction); it measures killing (cidal activity). A large excess of MBC over MIC indicates an essentially static agent.

Q6. State the factors that affect the heat resistance of microorganisms.

The principal factors are the species and form of the organism (spores far more resistant than vegetative cells); the water content of the environment (moist heat kills at a lower temperature than dry heat); the pH (resistance greatest near neutrality); the composition of the medium (fats, sugars and proteins protect against heat); and the initial population (which does not change the D-value but increases the time to reach a given survivor level). The D-value must therefore always be quoted with its conditions.

Q7. Explain the half-cycle method of validating an overkill sterilization process.

In the half-cycle method the process is shown to give complete destruction of a large population (typically 10^6 spores) of a resistant biological indicator in half the routine exposure time. If half the cycle destroys 10^6 spores, the full cycle delivers at least a further six logarithmic reductions, so the total reduction is at least twelve logarithms (a spore log reduction of 12), assuring an SAL of 10^{-6} even for a heavily contaminated load, without dependence on precise knowledge of the natural bioburden.

C. Long-answer questions (10 marks)

Q1. Explain the kinetics of microbial death and define the D-, z-, and F_0-values, with worked examples.

A complete answer states that death is exponential and the survivor curve log-linear, defines the three quantities with the relationships $N = N_0 \times 10^{(-t/D)}$, $D_T = D_{ref} \times 10^{((T_{ref} - T)/z)}$, and $L = 10^{((T - 121)/z)}$, and works numerical examples such as Worked Examples 8.1 to 8.3. Credit is given for noting that log reductions $= t/D = F_0/D_{121}$ and for mentioning shoulders and tails, the deviations from the ideal straight-line model that a real survivor curve may show. (High-yield: the death kinetics and the F_0 concept are frequently examined.)

Q2. Describe the validation of a sterilization process.

A complete answer explains that the process is validated rather than the product inspected, describes the three kinds of evidence (physical measurement with thermocouples and calculated F_0, chemical indicators, and biological indicators of resistant spores matched to the method), sets out the sequence of installation, operational and performance qualification, and notes parametric release as the outcome for a well-understood process.

Q3. Explain the sterility assurance level and the overkill approach, and relate them to the bioburden.

A complete answer defines the SAL as the probability that a sterilized item carries a viable organism, with 10^{-6} the standard for terminally sterilized products, explains that the passage from the bioburden to the SAL is a fixed number of logarithmic reductions ($\log N_0 - \log SAL$), describes the overkill approach as the delivery of at least a twelve-log reduction of a resistant spore

(validated by the half-cycle method) so that the SAL is assured regardless of the natural bioburden, and contrasts it with the bioburden-based approach used for fragile products, which measures the real bioburden and designs a milder cycle. Credit is given for relating the bioburden to contamination control.

D. Numerical practice problems (full recomputed solutions)

Problem 1 (worked). A load carries 1×10^5 spores per unit with $D_{121} = 1.5$ minutes, and the autoclave holds 121 degrees Celsius for 15 minutes. Find the logarithmic reductions delivered, the survivor level, and whether an SAL of 10^{-6} is achieved.

Logarithmic reductions = t/D = 15 ÷ 1.5 = 10. Survivor level $N = 10^5 \times 10^{(-10)} = 10^{(-5)}$, an SAL of 10^{-5}, which does not meet 10^{-6}. To reach 10^{-6} from 10^5 requires $5 - (-6) = 11$ reductions, so $t = 11 \times 1.5 = 16.5$ minutes, slightly longer than the 15-minute hold.

Problem 2 (faded). A population falls from 1×10^6 to 1×10^2 in an 8-minute hold at 121 degrees Celsius. Find the D-value, and the F_0 delivered if all lethality occurred at 121 degrees Celsius.

Logarithmic reductions = $\log(10^6) - \log(10^2) = 6 - 2 = 4$. D = t ÷ (reductions) = 8 ÷ 4 = 2 minutes. Because the killing occurred entirely at the reference temperature, F_0 = the real time at 121 degrees Celsius = 8 minutes, which meets the common minimum.

Problem 3 (independent; solution given). During a cycle a load is held for 10 minutes at 118 degrees Celsius (z = 10 degrees Celsius). How much F_0 does this contribute? If the target F_0 is 12 minutes, how much more is needed, and roughly how long at 121 degrees Celsius would supply it?

Lethal rate at 118 degrees Celsius: $L = 10^{((118 - 121)/10)} = 10^{(-0.3)} = 0.501$. Over 10 minutes, $F_0 = 0.501 \times 10 = 5.01$ minutes. The shortfall to 12 minutes is $12 - 5.01 = 6.99$ minutes of F_0. At 121 degrees Celsius the lethal rate is 1.0 per minute, so about 7 minutes at 121 degrees Celsius would supply the remainder. Each value was recomputed before printing, and the student is advised to reproduce each calculation independently, since the manipulation of logarithms is the commonest source of error in this topic.

Problem 4 (independent; solution given). A solution carries 1×10^8 organisms per container with $D_{121} = 1.0$ minute. How long at 121 degrees Celsius is needed to reach an SAL of 10^{-6}?

Logarithmic reductions = $\log(10^8) - \log(10^{-6}) = 8 - (-6) = 14$. Time = 14 × D = 14 × 1.0 = 14 minutes at 121 degrees Celsius.

Problem 5 (independent; solution given). An organism has $D_{121} = 1.5$ minutes and z = 10 degrees Celsius. Find its D-value at 110 degrees Celsius.

$D_{110} = D_{121} \times 10^{((121 - 110)/10)} = 1.5 \times 10^{(1.1)} = 1.5 \times 12.59 = 18.9$ minutes. The fall of 11 degrees, slightly more than one z, multiplies the killing time about thirteen-fold.

Problem 6 (independent; solution given). A validated cycle delivers an F_0 of 15 minutes. Against a reference spore of D_{121} = 1.5 minutes, how many logarithmic reductions does it achieve, and is this an overkill cycle?

Logarithmic reductions = $F_0 \div D_{121} = 15 \div 1.5 = 10$. Ten reductions is short of the twelve required for an overkill cycle, so this cycle, though it exceeds the minimum F_0 of 8 minutes, does not by itself meet the overkill criterion of a twelve-log spore reduction; either a higher F_0 or a less resistant reference would be required.

Write a note on biological indicators and chemical indicators in the validation of sterilization.

A biological indicator is a standardised preparation of resistant spores (Geobacillus stearothermophilus for moist heat, Bacillus atrophaeus for dry heat and ethylene oxide) placed at the most difficult position in the load and incubated after the cycle; no growth confirms that the process killed even this resistant challenge, making it the most direct evidence of efficacy, though its result is delayed by incubation. A chemical indicator changes colour on exposure to the sterilizing condition (autoclave tape, the Bowie-Dick test for steam penetration); it gives an immediate result and distinguishes treated from untreated items but does not by itself prove sterility. The two are used together, the chemical indicator for immediate confirmation and the biological indicator for proof of lethality.

Write a note on the validation of a steam sterilization process.

Validation shows that the autoclave reliably delivers the required lethality to every part of the load. It proceeds through installation qualification (correct installation, services and calibration), operational qualification (the empty machine operating to specification across its range), and performance qualification (the loaded machine delivering the required lethality, demonstrated by temperature mapping with thermocouples and by biological indicators of Geobacillus stearothermophilus at the coolest and least accessible points). Routine checks include a leak-rate test and, for porous loads, the daily Bowie-Dick test of air removal and steam penetration. The cycle is requalified periodically and after any change, and a fully validated cycle may be released parametrically on its physical record.

References

1. Denyer SP, Hodges NA, Gorman SP, editors. Hugo and Russell's Pharmaceutical Microbiology. 8th ed. Oxford: Wiley-Blackwell; 2011.
2. Hugo WB, Russell AD. Pharmaceutical Microbiology. Oxford: Blackwell Science.
3. Sandle T. Pharmaceutical Microbiology: Essentials for Quality Assurance and Quality Control. Cambridge: Woodhead Publishing/Elsevier; 2016.
4. Indian Pharmacopoeia. Ghaziabad: Indian Pharmacopoeia Commission. [latest edition]

Chapter 9: Microbial Limit Tests and Microbial Assay

Learning outcomes

After studying this chapter the student will be able to:

- State the purpose and limitations of the microbiological testing of finished products.
- Describe the **microbial limit tests**: the total counts and the tests for specified organisms.
- Describe the **microbial assay** of antibiotics by the cylinder-plate and turbidimetric methods.
- Describe the microbial assay of vitamins and amino acids.
- Explain the principle of comparison against a reference standard in a biological assay.

These outcomes serve Course Outcome CO5, the performance of microbial limit and assay tests in compliance with pharmacopoeial requirements.

9.1 Introduction

Microbiological quality control is the body of test by which a finished pharmaceutical product is shown to meet its microbiological specification, whether that specification is a limit on the number and type of organisms permitted, for a non-sterile product, or the absence of all viable organisms, for a sterile product. It is the final confirmation, at the end of the process, of the control built in throughout the earlier chapters. Two limitations of all such testing must be understood from the outset and govern its interpretation. First, a test is performed on a sample, not on the whole batch, so it can establish the quality only of the material examined, and its conclusion is extended to the batch by inference; a finished-product test cannot, by itself, guarantee the quality of every unit. Second, the test confirms quality but does not create it: a product is made of acceptable quality by the control of materials, environment and process, and the test is the verification, not the source, of that quality. These two limitations are not a counsel of despair but a statement of where assurance truly resides, namely in the controlled process, and they should be held in mind whenever a test result is interpreted. With these limitations in mind, this chapter treats the two tests applied to non-sterile products and to the biological products that require it: the microbial limit tests, which measure the number and exclude specified organisms, and the microbial assay, which measures the biological potency of an antibiotic, vitamin, or amino acid. The sterility test of sterile products is treated in Chapter 10. The microbiological tests of a finished product thus fall into three kinds: the enumeration and exclusion tests of the microbial limit test, which judge the microbial cleanliness of a non-sterile product; the sterility test, which judges the absolute freedom from organisms of a sterile product; and the microbial assay, which judges the potency of an active ingredient by its biological effect. The first two protect the patient from microorganisms in the medicine, the third ensures that the medicine contains the strength it is claimed to contain. All three share the discipline

of testing against a defined acceptance criterion on a representative sample under controlled conditions, and all three are prescribed in detail by the pharmacopoeia so that the result of one laboratory may be relied upon by another.

9.2 Microbial limit tests

A non-sterile product is not required to be free of microorganisms, but it must not contain more than a specified number, and it must be free of organisms that would endanger the patient. The microbial limit tests of the pharmacopoeia establish both, and they comprise two distinct determinations: the enumeration of the total viable count, and the test for specified (objectionable) organisms. The test begins with the preparation of the sample: a representative quantity of the product is dissolved or suspended in a sterile diluent, commonly a buffered saline with a wetting agent and, where needed, a neutraliser for any preservative, so that the organisms are released into a form that can be plated or filtered. A water-soluble product is dissolved, a water-insoluble solid is suspended, and an oily product is emulsified, the method of preparation being chosen so that the organisms are recovered without being killed or hidden.

9.2.1 Enumeration of the total viable count

The total viable count is determined as two separate figures. The **total aerobic microbial count (TAMC)** is the number of aerobic bacteria, and the **total combined yeasts and moulds count (TYMC)** is the number of fungi, each expressed in colony-forming units per gram or per millilitre of product. The two are counted separately because bacteria and fungi require different media and conditions, the fungi being counted on a medium of acidic pH or containing an antibiotic that suppresses bacteria. The count is performed by one of the methods of Chapter 1, namely the pour-plate or spread-plate method, or, for a filterable liquid, by membrane filtration, the chosen method being the one that best recovers the organisms from the particular product. Where the expected count is very low, the **most probable number (MPN)** method may be used: the product is added to several tubes of liquid medium in a dilution series, the pattern of tubes showing growth after incubation is recorded, and the most probable number of organisms in the sample is read from a standard statistical table. The medium for the total aerobic count is a general-purpose agar incubated at 30 to 35 degrees Celsius, and that for the yeasts and moulds an agar of acidic pH or one containing an antibiotic to suppress bacteria, incubated at 20 to 25 degrees Celsius; the two are counted on separate plates because the conditions that favour one group suppress the other. The quantity of product examined is specified, and is larger for a product expected to have a low count, so that the test is sensitive enough to detect a breach of the limit. The result is compared against the limit laid down for that category of product, the limit being stricter for a product used by a more vulnerable route: an oral solid is permitted a higher count than an oral liquid, and a product for topical or oromucosal use a lower one still.

A critical preliminary, common to all microbiological testing of a product that contains an antimicrobial substance, is the removal or neutralisation of that substance before the count. A preservative or an antibiotic carried into the test would inhibit the organisms and give a falsely low count, so the product is recovered by membrane filtration with washing of the membrane, or a specific neutraliser is added, and the adequacy of the recovery is confirmed by a **method suitability test** in which the product, inoculated with a known number of organisms, is shown to permit their recovery.

9.2.2 Test for specified organisms

Certain organisms are not permitted in particular products at all, irrespective of the total count, because of the hazard they present by the route of use, the objectionable organisms introduced in Chapter 3. The product is therefore tested for the absence of named organisms, the list being chosen according to the route of administration. The organisms commonly specified are ***Escherichia coli*** *(an indicator of faecal contamination, excluded from oral products),* ***Salmonella*** *species,* ***Pseudomonas aeruginosa*** *and* ***Staphylococcus aureus*** *(excluded from topical and several other products), bile-tolerant Gram-negative bacteria, and, for particular products,* Clostridia *and* Candida albicans*. The test proceeds by first growing the product in a non-selective enrichment broth, which allows a small number of the target organism to multiply, and then subculturing onto a selective and differential medium on which that organism gives a characteristic colony; a suspect colony is confirmed by the staining and biochemical methods of Chapter 2. The procedure for each organism follows the same pattern of enrichment then selective culture, with the medium chosen for the organism: a lactose medium and a bile-salt medium for* Escherichia coli *and the bile-tolerant Gram-negative bacteria, a selective enrichment and a selective agar for* Salmonella*, a selective agar for* Pseudomonas aeruginosa *and for* Staphylococcus aureus*, and so on. The identity of a colony with the expected appearance is confirmed by the Gram reaction and the biochemical tests of Chapter 2, since a colony of the right appearance is presumptive only. The product passes if the specified organism is not detected in the quantity of product examined, and fails if it is, irrespective of the total count, because the presence of an objectionable organism is by itself a failure of the product.*

Two related quality-control tests of parenteral products, although strictly tests of the absence of a pyrogenic response rather than of microbial number, are conveniently noted here. The **bacterial endotoxin test (LAL test)** detects the heat-stable endotoxin of Gram-negative bacteria by the gelation or colour change of a reagent prepared from the blood of the horseshoe crab, and has largely replaced the older **rabbit pyrogen test**, in which the rise in the temperature of test rabbits given the product is measured. Both are applied to injections and to Water for Injection, and both address the fact, met in Chapter 3, that a product may be sterile yet pyrogenic because endotoxin survives the death of the organism. The endotoxin test is treated further with water monitoring in Chapter 10.

> **Quick recall.** The microbial limit tests have two parts: enumeration of the total aerobic microbial count (TAMC) and the total yeasts and moulds count (TYMC), in CFU per g or mL against a route-based limit; and the test for the absence of specified organisms (E. coli, Salmonella, P. aeruginosa, S. aureus) by enrichment and selective culture. Any antimicrobial substance in the product must first be neutralised, and the method validated by a suitability test.

9.2.3 Interpretation and action

A product complies with the microbial limit tests if the total counts are within the limits for its category and the specified organisms are absent in the quantity examined. A result that exceeds a count limit, or that detects a specified organism, is a failure, and the batch is rejected pending investigation; because the test only samples the batch, a failure is treated as evidence of a real loss of control rather than as an isolated event, and the investigation extends to the materials, the water, the environment, and the process that produced the batch. The limits themselves are set with the route of administration in mind, and the student should appreciate that the same count that is acceptable in an oral powder may be unacceptable in a product for the eye or for application to a wound, a principle already met in the discussion of objectionable organisms.

9.3 Microbial assay

Certain substances cannot be measured satisfactorily by chemical analysis, and their content is determined instead by their biological activity, in a microbial assay (biological assay). The pharmacopoeias prescribe a microbial assay for several antibiotics, vitamins and amino acids, either because a chemical method cannot distinguish the active substance from inactive but chemically similar impurities, or because the biological activity is precisely the property of interest and is the established measure.

The governing principle of every biological assay is comparison against a reference standard. The activity of the test substance is never read as an absolute value from an instrument; it is compared with that of a reference standard of known, defined potency, the two being treated identically and in parallel within the same assay, so that any factor affecting the test affects the standard equally and cancels out. The potency of the test substance is then expressed relative to the standard. Because the test organism is a living thing whose response varies, the assay is designed and analysed statistically, with replication and with a check that the dose-response relationship of the test substance is parallel to that of the standard; a lack of parallelism indicates that the two are not behaving as the same substance and that the result is invalid. To secure this validity the assay is laid out in a balanced, symmetrical design, in which the standard and the test are each applied at two or more dose levels, equally spaced on the logarithmic scale, and arranged on the plate or among the tubes so that systematic variations of position or time affect standard and test alike. On

a cylinder-plate assay, for example, the doses are commonly distributed in a regular pattern over several plates, and the responses are combined and analysed by the statistical methods the pharmacopoeia prescribes, which estimate the potency and its confidence limits and test the assay for validity (linearity and parallelism of the dose-response lines). The potency of an antibiotic determined in this way is expressed in **International Units (IU)** or in micrograms of the reference standard per milligram, the unit being defined by reference to an international or pharmacopoeial standard preparation held for the purpose.

9.3.1 Microbial assay of antibiotics

The microbial assay of an antibiotic measures its potency by its power to inhibit a sensitive test organism, and is conducted by one of two methods.

The **cylinder-plate (cup-plate) method**, an agar-diffusion method, is the more widely used and is shown in Figure 9.1. A plate of agar is seeded uniformly with a test organism sensitive to the antibiotic. Small cylinders, or cups cut in the agar, are charged with solutions of the antibiotic, namely known concentrations of the reference standard and the test solution, and the plate is incubated. The antibiotic diffuses outward into the agar, and around each cup a clear **zone of inhibition** forms, within which the concentration was sufficient to prevent growth. The diameter of the zone increases with the logarithm of the concentration, so that a calibration line of zone diameter against the logarithm of the standard concentration allows the concentration, and hence the potency, of the test solution to be read from its zone. The same zone-of-inhibition principle was met in the disc-diffusion susceptibility test of Chapter 2, applied here to measurement rather than to screening. The antibiotics assayed microbially include the aminoglycosides such as streptomycin, gentamicin and neomycin, and others for which the biological potency is the property of interest; each is assayed against a test organism sensitive to it and against the corresponding reference standard. The conditions, the test organism, and the standard for each antibiotic are prescribed by the pharmacopoeia, so that an assay performed in any laboratory is comparable with that performed in another.

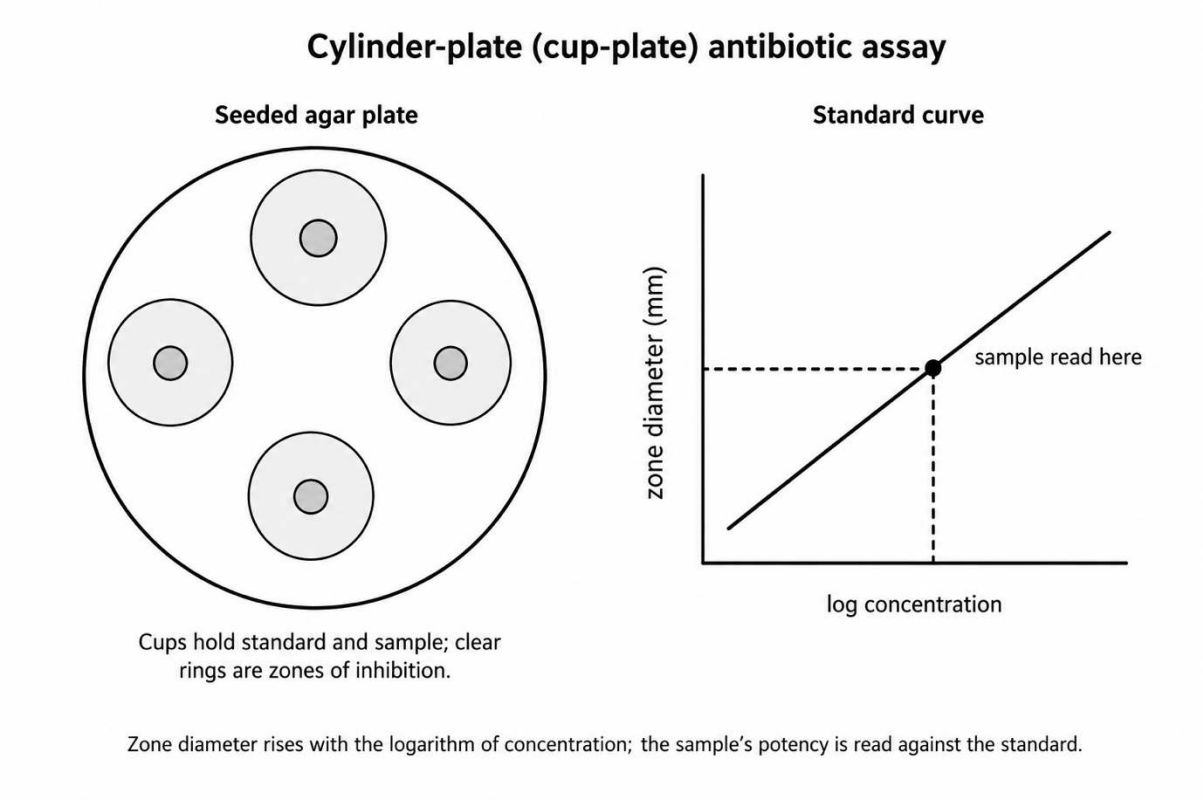


Figure 9.1 *The cylinder-plate antibiotic assay and its standard curve.*

> **Worked illustration.** In a cylinder-plate assay the reference standard at 10 units per millilitre gives a mean zone of 18 mm and at 20 units per millilitre a mean zone of 22 mm, so that a doubling of concentration (an increase of 0.30 in the logarithm) widens the zone by 4 mm. A test solution gives a mean zone of 20 mm. Interpolating on the line of zone diameter against the logarithm of concentration, a 20 mm zone lies midway between the two standard points, corresponding to log concentration midway between log 10 and log 20, that is to a concentration of about 14 units per millilitre. The potency of the test solution is then calculated from this concentration and the dilution made, and is expressed relative to the standard. In practice the reading is made from a fitted line over several doses and replicates, not from two points, but the principle is this interpolation on the log-concentration scale.

The **turbidimetric (tube) method** is more rapid and uses liquid medium. The antibiotic is added, in graded amounts, to tubes of broth seeded with the test organism, and the tubes are incubated. The greater the concentration of antibiotic, the more growth is inhibited and the less the broth becomes turbid; the turbidity of each tube, measured photometrically, is compared with the turbidity produced by known concentrations of the standard to determine the potency of the test solution. The turbidimetric method is faster than the cylinder-plate method, giving a result in a few hours rather than the time required for zones to develop on a plate, but it is more sensitive to any other factor that affects the growth of the organism, such as a trace of an inhibitory substance in the test solution, and it therefore requires careful control and is less robust to a coloured or turbid

test solution that would interfere with the photometric reading. The choice between the two methods rests on these considerations and on the requirement of the particular monograph.

9.3.2 Microbial assay of vitamins and amino acids

For a vitamin or an amino acid the assay is conducted on the opposite principle: instead of an organism that the substance inhibits, it employs an organism that requires the substance for growth. A test organism, commonly a species of *Lactobacillus*, that is unable to synthesise the vitamin or amino acid for itself is grown in a medium complete in every respect except for that one nutrient. The amount of growth obtained then depends on the amount of the nutrient supplied, over a range, so that the growth produced by the test substance, measured as turbidity, is compared with the growth produced by known amounts of the standard to determine the content of the test substance. The assay thus measures the nutrient by the quantity of growth it supports, the mirror image of the antibiotic assay, which measures the inhibitor by the growth it prevents. Particular examples are the assay of riboflavin and of folic acid with a species of *Lactobacillus* that requires the vitamin, and of cyanocobalamin (vitamin B12) with *Lactobacillus leichmannii*; amino acids are assayed in the same way with organisms requiring the particular amino acid. As with the antibiotics, the test organism, the medium deficient in the single nutrient, and the reference standard are prescribed, and the result is obtained by comparison of the growth produced by the test with that produced by the standard. The two types of microbial assay are compared in Table 9.1.

Table 9.1: Types of microbial assay

Feature	Antibiotic assay	Vitamin / amino-acid assay
Property measured	Inhibition of growth	Promotion of growth
Test organism	Sensitive to the antibiotic	Requires the nutrient to grow
Read by	Zone of inhibition (cylinder-plate) or reduced turbidity	Growth (turbidity) produced
Basis	More antibiotic, less growth	More nutrient, more growth

> **Quick recall.** A microbial assay measures biological potency by comparison with a reference standard run in parallel. Antibiotics are assayed by inhibition: the cylinder-plate method reads the zone of inhibition against the logarithm of concentration, the turbidimetric method reads reduced turbidity. Vitamins and amino acids are assayed by promotion of growth in an organism that requires them. Validity requires parallel dose-response lines.

9.3.3 Validity and sources of error

The reliability of a microbial assay depends on the control of the many factors that affect the response of a living test organism. The inoculum must be standardised in number and in

physiological state; the medium must be of constant composition, since traces of the nutrient assayed, or of an inhibitor, distort the result; the temperature and time of incubation must be held constant; and, in the cylinder-plate method, the depth and the setting of the agar and the volume delivered to each cup must be uniform, since these affect the size of the zone. The symmetrical design and the parallel-line analysis are precisely the means of detecting and allowing for such variation: if the test and standard do not give parallel dose-response lines, some factor has affected them unequally and the assay is rejected. It is for these reasons that a biological assay, although less precise than a chemical one, is accepted where it measures the true biological activity that a chemical method cannot, and that its conduct is specified in exact detail by the pharmacopoeia.

9.3a Scope of the microbiological tests

It is useful to set the tests of this chapter against one another and against the sterility test of Chapter 10. The microbial limit tests apply to non-sterile products, establishing that the microbial load is within limits and that objectionable organisms are absent; the sterility test applies to products required to be sterile, establishing the absence of all viable organisms. The microbial assay is a different kind of test altogether, measuring not the contamination of a product but the potency of its active ingredient, and is applied to those antibiotics, vitamins and amino acids whose activity is determined biologically. A given product is subject to whichever of these its nature requires: a non-sterile oral antibiotic syrup, for example, is subject both to the microbial limit tests, for its microbial quality, and to the microbial assay, for the potency of its antibiotic, while a sterile antibiotic injection is subject to the sterility test and the assay. The common thread is that each test verifies, on a sample, a particular attribute of quality that has been built into the product by its manufacture.

9.4 Summary

Microbiological quality control is the final stage of the assurance of quality, confirming by test on a representative sample that a finished product meets its microbiological specification, with the standing limitations that a sample cannot speak with certainty for the whole batch and that testing verifies but does not create quality. For non-sterile products the microbial limit tests determine the total aerobic microbial count and the total yeasts and moulds count against route-based limits, and test for the absence of specified objectionable organisms such as *Escherichia coli*, *Salmonella*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, any antimicrobial substance in the product being neutralised first and the method validated by a suitability test.

Where a substance's activity is biological, it is measured by microbial assay, on the governing principle of comparison with a reference standard run in parallel under identical conditions, with parallel dose-response lines required for validity. An antibiotic is assayed by its inhibition of a sensitive organism, by the cylinder-plate method, in which the zone of inhibition increases with the logarithm of concentration, or by the faster turbidimetric method; a vitamin or amino acid is

assayed by the growth it supports in an organism that requires it. These tests are prescribed in detail by the pharmacopoeia, so that each verifies a defined attribute of quality on a representative sample under controlled conditions, the limit tests and the assay together establishing that a non-sterile product is both microbiologically clean and of the correct potency, just as the sterility test and the assay together establish the same of a sterile one. The next chapter completes the account of quality control with the sterility test, the monitoring of water and air, and the cell-culture techniques on which modern biological products depend.

9.5 Self-assessment

A. Multiple-choice questions

1. The total aerobic microbial count and the total yeasts and moulds count are reported in:

(a) grams per litre (b) colony-forming units per gram or millilitre (c) optical density (d) parts per million. Answer: (b).

2. Before counting a preserved product, the preservative must be:

(a) increased (b) neutralised or removed (c) heated (d) ignored. Answer: (b).

3. An organism that must be absent from a product irrespective of the total count is termed:

(a) an indicator organism (b) a specified (objectionable) organism (c) a commensal (d) a spore-former. Answer: (b).

4. In the cylinder-plate assay of an antibiotic, the diameter of the zone of inhibition increases with:

(a) the square of the concentration (b) the logarithm of the concentration (c) the temperature (d) the agar depth. Answer: (b).

5. The microbial assay of a vitamin uses a test organism that:

(a) is inhibited by the vitamin (b) requires the vitamin for growth (c) produces the vitamin (d) destroys the vitamin. Answer: (b).

6. The governing principle of a biological assay is:

(a) absolute measurement by instrument (b) comparison with a reference standard run in parallel (c) a single concentration only (d) the use of a chemical indicator. Answer: (b).

7. A microbial assay result is invalid if the dose-response lines of test and standard are:

(a) parallel (b) not parallel (c) straight (d) reproducible. Answer: (b).

8. The most probable number (MPN) method is used to enumerate organisms when the expected count is:

(a) very high (b) very low (c) zero (d) unknown. Answer: (b).

9. The potency of an antibiotic determined by microbial assay is expressed in:

(a) colony-forming units (b) International Units or micrograms per milligram (c) parts per million (d) zone diameter only. Answer: (b).

10. Vitamin B12 is assayed microbially using a strain of:

(a) Staphylococcus aureus (b) Lactobacillus (c) Pseudomonas aeruginosa (d) Aspergillus niger. Answer: (b).

11. A microbial assay is laid out in a symmetrical design chiefly to:

(a) save medium (b) allow systematic variation to affect test and standard equally (c) speed the result (d) avoid a standard. Answer: (b).

12. The bacterial endotoxin test (LAL) uses a reagent from the:

(a) rabbit (b) horseshoe crab (c) horse (d) sheep. Answer: (b).

13. A non-sterile oral antibiotic syrup is subject to:

(a) the sterility test only (b) the microbial limit tests only (c) both the microbial limit tests and the microbial assay (d) neither test. Answer: (c).

14. In the cylinder-plate assay, zone diameter is plotted against:

(a) concentration (b) the logarithm of concentration (c) time (d) temperature. Answer: (b).

15. The total yeasts and moulds count is performed on a medium that is:

(a) strongly alkaline (b) acidic or contains an antibiotic to suppress bacteria (c) the same as for bacteria (d) free of nutrients. Answer: (b).

B. Short-answer questions (2 to 5 marks)

Q1. State the two parts of the microbial limit test.

The first part is the enumeration of the total viable count as two figures: the total aerobic microbial count (bacteria) and the total combined yeasts and moulds count (fungi), in CFU per gram or millilitre, compared against the limit for the product category. The second part is the test for specified organisms, in which the product is tested for the absence of named objectionable organisms chosen by the route of use.

Q2. Why must an antimicrobial substance be neutralised before a microbial limit test, and how is the method validated?

A preservative or antibiotic carried into the test would inhibit the organisms and give a falsely low count or a false absence. It is therefore removed by membrane filtration with washing, or neutralised by a specific agent. The method is validated by a method suitability test, in which the product is inoculated with a known number of organisms and shown to permit their recovery.

Q3. Describe the cylinder-plate method for the assay of an antibiotic.

A plate of agar is seeded uniformly with a sensitive organism. Cylinders or cups on the agar are charged with known concentrations of the reference standard and with the test solution. On incubation the antibiotic diffuses out and forms a clear zone of inhibition around each cup, the diameter increasing with the logarithm of the concentration. A calibration line of zone diameter against log concentration of the standard allows the potency of the test solution to be read from its zone.

Q4. How does the microbial assay of a vitamin differ from that of an antibiotic?

An antibiotic is assayed by its inhibition of a sensitive organism: more antibiotic gives less growth. A vitamin is assayed by the growth it supports in an organism that requires it: more vitamin gives more growth. The antibiotic assay measures an inhibitor by the growth it prevents, the vitamin assay a nutrient by the growth it promotes, both by comparison with a reference standard.

Q5. State the limitations of finished-product microbiological testing.

The test is performed on a sample, so it establishes the quality only of the material examined and cannot guarantee every unit of the batch with certainty. And the test verifies quality but does not create it; quality is built in by the control of materials, environment and process, the test being the final confirmation. Both limitations require that good manufacturing control precede and underpin the test, so that the test is relied upon to detect a failure of control rather than to substitute for it.

Q6. How is a sample prepared for the microbial limit test, and why is preparation important?

A representative quantity of product is dissolved or suspended in a sterile diluent, commonly buffered saline with a wetting agent and, where needed, a neutraliser for any preservative; a soluble product is dissolved, an insoluble solid suspended, and an oily product emulsified. The preparation is important because the organisms must be released into a form that can be plated or filtered, without being killed or hidden, and any antimicrobial substance must be neutralised, so that the count reflects the true microbial load.

Q7. What is the bacterial endotoxin (LAL) test, and why is it needed in addition to a test for viable organisms?

The bacterial endotoxin test detects the heat-stable lipopolysaccharide endotoxin of Gram-negative bacteria by the gelation or colour change of a lysate prepared from the blood of the horseshoe crab. It is needed in addition to a test for viable organisms because endotoxin causes fever when injected and survives the death of the organism that produced it, so a parenteral product or Water for Injection may be sterile yet pyrogenic; the test has largely replaced the older rabbit pyrogen test.

Q8. Why is a microbial assay analysed statistically, and what is the requirement of parallelism?

The response of a living test organism varies, so a single measurement is unreliable; the assay is therefore replicated in a symmetrical design and analysed statistically to estimate the potency and its confidence limits. The dose-response lines of the test and the standard must be parallel, because parallelism shows that the test substance behaves as a dilution of the standard, that is, as the same active substance; if the lines are not parallel, some factor has affected them unequally and the assay is invalid.

Q9. Why are the total aerobic microbial count and the total yeasts and moulds count determined separately?

Bacteria and fungi require different media and conditions: bacteria grow best on a general-purpose agar at 30 to 35 degrees Celsius, whereas fungi are favoured by an acidic medium or one containing an antibiotic that suppresses bacteria, incubated at 20 to 25 degrees Celsius. Counting them on separate plates under their own optimal conditions gives a true count of each group, since a single condition would favour one and suppress the other and so misrepresent the load.

C. Long-answer questions (10 marks)

Q1. Describe the microbial limit tests applied to a non-sterile pharmaceutical product.

A complete answer describes the enumeration of the total aerobic microbial count and the total yeasts and moulds count (separate media and conditions, CFU per g or mL, route-based limits) and the test for specified organisms (enrichment then selective and differential culture, confirmation by staining and biochemical tests, the organisms specified by route), and explains the neutralisation of antimicrobial substances and the method suitability test. (High-yield: the microbial limit tests are frequently examined.)

Q2. Explain the microbial assay of antibiotics, describing both methods and the principle of comparison with a standard.

A complete answer states the principle of comparison with a reference standard run in parallel, with parallel dose-response lines for validity, and describes the cylinder-plate method (seeded plate, cups, zone of inhibition increasing with log concentration, calibration line) and the turbidimetric method (graded antibiotic in seeded broth, reduced turbidity measured photometrically), noting the greater speed but greater sensitivity to interference of the latter.

Q3. Compare the microbial assay of an antibiotic with that of a vitamin.

A complete answer states the common principle of comparison with a reference standard in a symmetrical, statistically analysed design, then contrasts the two: the antibiotic is assayed by its inhibition of a sensitive organism (cylinder-plate zone of inhibition increasing with log concentration, or reduced turbidity), so that more antibiotic gives less growth; the vitamin is

assayed by the growth it supports in an organism that requires it (a Lactobacillus in a medium deficient in the vitamin), so that more vitamin gives more growth. The first measures an inhibitor by the growth prevented, the second a nutrient by the growth promoted.

Q4. The reference standard of an antibiotic gives zones of 16 mm at 5 units/mL and 20 mm at 10 units/mL; a test solution gives a zone of 18 mm. Estimate the concentration of the test solution and explain the basis of the estimate.

Zone diameter increases with the logarithm of concentration, and a 18 mm zone lies midway between the two standard zones (16 mm and 20 mm), so it corresponds to a log concentration midway between log 5 and log 10, that is to a concentration of about 7 units/mL (the geometric mean of 5 and 10 is approximately 7.1). The basis is interpolation on the line of zone diameter against log concentration, the standard being run in the same assay so that conditions cancel.

Q5. Set the microbial limit test, the sterility test, and the microbial assay against one another, stating what each measures and to what it applies.

The microbial limit tests apply to non-sterile products and measure the total viable count and the absence of specified organisms, judging microbial cleanliness. The sterility test applies to products required to be sterile and measures the absence of all viable organisms. The microbial assay applies to antibiotics, vitamins and amino acids whose activity is biological, and measures the potency of the active ingredient against a reference standard rather than the contamination of the product. A given product undergoes whichever of these its nature requires, each verifying on a sample an attribute built in by manufacture.

Q6. Discuss the principle and the validity requirements of a biological (microbial) assay.

A complete answer states that a biological assay measures potency by biological effect where chemistry cannot, on the principle of comparison with a reference standard run in parallel under identical conditions, so that interfering factors affect both equally. It explains the symmetrical, multi-dose design and the statistical analysis that estimates the potency and its confidence limits, and the requirement that the dose-response lines of test and standard be linear and parallel, a lack of parallelism rendering the assay invalid because the test substance is then not behaving as a dilution of the standard.

D. Structured note

Write a note on the principle of biological assay.

A biological assay measures the potency of a substance by its biological effect rather than by chemical analysis, and is used where chemistry cannot capture the activity. Its governing principle is comparison: the test substance and a reference standard of known potency are assayed in parallel under identical conditions, so that any factor affecting the test affects the standard equally, and the potency of the test is expressed relative to the standard. Because the response of the living

test system varies, the assay is replicated and analysed statistically, and the dose-response lines of test and standard must be parallel for the result to be valid.

Write a note on the most probable number (MPN) method.

The most probable number method estimates a low microbial count by a statistical, rather than a direct, count. The product is added, in a dilution series, to several tubes of liquid medium at each dilution; after incubation the pattern of tubes showing growth is recorded, and the most probable number of organisms in the original sample is read from a standard statistical table corresponding to that pattern. The method is used when the expected count is too low for a reliable plate count, and gives an estimate with stated probability rather than an exact figure.

Write a note on the test for specified (objectionable) organisms.

The test for specified organisms establishes the absence from a product of named organisms that are unacceptable irrespective of the total count, the list being set by the route of administration (for example Escherichia coli in oral products, and Pseudomonas aeruginosa and Staphylococcus aureus in topical products). The product is grown in a non-selective enrichment broth to allow a small number of the organism to multiply, then subcultured onto a selective and differential medium on which the organism gives a characteristic colony, which is confirmed by Gram staining and biochemical tests. Detection of the organism in the quantity examined is a failure of the product.

Write a note on the limitations of finished-product microbiological testing.

Finished-product testing has two inherent limitations. It is performed on a sample, so it establishes the quality only of the units examined and extends to the batch by inference; a small sample is likely to miss a contamination present in a small proportion of units, so the test confirms but cannot guarantee whole-batch quality. And the test verifies quality but does not create it: quality is built into the product by the control of materials, environment and process, the test being the final confirmation. Both limitations require that good manufacturing control precede and underpin the test, and they are the reason that assurance, especially of sterility, rests on the validated process rather than on the end-product test alone.

References

1. Indian Pharmacopoeia. Microbial limit tests and microbiological assay of antibiotics. Ghaziabad: Indian Pharmacopoeia Commission. [latest edition]
2. Denyer SP, Hodges NA, Gorman SP, editors. Hugo and Russell's Pharmaceutical Microbiology. 8th ed. Oxford: Wiley-Blackwell; 2011.
3. Sandle T. Pharmaceutical Microbiology: Essentials for Quality Assurance and Quality Control. Cambridge: Woodhead Publishing/Elsevier; 2016.
4. Hugo WB, Russell AD. Pharmaceutical Microbiology. Oxford: Blackwell Science.

Chapter 10: Sterility Testing, Environmental Monitoring and Cell Culture

Learning outcomes

After studying this chapter the student will be able to:

- Describe the sterility test by **membrane filtration** and **direct inoculation**, with the media and conditions of the IP, BP and USP.
- Explain the statistical limitation of the sterility test.
- Describe the methods for monitoring water and air quality.
- Describe the general procedure of in vitro cell culture.
- State the applications of cell culture in the pharmaceutical industry and research.

These outcomes serve Course Outcome CO5, the performance of the sterility test in compliance with the pharmacopoeia, and Course Outcome CO1, the role of microorganisms and cultured cells in pharmaceutical research and manufacture.

10.1 Introduction

The sterility test is the pharmacopoeial test that examines a product required to be sterile for the presence of viable microorganisms. It completes the chain of control of sterile products, which begins with clean manufacture (Chapters 3 and 5), proceeds through sterilization (Chapters 7 and 8), and ends with this final examination. Its character is governed by the limitation that applies to all finished-product testing, but with peculiar force: the test is performed on a small sample, yet the property it seeks, the presence of even a single organism, is rare and unevenly distributed, so that a sterility test can never prove a batch sterile, only fail to detect contamination in the units examined. The assurance of sterility therefore rests, as the earlier chapters insisted, on the validated process, and the sterility test is a confirmation of that assurance, not a substitute for it. This chapter describes the sterility test and its limitation, the monitoring of the water and air on which sterile manufacture depends, and, finally, the in vitro culture of animal cells, a technique that has become central to the manufacture of **vaccines** and biological medicines. The three topics are united by the theme of the whole unit: that the microbiological quality of a medicine is assured by a combination of controlled process and confirmatory test, neither sufficient alone, and that the same microbiological discipline now extends from the exclusion of organisms to the deliberate culture of mammalian cells for therapeutic ends.

10.2 The sterility test

For a product required to be sterile, the pharmacopoeias prescribe a sterility test that attempts to cultivate any organism present in the sample. The test is set out in closely harmonised form in the Indian Pharmacopoeia, the British Pharmacopoeia (and European Pharmacopoeia), and the United States Pharmacopeia. Two methods are recognised, and the choice between them depends on the product (Figure 10.1).

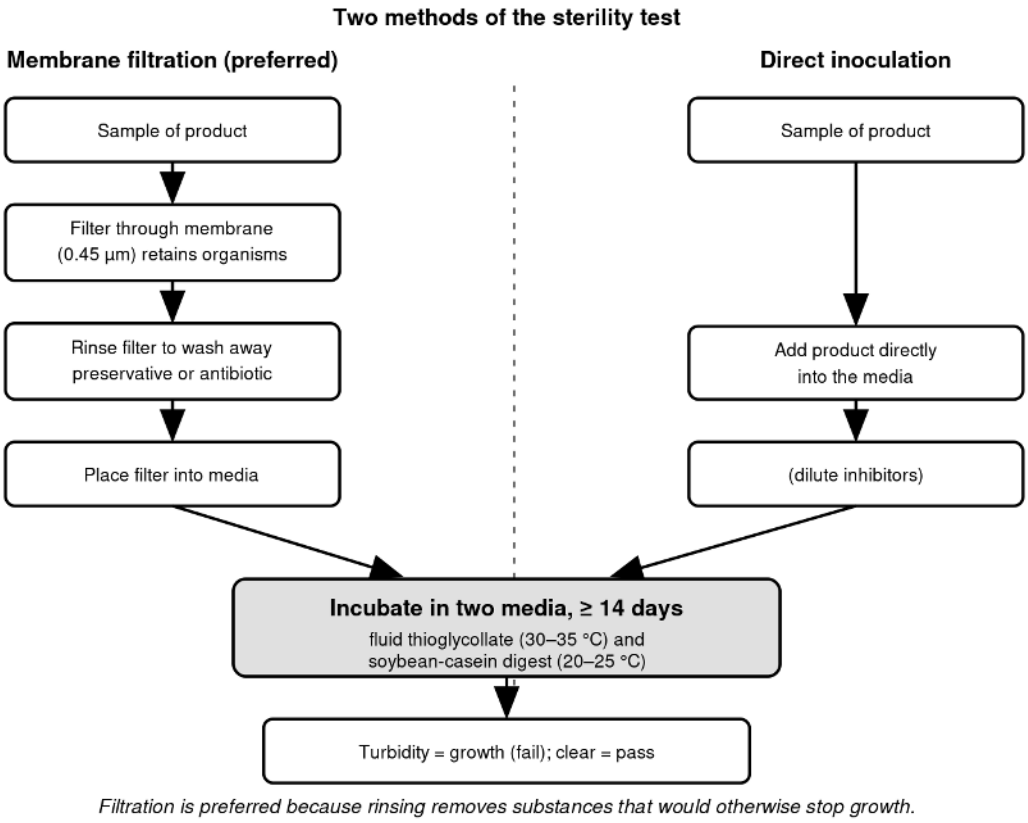


Figure 10.1 *The two methods of the sterility test.*

10.2.1 Membrane filtration

The membrane filtration method is the method of choice wherever the product can be filtered. A measured volume of the product is passed through a sterile membrane filter of pore size 0.45 micrometre, which retains any microorganisms on its surface while the product itself passes through. The filter, and the organisms on it, are then washed to remove any antimicrobial substance carried by the product, and this is the decisive advantage of the method: a preserved or antibiotic product would otherwise inhibit the very growth the test seeks to detect, and washing the filter frees the retained organisms from that inhibition. The washed filter is transferred to culture medium and incubated; commonly the filter is divided, or two filters are used, so that one portion is incubated in each of the two media described below. Because the whole sampled volume is filtered, the method also concentrates onto the filter any organisms from a large volume of product, so that the

sensitivity of the test is increased. The pharmacopoeia prescribes the number of units to be taken from the batch and the quantity of product to be tested from each, both increasing with the batch size and with the volume of the individual container, so that the sample, although small in relation to the batch, is defined and consistent; the larger the sample tested, the greater the chance of detecting a low level of contamination, within the limit discussed below.

10.2.2 Direct inoculation

The direct inoculation method is used when the product cannot be filtered. A measured volume of the product is transferred directly into the culture medium and incubated. Its weakness is the converse of filtration's strength: any antimicrobial substance in the product enters the medium with it and may suppress the growth of an organism present, to give a falsely negative result. The medium volume must therefore be large enough to dilute the antimicrobial substance below an inhibitory concentration, or a specific neutraliser must be included, and the capacity of the method to detect organisms in the presence of the product must be demonstrated by validation.

10.2.3 Media, conditions and interpretation

Both methods employ two culture media, to recover the range of possible contaminants (Table 10.1). **Fluid thioglycollate medium** is incubated at about 30 to 35 degrees Celsius and is designed for the growth of anaerobic and aerobic bacteria; **soybean-casein digest medium** is incubated at about 20 to 25 degrees Celsius and grows fungi and aerobic bacteria. The cultures are incubated for **not less than 14 days** and examined for turbidity, which indicates microbial growth. If no growth appears in either medium, the sample complies with the test. Two media at two temperatures are used so that the range of possible contaminants is covered: the fluid thioglycollate medium, which is partly reduced and incubated warm, recovers anaerobic and aerobic bacteria, while the soybean-casein digest medium, incubated cooler, recovers fungi and aerobic bacteria. The 14-day incubation is required because a small number of organisms, or organisms injured by the manufacturing process, may grow slowly and would be missed by a shorter incubation; the cultures are examined at intervals and finally at the end of the period.

Table 10.1: Media used in the sterility test

Medium	Incubation temperature	Organisms grown
Fluid thioglycollate medium	about 30–35 °C	Anaerobic and aerobic bacteria
Soybean-casein digest medium	about 20–25 °C	Fungi and aerobic bacteria

Both media are incubated for not less than 14 days.

The validity of the test depends on a set of controls. A **negative control** confirms that the media and the technique do not themselves introduce organisms, for a false positive from faulty technique

would wrongly condemn an acceptable batch. A **growth-promotion test** confirms that each medium will support the growth of a small inoculum of test organisms, so that a clear medium signifies the absence of organisms rather than a medium unable to grow them. A **method suitability (validation) test** confirms that the product, after filtration and washing or after dilution, does not prevent the growth of organisms. The test is performed under conditions of the highest cleanliness, in a Grade A environment within a Grade B background or, increasingly, within an isolator, so that organisms from the testing environment are not mistaken for contamination of the product; the use of an isolator is now preferred precisely because it removes the operator and the room as sources of the false positive that is the bane of the test. The integrity of the testing environment is itself monitored, by the methods of Section 10.3, during the test. Where growth occurs, the result is investigated, and a retest is permitted only when the initial growth can be attributed with evidence to a flaw in the conduct of the test rather than to the product.

10.2.4 The limitation of the sterility test

The sterility test is a test of a sample, and its limitation follows from the statistics of sampling. If only a small proportion of the units in a batch is contaminated, that proportion is very likely to be absent from the small sample tested, so that a contaminated batch will frequently pass. For example, if one unit in a thousand is contaminated, and twenty units are tested, the probability that the sample contains at least one contaminated unit is only about two per cent; a batch at this level of contamination would pass the sterility test approximately ninety-eight times in a hundred. The same arithmetic shows why testing more units helps only slowly: at a contamination level of one in a thousand, raising the sample from twenty to forty units only raises the chance of detection from about two to about four per cent, and even at a higher contamination of one per cent, twenty units give only about an eighteen per cent chance of detection. No practical sample size removes this limitation. The sterility test therefore confirms sterility at the level of the sample but cannot establish the sterility of the whole batch, and the assurance of sterility must rest on the validated process, of which the test is the final confirmation. This is the reason that a well-understood process may be released parametrically, on the evidence of the validated cycle, as described in Chapter 8.

> **Quick recall.** The sterility test grows any organism present. Membrane filtration (0.45 µm filter, then washing to remove inhibitory substances) is preferred; direct inoculation is used for non-filterable products, with dilution or neutralisation of any antimicrobial substance. Two media (fluid thioglycollate at 30–35 °C; soybean-casein digest at 20–25 °C), incubated not less than 14 days. The test samples the batch and so confirms, but cannot guarantee, whole-batch sterility.

10.2.5 The sterility test and process assurance

The limitation of the sterility test does not make it useless, but it fixes its proper place. The test will detect a gross failure of sterilization or a major breakdown of aseptic technique, in which a large

proportion of units is contaminated, and it provides a continuing check that the process has not departed grossly from its validated state. What it cannot do is provide, by itself, the assurance that a product is sterile to the level of one non-sterile item in a million; that assurance comes from the design, validation and routine control of the sterilization process described in Chapter 8. The two are complementary: the validated process furnishes the assurance, and the sterility test confirms that nothing gross has gone wrong. It is for this reason that the pharmacopoeias permit parametric release of a terminally sterilized product, the release of the batch on the evidence of the validated physical cycle, when the process is sufficiently understood and controlled, the sterility test then adding little to the assurance the process already gives. For an aseptically filled product, which has no such robust physical record, the sterility test retains a larger role, supported by the rigorous environmental monitoring of the filling.

10.3 Monitoring of water and air

Two materials enter almost every product and cannot be sampled in their entirety: the water with which it is made and the air in which it is made. Both are therefore monitored continuously, since either may pass from an acceptable to an unacceptable state between one check and the next.

10.3.1 Water

The microbiological quality of pharmaceutical water, whose grades and system were described in Chapter 3, is monitored on a defined schedule that covers every point of use in turn. The viable count is determined by filtering or plating a sample onto a low-nutrient medium suited to the slow-growing organisms of a purified-water system, and counting the colonies against an established limit. For Water for Injection the bacterial **endotoxin** is determined in addition, by the **limulus amoebocyte lysate (LAL) test**, in which a reagent prepared from the blood of the horseshoe crab gels or develops colour in the presence of endotoxin; this is necessary because endotoxin survives the death of the organism that produced it, so that water may be sterile and yet pyrogenic. The chemical measures of conductivity and total organic carbon complete the control of the water, but the viable count and the endotoxin are the determinations this subject owns. The sample is taken from each point of use in turn, on a rotating schedule, so that the whole system is covered over time, and it is examined promptly, since organisms may multiply or die in a stored sample. A low-nutrient medium, incubated for several days, is used because the organisms of a purified-water system grow slowly and are easily overgrown or missed on a rich medium. The results are judged against two tiers, an **alert level** that warns of a drift and an **action level** that requires a defined response, so that a problem is corrected while it is still small. A persistent rise prompts investigation and sanitisation of the system, since a water system that has lost control will contaminate every product made with it.

10.3.2 Air

The microbiological quality of the air of a clean area, classified as described in Chapter 5, is monitored as routine surveillance of the working environment. The methods are several and complementary. Settle plates, exposed open for a measured time, collect the organisms that settle from the air under gravity. Active air samplers draw a measured volume of air onto the surface of a culture medium, to give a count of colony-forming units per cubic metre. Surface and contact plates, and swabs, sample the surfaces and the gloved hands of operators. A particle counter measures the non-viable particle concentration that defines the grade of the area. The results are trended against alert and action limits, the monitoring being most intensive in and around the Grade A zone during actual filling, when the environment is most heavily loaded by personnel and activity. The frequency and the limits of air monitoring follow the grade of the area, the Grade A zone being monitored continuously or near-continuously during a fill and held to the strictest limits, the supporting grades less often and less strictly. The area is first qualified by classification, as described in Chapter 5, and then monitored routinely to confirm that it remains in its qualified state. The monitoring of water and air together constitutes the early-warning system of sterile manufacture, detecting a loss of control before it reaches a product; the place of these tests within the whole scheme of microbiological quality control is shown in Figure 10.2.

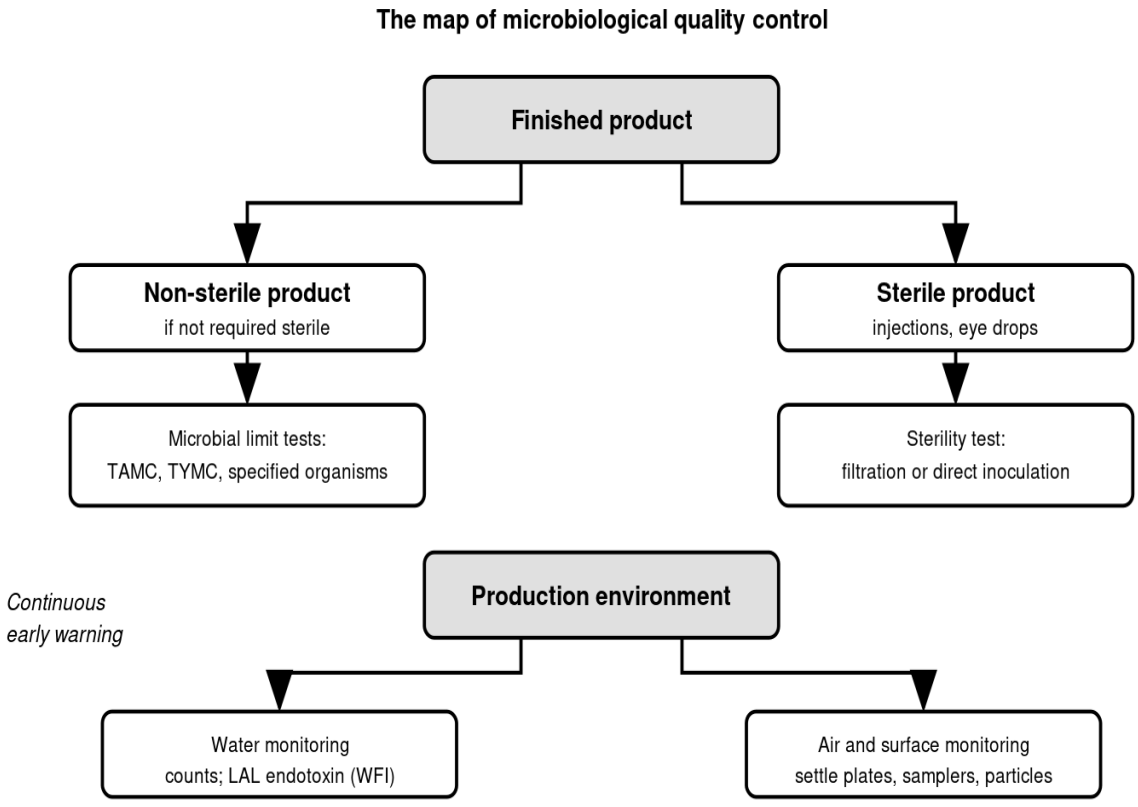


Figure 10.2 *The scheme of microbiological quality control.*

A note on speed is in order. The classical tests of this chapter are slow: the sterility test requires fourteen days and a viable count several days. **Rapid microbiological methods**, which detect growth or cellular markers far sooner, are increasingly adopted and are accepted by the pharmacopoeias once validated, because a result obtained in hours rather than days allows a problem to be corrected while it still matters. The principles are unchanged; only the speed of the answer improves.

10.4 In vitro cell culture

The final topic of the syllabus looks beyond the control of microorganisms to the cultivation of animal and human cells, a technique that has grown from a research tool into a manufacturing platform for medicines that can be made in no other way. **Cell culture** is the growth of cells derived from a multicellular organism outside the body, in a controlled artificial environment.

10.4.1 General procedure

The general procedure is shown in Figure 10.3. The work begins with the establishment of a culture from a tissue. A **primary culture** is obtained directly from a tissue, which is dissociated into its constituent cells, usually by an enzyme, and seeded into a vessel with a nutrient medium. Primary cells resemble the tissue of origin but divide only a limited number of times. A **cell line** is a culture that has been adapted to grow and divide through many generations, in some cases indefinitely, and provides a consistent and renewable supply of cells for repeated work.

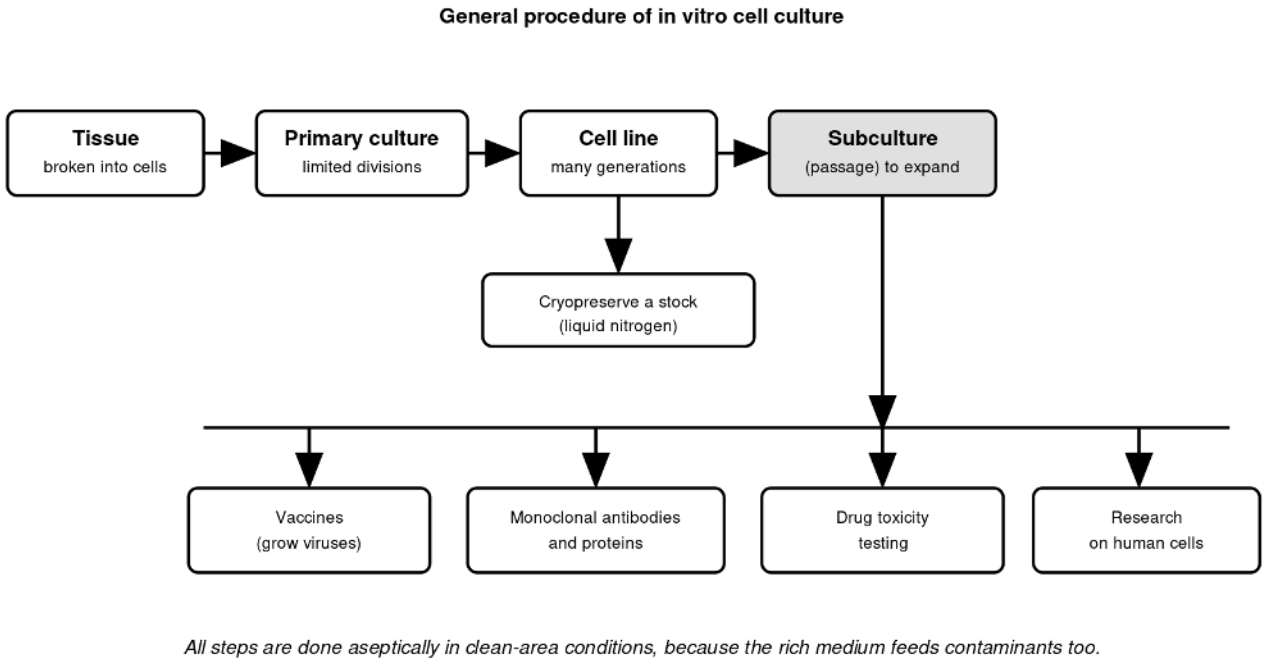


Figure 10.3 *General procedure of in vitro cell culture.*

The cells are grown in a medium that supplies amino acids, vitamins, salts, glucose, and, usually, animal serum as a source of growth factors, and they are maintained at body temperature, about 37 degrees Celsius, in an atmosphere controlled, often with added carbon dioxide, to maintain the pH. The composition of the medium is exact, and chemically defined, serum-free media are increasingly used in manufacture to remove the variability and the risk of contamination that animal serum carries. An antibiotic is sometimes added to the medium to suppress bacterial contamination, but it is no substitute for aseptic technique and may mask a low-level contamination, so its use in a production process is restricted. Because this rich medium is at least as favourable to bacteria and fungi as to the cells, and the cells lack the defences of the living body, the work must be conducted under wholly aseptic conditions, in the laminar-flow and clean-area conditions of Chapter 5; the operator works in a Class 2 biological safety cabinet, which protects both the culture and the worker. As the cells grow and occupy the available surface or volume, they are **subcultured (passaged)**, that is, detached, diluted and reseeded into fresh vessels, so that the population can be expanded. Cells fall into two styles of growth that determine the vessel: anchorage-dependent cells grow attached to a surface as a single layer, whereas suspension cells grow freely in the medium and can be cultured in stirred vessels resembling the fermenters of Chapter 4. Cell lines are further distinguished by their capacity for division: a **finite cell line** divides only a limited number of times before it ages and dies, whereas a **continuous (immortal) cell line**, often derived from a tumour or by transformation, divides indefinitely and so provides an unlimited and uniform supply, at the cost of differing in some respects from the normal cell.

For the manufacture of a medicine the supply of cells must be defined and consistent over the life of the product, and this is achieved by the cell bank system. A single well-characterised culture is expanded and divided into many identical portions that are frozen as the **master cell bank**; from this, further portions are grown and frozen as the **working cell bank**, and it is from the working bank that each production batch is started. The two-tier bank ensures that every batch derives from the same characterised origin and that the supply is secure for many years, while the master bank is held in reserve. The cells of the bank are characterised for their identity, their freedom from contaminants including **mycoplasma** and adventitious viruses, and their stability, and the whole system is conducted under Good Manufacturing Practice when it supports a medicinal product.

Contamination is the constant hazard, and bacteria and fungi announce themselves by clouding the medium; the insidious danger is the mycoplasma, a minute wall-less bacterium that grows without obvious turbidity and may corrupt a culture or a product undetected, so that cultures are tested specifically for it. Valuable cultures are preserved by freezing in liquid nitrogen, a procedure called **cryopreservation**, which banks a stock at a low passage number from which a clean culture can be recovered should the working culture be lost or altered.

10.4.2 Applications

For manufacture, cell culture is scaled up from the flask to large bioreactors, suspension cells being grown in stirred or airlift vessels much like those of a microbial fermentation, and anchorage-dependent cells on microcarrier beads or in special vessels that provide the large surface they require. The control of the bioreactor, of its temperature, pH, dissolved oxygen, and sterility, follows the same principles as the microbial fermentation of Chapter 4, with the added difficulty that animal cells are larger, more fragile, and slower-growing than microorganisms, and so demand gentler agitation and a longer, more vulnerable culture. The applications of cell culture justify the exacting technique it demands. Cell cultures are used to grow viruses for the manufacture of vaccines, since a virus can multiply only within a living cell; the vaccines against poliomyelitis and influenza are produced in this way. They are the production system for many modern biological medicines, including **monoclonal antibodies** and other therapeutic proteins, manufactured by engineered cells grown in large bioreactors. They provide a means of testing the toxicity and the action of new drugs on human cells without the prior use of whole animals, a faster, cheaper and more humane approach, and a means of studying disease in human cells directly. They are used to manufacture other therapeutic proteins, such as hormones and blood factors, by cells engineered to secrete them; they are the basis of the newer cell- and gene-based therapies, in which cells themselves, or genes delivered to cells, are the medicine; and they are used in the production and testing of biological products generally, including the potency and safety testing of vaccines. And they are a fundamental tool of the biological and pharmaceutical research on which all of these rest, allowing the study of the action of drugs, the mechanisms of disease, and the behaviour of human cells under controlled conditions. The microorganism, which began this book as a contaminant to be excluded and became, in fermentation, a producer to be exploited, is thus joined at its close by the cultured human cell as a third kind of living system that the pharmacist must understand, harness and protect.

> **Quick recall.** Cell culture grows animal or human cells outside the body. A primary culture comes directly from tissue and divides a limited number of times; a cell line is adapted to divide for many generations. The cells need a rich serum-containing medium at 37 °C with controlled carbon dioxide, and wholly aseptic handling, since they have no defences; mycoplasma is the insidious contaminant, and stocks are cryopreserved. Applications: vaccine production (growing viruses), monoclonal antibodies and therapeutic proteins, drug toxicity testing, and research.

10.5 Summary

The sterility test examines a product required to be sterile for any viable organism, by membrane filtration through a 0.45 micrometre filter, preferred because washing the filter removes inhibitory substances, or by direct inoculation for non-filterable products, with dilution or neutralisation of

any antimicrobial substance; two media, fluid thioglycollate at 30 to 35 degrees Celsius and soybean-casein digest at 20 to 25 degrees Celsius, are incubated for not less than 14 days, under the controls of negative control, growth promotion, and method suitability, and in an environment of the highest grade. The governing limitation is statistical: the test samples the batch and so can confirm but never guarantee whole-batch sterility, which is the reason sterility is assured by the validated process.

The water and air of sterile manufacture are monitored continuously: water by viable count on a low-nutrient medium and, for Water for Injection, by the LAL test for endotoxin; air by **settle plates**, active samplers, contact plates, and particle counters, all trended against alert and action limits. In vitro cell culture grows animal and human cells outside the body, from primary cultures and cell lines, in a rich serum-containing medium under aseptic conditions, with mycoplasma as the insidious contaminant and cryopreservation as the means of preserving a stock; its applications are the production of vaccines and biological medicines, the testing of drugs, and research. The sterility test, the monitoring of water and air, and the culture of cells thus complete the microbiological quality control of the medicine, the first confirming the absence of organisms, the second guarding the materials and environment that determine it, and the third extending the discipline to the manufacture of biological medicines. With this the syllabus is complete: the book has followed the microorganism from its structure and identification, through its control and its constructive use, to the destruction and the detection that assure the quality of the medicine, and finally to the cultured cell that extends the same microbiological discipline into the manufacture of the biological medicines of the present day.

10.6 Self-assessment

A. Multiple-choice questions

1. The membrane filter used in the sterility test has a pore size of:

(a) 0.22 µm (b) 0.45 µm (c) 1.0 µm (d) 5.0 µm. Answer: (b).

2. Membrane filtration is preferred for a preserved product because:

(a) it is faster (b) washing the filter removes the inhibitory substance (c) it needs no medium (d) it sterilizes the product. Answer: (b).

16. The two media of the sterility test are fluid thioglycollate medium and:

(a) nutrient agar (b) soybean-casein digest medium (c) MacConkey agar (d) blood agar. Answer: (b).

17. The minimum incubation period of the sterility test is:

(a) 24 hours (b) 7 days (c) 14 days (d) 28 days. Answer: (c).

18. Bacterial endotoxin in Water for Injection is detected by the:

(a) Gram stain (b) limulus amoebocyte lysate (LAL) test (c) phenol coefficient (d) Bowie-Dick test. Answer: (b).

19. The insidious contaminant of animal cell cultures, growing without obvious turbidity, is:

(a) Aspergillus (b) mycoplasma (c) Pseudomonas (d) yeast. Answer: (b).

20. Viral vaccines are produced in cell culture because viruses:

(a) are too small to filter (b) can multiply only within living cells (c) grow on nutrient agar (d) are not living. Answer: (b).

21. The medium used to count organisms in a purified-water system is:

(a) a rich nutrient medium (b) a low-nutrient medium incubated for several days (c) fluid thioglycollate medium (d) blood agar. Answer: (b).

22. The supply of cells for the manufacture of a biological medicine is secured by:

(a) a single fresh culture each time (b) a master and working cell-bank system (c) buying cells as needed (d) primary culture only. Answer: (b).

23. A continuous (immortal) cell line, unlike a finite cell line:

(a) divides only a few times (b) divides indefinitely (c) cannot be frozen (d) needs no medium. Answer: (b).

24. Serum-free defined media are increasingly used in manufacture because they:

(a) are cheaper only (b) remove the variability and contamination risk of animal serum (c) need no carbon dioxide (d) require no aseptic technique. Answer: (b).

25. Monitoring of a Grade A filling zone is most intensive:

(a) when the area is empty (b) during the actual fill (c) once a year (d) only at qualification. Answer: (b).

26. Parametric release of a terminally sterilized product is release on the basis of:

(a) the sterility test alone (b) the validated physical record of the sterilization cycle (c) the appearance of the product (d) the bioburden only. Answer: (b).

27. Anchorage-dependent cells are scaled up in a bioreactor by growing them on:

(a) the bare vessel wall (b) microcarrier beads or large-surface vessels (c) the surface of the medium (d) filter paper. Answer: (b).

28. Compared with microorganisms, animal cells in culture are:

(a) smaller and hardier (b) larger, more fragile, and slower-growing (c) identical in requirements (d) free of contamination risk. Answer: (b).

B. Short-answer questions (2 to 5 marks)

Q1. Explain why a finished-product sterility test cannot guarantee that a batch is sterile.

The test examines only a small sample of the batch. If only a small proportion of units is contaminated, that proportion is likely to be absent from the sample, so a contaminated batch frequently passes; for example, with one unit in a thousand contaminated, testing twenty units gives only about a two per cent chance of detecting it. The test therefore confirms sterility only at the level of the sample, and whole-batch sterility must be assured by the validated process.

Q2. Why is membrane filtration preferred to direct inoculation, and what two media are used?

Membrane filtration retains organisms on a 0.45 micrometre filter and allows any antimicrobial substance in the product to be washed away before the filter is cultured, so a preserved or antibiotic product does not inhibit the growth the test seeks; it also concentrates organisms from a large volume. The two media are fluid thioglycollate medium (30 to 35 degrees Celsius, anaerobic and aerobic bacteria) and soybean-casein digest medium (20 to 25 degrees Celsius, fungi and aerobic bacteria), incubated for not less than 14 days.

Q3. How is the microbiological quality of pharmaceutical water monitored?

The viable count is determined on a schedule covering every point of use, by filtering or plating a sample onto a low-nutrient medium and counting against a limit. For Water for Injection the bacterial endotoxin is determined in addition by the LAL test, because endotoxin survives the death of the organism. Results are judged against alert and action levels so that a drift is corrected early, and the water system is kept circulating and sanitised to prevent growth.

Q4. Describe the general procedure of in vitro cell culture.

Cells are obtained from a tissue as a primary culture (dissociated and seeded, dividing a limited number of times) or maintained as a cell line (adapted to divide for many generations). They are grown in a rich medium with amino acids, vitamins, salts, glucose and serum, at about 37 degrees Celsius with controlled carbon dioxide, under wholly aseptic conditions, and are subcultured (passaged) by detaching, diluting and reseeding as they grow. Mycoplasma is the insidious contaminant, and stocks are preserved by cryopreservation in liquid nitrogen.

Q5. State the applications of cell culture in pharmacy.

Cell cultures are used to grow viruses for vaccine manufacture (for example against poliomyelitis and influenza); to produce monoclonal antibodies and other therapeutic proteins by engineered cells in bioreactors; to test the toxicity and action of new drugs on human cells without the prior use of whole animals; and as a fundamental tool of biological and pharmaceutical research.

Q6. What is the cell-bank system, and why is it used in the manufacture of a biological medicine?

The cell-bank system secures a defined and consistent supply of cells over the life of a product. A single characterised culture is expanded and frozen in identical portions as the master cell bank; portions of this are grown and frozen as the working cell bank, from which each production batch is started. It is used because it ensures that every batch derives from the same characterised origin, secures the supply for many years, and allows the cells to be tested for identity, freedom from contaminants, and stability, the master bank being held in reserve.

Q7. Why is the testing environment for the sterility test of the highest grade, and why is an isolator preferred?

A sterility test seeks the rare presence of an organism, so an organism introduced from the testing environment would be mistaken for contamination of the product and would wrongly fail an acceptable batch. The test is therefore conducted in a Grade A environment within a Grade B background, with its own environmental monitoring. An isolator is preferred because it encloses the test completely and removes the operator and the room, the chief sources of the false positive, giving a more reliable result.

Q8. Distinguish a finite cell line from a continuous cell line.

A finite cell line divides only a limited number of times before it ages and dies, and tends to retain the characteristics of the normal cell from which it derived. A continuous (immortal) cell line, often derived from a tumour or by transformation, divides indefinitely and provides an unlimited and uniform supply, but differs in some respects from the normal cell. The choice between them in manufacture balances fidelity to the normal cell against the convenience of an unlimited supply.

Q9. Why may a terminally sterilized product be released parametrically, whereas an aseptically filled product relies more on the sterility test?

A terminally sterilized product has a validated physical sterilization cycle whose delivered lethality can be measured and recorded for each batch, giving direct assurance of an SAL of 10^{-6}; the sterility test adds little to this, so the batch may be released parametrically on the physical record. An aseptically filled product has no such terminal cycle and no equivalent physical record of lethality, so the sterility test, together with rigorous environmental monitoring of the filling, retains a larger part of the assurance, limited though the test is.

Q10. How is cell culture scaled up for the manufacture of a biological medicine?

Suspension cells are grown in large stirred or airlift bioreactors resembling those of a microbial fermentation; anchorage-dependent cells are grown on microcarrier beads or in vessels providing a large surface. The bioreactor controls temperature, pH, dissolved oxygen, and sterility on the same principles as a microbial fermentation, but with gentler agitation and greater care, because animal cells are larger, more fragile, and slower-growing, which makes the long culture more vulnerable to contamination.

Q11. Why are two media incubated at two different temperatures in the sterility test?

The two media at two temperatures together cover the range of possible contaminants. Fluid thioglycollate medium, partly reduced and incubated at 30 to 35 degrees Celsius, recovers anaerobic and aerobic bacteria; soybean-casein digest medium, incubated at 20 to 25 degrees Celsius, recovers fungi and aerobic bacteria. A single medium at one temperature would favour some organisms and miss others, so both are required for a valid test.

Q12. Why is the sterility test incubated for not less than 14 days?

A small number of organisms, or organisms injured and slowed by the manufacturing process, may grow slowly and would not produce visible turbidity within a few days. The 14-day incubation allows such organisms time to multiply to a detectable level, so that a clear medium at the end of the period can be taken to indicate the absence of viable organisms rather than merely their slow growth.

C. Long-answer questions (10 marks)

Q1. Describe the sterility test according to the pharmacopoeia, with its methods, media, conditions and limitations.

A complete answer describes membrane filtration (0.45 micrometre filter, washing to remove inhibitory substances, preferred for filterable and preserved products) and direct inoculation (for non-filterable products, with dilution or neutralisation), names the two media (fluid thioglycollate at 30 to 35 degrees Celsius; soybean-casein digest at 20 to 25 degrees Celsius) and the incubation of not less than 14 days, describes the negative control, growth-promotion, and method-suitability controls and the Grade A environment, and discusses the statistical limitation that the test samples the batch and cannot guarantee whole-batch sterility. (High-yield: sterility testing is among the most frequently examined topics.)

Q2. Describe in vitro cell culture and its applications in the pharmaceutical industry and research.

A complete answer defines cell culture, distinguishes primary cultures from cell lines and anchorage-dependent from suspension cells, describes the medium and conditions and the necessity of aseptic technique, notes mycoplasma contamination and cryopreservation, and sets out the applications: vaccine production by growing viruses, the manufacture of monoclonal antibodies and therapeutic proteins, drug toxicity testing, and research.

Q3. Describe the monitoring of water and air quality in sterile manufacture.

A complete answer describes water monitoring by viable count on a low-nutrient medium, sampled from each point of use on a schedule, with the LAL endotoxin test in addition for Water for Injection, judged against alert and action levels; and air monitoring by settle plates, **active air samplers** (CFU per cubic metre), contact plates and swabs for surfaces and gloved hands, and a

particle counter for the non-viable count, trended against alert and action limits, most intensively in the Grade A zone during filling. It notes that the two together form the early-warning system of sterile manufacture.

Q4. Give an account of in vitro cell culture and its applications.

A complete answer defines cell culture, distinguishes primary cultures, finite cell lines, and continuous cell lines, and anchorage-dependent from suspension growth, describes the medium and conditions, the necessity of aseptic technique, the hazard of mycoplasma, the cell-bank system, and cryopreservation, and sets out the applications: the production of viral vaccines, monoclonal antibodies and therapeutic proteins, cell- and gene-based therapies, drug toxicity testing, and research. (High-yield: in vitro cell culture and its applications are frequently examined.)

Q13. State the controls that ensure the validity of the sterility test.

A negative control confirms that the media and technique do not themselves introduce organisms, so that a positive result is not a false one from faulty technique. A growth-promotion test confirms that each medium will support a small inoculum, so that a clear medium means absence of organisms and not an inadequate medium. A method suitability test confirms that the product, after filtration and washing or after dilution, does not inhibit growth. The test is conducted in a Grade A environment, increasingly an isolator, with its own monitoring, so that environmental organisms are not mistaken for product contamination.

D. Structured note

Write a note on the monitoring of air quality in a clean area.

The air of a clean area is monitored as routine surveillance by several complementary methods: settle plates exposed for a measured time collect organisms falling under gravity; active air samplers draw a measured volume of air onto a medium to give a count per cubic metre; contact plates and swabs sample surfaces and gloved hands; and a particle counter measures the non-viable particle concentration that defines the grade. The results are trended against alert and action limits, most intensively in and around the Grade A zone during filling, so that a loss of control is detected before it reaches a product.

Write a note on mycoplasma contamination of cell cultures.

Mycoplasma are minute, wall-less bacteria that are a particularly insidious contaminant of animal cell cultures, because they grow without producing the obvious turbidity by which bacterial and fungal contamination is recognised, and so may persist undetected while altering the behaviour of the cells. They can corrupt an experiment or a product without warning, and being wall-less they are unaffected by the cell-wall-active antibiotics sometimes added to media. Cultures are therefore tested specifically for mycoplasma, and the cells of a manufacturing cell bank are certified free of it as part of their characterisation.

Write a note on rapid microbiological methods.

Rapid microbiological methods are newer techniques that detect microbial growth or cellular markers far sooner than the classical tests, which are slow (the sterility test takes 14 days and a viable count several days). They include methods based on the detection of cellular adenosine triphosphate, on the measurement of carbon dioxide or other products of growth, and on the staining and automated counting of viable cells. The pharmacopoeias accept such methods once they are validated as equivalent to the classical test. Their value is that a result obtained in hours rather than days allows a problem to be detected and corrected while it still matters, without any change in the underlying principles.

References

1. Indian Pharmacopoeia. Sterility testing. Ghaziabad: Indian Pharmacopoeia Commission. [latest edition]
2. Denyer SP, Hodges NA, Gorman SP, editors. Hugo and Russell's Pharmaceutical Microbiology. 8th ed. Oxford: Wiley-Blackwell; 2011.
3. Sandle T. Pharmaceutical Microbiology: Essentials for Quality Assurance and Quality Control. Cambridge: Woodhead Publishing/Elsevier; 2016.
4. Freshney RI. Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications. Hoboken (NJ): Wiley-Blackwell.

Glossary

Aseptic processing. Manufacture by combining separately sterilized components under conditions clean enough that no organism is added; used when terminal sterilization is impossible.

Bioburden. The number of viable organisms on or in a product before sterilization.

Colony-forming unit (CFU). The unit of a viable count, taken to arise from one viable cell or clump.

D-value (decimal reduction time). The time, at a stated temperature, to reduce a population by 90 per cent (one log).

z-value. The temperature rise that reduces the D-value tenfold; about 10 degrees Celsius for steam.

F0. The lethality of a moist-heat cycle as equivalent minutes at 121 degrees Celsius (z = 10).

Disinfectant / antiseptic / preservative. Antimicrobial agents for, respectively, inanimate surfaces, living tissue, and the interior of a product.

Endotoxin. Heat-stable lipopolysaccharide of Gram-negative cell walls; pyrogenic when injected.

Gram stain. A differential stain dividing bacteria into Gram-positive and Gram-negative by cell-wall structure.

HEPA filter. A high-efficiency particulate air filter removing essentially all airborne particles.

IMViC. The Indole, Methyl-red, Voges-Proskauer, and Citrate tests for enteric bacteria.

Laminar air flow. A unidirectional stream of HEPA-filtered air that sweeps contamination from a critical zone.

MIC / MBC. The lowest concentration of an agent that inhibits growth (MIC) or kills the organism (MBC).

Peptidoglycan. The cell-wall polymer whose thickness underlies the Gram reaction.

Sterility assurance level (SAL). The probability that a sterilized item carries a viable organism; 10 to the minus 6 is standard.

TAMC / TYMC. Total aerobic microbial count and total combined yeasts and moulds count.

Water activity (aw). The water available to microorganisms, on a scale where pure water is 1.0.

Consolidated formula sheet

Survivor count: N = N0 x 10^(-t/D)

Logarithmic reductions: t / D = F0 / D121

D-value at temperature T: D_T = D_ref x 10^((T_ref - T) / z)

Lethal rate (moist heat, ref 121 C, z = 10): L = 10^((T - 121) / z)

Accumulated lethality: F0 = sum of (L x time interval)

Logarithmic reductions to an SAL: log(N0) - log(SAL)

Phenol coefficient = (killing dilution of disinfectant) / (killing dilution of phenol)

Plate count = colonies x dilution factor / volume plated (CFU per mL or g)

Generation time: g = t / n, where 2^n = N / N0

Abbreviations

BSL - Biological safety level

CFU - Colony-forming unit

EO - Ethylene oxide

GMP - Good Manufacturing Practice

HEPA - High-efficiency particulate air

IP / BP / USP - Indian / British / United States Pharmacopoeia

LAF - Laminar air flow

LAL - Limulus amoebocyte lysate

MBC - Minimum bactericidal concentration

MIC - Minimum inhibitory concentration

MPN - Most probable number

SAL - Sterility assurance level

TAMC - Total aerobic microbial count

TYMC - Total combined yeasts and moulds count

WFI - Water for injection

Consolidated references

1. Pelczar MJ, Chan ECS, Krieg NR. Microbiology: Concepts and Applications. New York: McGraw-Hill.

2. Prescott LM, Harley JP, Klein DA. Microbiology. New York: McGraw-Hill.

3. Hugo WB, Russell AD. Pharmaceutical Microbiology. Oxford: Blackwell Science.

4. Waites MJ, Morgan NL, Rockey JS, Higton G. Industrial Microbiology: An Introduction. Oxford: Blackwell Science; 2001.

5. Denyer SP, Hodges NA, Gorman SP, editors. Hugo and Russell's Pharmaceutical Microbiology. 8th ed. Oxford: Wiley-Blackwell; 2011.

6. Sandle T. Pharmaceutical Microbiology: Essentials for Quality Assurance and Quality Control. Cambridge: Woodhead Publishing/Elsevier; 2016.

7. Sutton SVW. Microbiology in Pharmaceutical Manufacturing. Bethesda: PDA/DHI Publishing.

8. Indian Pharmacopoeia. Ghaziabad: Indian Pharmacopoeia Commission. [latest edition]

9. British Pharmacopoeia. London: The Stationery Office. [latest edition]

10. United States Pharmacopeia (USP-NF). Rockville (MD): USP. [latest edition]

11. The Drugs and Cosmetics Act 1940 and Rules 1945, Schedule M. Government of India. [latest amendment]