FUNDAMENTALS OF MICROBIOLOGY

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Lesson 1
HISTORICAL PERSPECTIVE OF MICROBIOLOGY

1.1 Introduction

The history of microbiology can be traced to 'First Epidemics' on earth, which affected Caveman and were probably waterborne. People had no real understanding of why disease occurred. As the civilization progressed, people started clustering into cities. They increasingly shared communal water, handled unwashed food, and stepped in excrement from casual discharge. The crowding increased and spread water-borne, insect-borne and skin-to-skin infectious diseases. Yet there was no general understanding of why disease occurred. By the 13th century fear of the diseased took a drastic turn in the formation of small leper colonies intended to isolate people carrying the devastating disease caused by *Mycobacterium leprae*. In 1348, a mass epidemic caused by a single organism, *Yersinia pestis*, wiped out nearly one third of Europe's population. The Plague spread rapidly in the unsanitary conditions of the Middle Ages, leaving Medieval Europeans defenseless against its devastation. Entire towns succumbed to the disease, leaving the living to dispose of thousands of contaminated corpses. Perhaps the deadliest disease in history, the plague or 'Black Death', claimed over 20 million lives and contributed to the fall of empires.

1.1.1 Bubonic plague

The social and political dislocations as a result of Bubonic plague were immense, and the resulting terror was particularly intense because the cause of the disaster was unknown. Not until 500 years later, in 1890, did microbiologists identify the causative organism,*Yersinia pestis*, carried by infected fleas. Infection spreads among the rats as fleas bite them. But rats have little resistance to plague and usually die. When rats become scarce, the fleas move to humans and a plague epidemic is under way. Although rare today, Bubonic plague still occurs in parts of the world.

1.1.2 Potato blight

The Irish famine Potato blight, a disease of plants, rather than humans, caused by a fungus rather than a bacterium, had even greater impact than the plague. Potato blight was responsible for the great Irish famine of the 1800s. Potatoes were the staple of the Irish diet, so when the fungus *Phytophthora infestans* infected potatoes, causing them to rot in the fields, the result was devastating. By 1846, the potato harvest was so meager that starvation and hunger-based diseases were widespread. An estimated 1,240,000 people had died, and 1,200,000 more had migrated to other countries. Potato blight remains an economic threat to potato farmers even today.

1.2 Disease and Warfare

Warfare and infectious diseases have always been intimately connected. The poor sanitation, movement of peoples, and malnutrition that a war brings, all foster outbreaks of disease. For example, in 1812 when Napoleon invaded Russia he lost more of his troops to typhus (a bacterial

disease) than to all other causes combined. And more of his soldiers died from wound-related bacterial infections (such as tetanus and gas gangrene) than from the wound itself.

1.3 Optical Visual Aids

The first vision aid was invented (inventor unknown- possibly a monk) called a reading stone around 1000 AD. It was a glass sphere that magnified when laid on top of reading materials. In the year 1590, two Dutch eye-glass makers, Zaccharias Janssen and son Hans Janssen experimented with multiple lenses placed in a tube. The Janssens observed that viewed objects in front of the tube appeared greatly enlarged, creating both the forerunner of the compound microscope and the telescope.

1.3.1 Microscopy

Robert Hooke (1635-1703): Robert Hooke, a young English scientist, became the first person to view and describe fungi using a simple compound microscope. In 1665, Hooke published Micrographia, which detailed his observations of tiny cork-like cells resembling 'little boxes'. Hooke is known principally for his law of elasticity (Hooke's Law) and for his work as 'the father of microscopy' - it was Hooke who coined the term 'cell' to describe the basic unit of life.

Antony van Leeuwenhoek (1632-1723): Antony van Leeuwenhoek, a Dutch merchant, made small hand-held microscopes as a hobby. Squinting through the lens at specimens held on a pin, he discovered a world of invisible creatures he called animalcules (small animals). He found them almost everywhere he looked: in water droplets, particles of soil and his teeth scrapings. In 1674, Leeuwenhoek communicated his discoveries to the Royal Society of London, sending detailed drawings. His primary observations shook the scientific community and led to expanded use of microscopy as a standard scientific tool (Fig. 1.1). All his drawings and nine of the estimated 500 microscopes that Leeuwenhoek made still exist. The most powerful of these has a magnification of 266, powerful enough to magnify an average-sized bacterial cell to the size of the period at the end of this sentence.

Fig. 1.1 Leeuwenhoek's microscope

1.4 Spontaneous Generation

The idea that life routinely arises from non-life was supported by Aristotle (Circa 350 BC). According to him, it was: "readily observable that aphids arise from the dew which falls on plants, fleas from putrid matter, mice from dirty hay". This belief remained unchallenged for more than 2000 years. From ancient times, spontaneous generation was thought to be the origin of many organisms (such as rats and flies) that routinely appeared in certain materials. Microorganisms always appeared suddenly in certain materials (meat juices or plant extracts, for example) that had previously been free of them. It seemed logical that microbes were products of spontaneous generation (the formation of living things from inanimate matter).

Needham versus Spallanzani: For 80 years the above debate continued. Then the proponents of spontaneous generation seemed to gain ground when in 1745 an English clergyman named John Needham did a well-publicized experiment. Everyone knew boiling killed microorganisms. Therefore, he boiled chicken broth, put it in a flask, and sealed it. Microorganisms could develop in it only by spontaneous generation. Experiments with gravy seemed to show that life could be generated from non-living materials.

But an Italian priest and professor named Lazzaro Spallanzani was not convinced. According to him, perhaps microorganisms entered the broth after boiling but before sealing. Therefore, Spallanzani put broth in a flask, sealed it, and then boiled it. No microorganisms appeared in the cooled broth. Still the critics were not persuaded. Spallanzani didn't disprove spontaneous generation, they said, he just proved that spontaneous generation required air.

Gradually, spontaneous generation was rejected as the origin of visible organisms. Francesco Redi, a physician in Italy, played a major role. In 1665 he did experiments with covered and uncovered jars of meat. He showed that maggots (fly larvae) developed only in meat that flies could reach to lay eggs on. Apparently, spontaneous generation did not occur, at least in the case of flies. Instead, flies and by extension all living things come only from preexisting living things. Still, many people believed that microorganisms were an exception to this rule. They are very simple and they always appear, in large numbers, soon after a plant or animal dies. Could decomposition form microorganisms instead of microorganisms causing decomposition? Controversy of spontaneous generation gained momentum during the late 18th and 19th centuries, when further advances in microscopy allowed people to view bacteria and other microorganisms.

1.5 Pasteur's Experiments

Remarkably, the controversy continued another 100 years and became a significant barrier to the development of microbiology as a science. Finally, in 1859 the French Academy of Science sponsored a competition to prove or disprove the theory of spontaneous generation of microbes. A young French chemist named Louis Pasteur (1822-1895) entered to counter the argument that air was necessary for spontaneous generation. Pasteur used barriers that allowed free passage of air but not microorganisms. In his most famous experiment, Pasteur boiled meat broth in a flask and then drew out and curved the neck of the flask in a flame in the shape of a swan's neck (Fig. 1.2). No microorganisms grew in the flask. But when he tilted the flask so some broth flowed into the curved neck and then tilted it back so the broth was returned to the base of the flask, the broth quickly became cloudy with the growth of microbial cells. Gravity had caused the microbial cells that had entered the flask to settle at the low point of the neck. They never reached the broth in the base until

they were washed into it. Thus, Pasteur convinced the scientific world that spontaneous generation of microorganisms does not occur even in the presence of air. Pasteur's simple but elegant experiments grounded microbiology in scientific reality. Microorganisms could now be studied by rational scientific means. Probably the most famous contribution to microbiology by Pasteur is the heating process he developed to kill spoilage microbes while still preserving flavor.
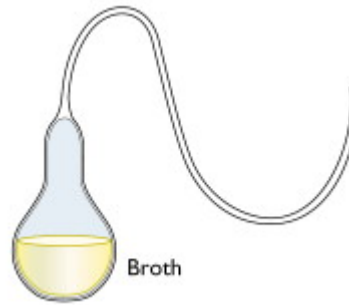
Fig. 1.2 Pasteur's swan-neck flask

1.6 Fermentation

Another important contribution of Pasteur was defining fermentation. In 1856, the father of one of Pasteur's chemistry students asked him to help him solve some problems he was encountering in his attempt to make alcohol by fermenting beetroot. Often, instead of alcohol, the fermentations yielded lactic acid. At the time, fermentation was believed to be a pure chemical process in which sugar was transformed into alcohol. But in 1857, Pasteur proved that a microscopic plant caused the souring of milk (lactic acid fermentation). Pasteur was able to prove that living cells, the yeast, were responsible for forming alcohol from sugar, and that contaminating microorganisms found in ordinary air could turn the ferments sour. Next, he identified the microorganisms responsible for both normal and abnormal fermentations, and found that through heating wine, beer, milk, or vinegar briefly, certain living organisms could be killed, thereby sterilizing or 'pasteurizing' the substances. He reported his findings in "Mémoire sur la fermentation appelée lactique" (Memoir on the fermentation of lactic acid) in 1857, and "Mémoire sur la fermentation alcoholique" (Memoir on the fermentation of alcohol) in 1860. Pasteur also made notable contributions in the field of vaccination and immunity. Studying cholera, Pasteur found that attenuated organisms, inoculated into poultry, offered protection against virulent strains. Based on this research, he developed the first rabies and anthrax vaccines.

1.7 Immunology

Edward Anthony Jenner (1749-1823) was an English scientist who studied his natural surroundings in Berkeley, Gloucestershire. Jenner is widely credited as the pioneer of smallpox vaccine, and is sometimes referred to as the 'Father of Immunology'. Though Pasteur's achievements in microbial immunity were revolutionary, Jenner is credited with inventing the first vaccine against smallpox in late 1700s. In 1796, Jenner developed a controversial experiment to determine the validity of rumours that were circulating in rural communities. Milkmaids and villagers often recanted, "if you want to marry a woman who will never be scarred by the pox, marry a milkmaid." Jenner speculated that becoming infected with cowpox could offer protection against the more virulent smallpox. To test his hypothesis, he created an inoculation with scrapings of cowpox lesions from the fingers of

Sarah Nelmes, a young milkmaid and injected it into an 8-year old boy named James Phipps. As expected, James developed the mild fever and cowpox lesions typical of the disease. After a few weeks of recovery, Jenner injected James with the live smallpox virus and found that the boy was indeed protected from the disease. In 1798, Jenner published his findings and presented them to the Royal Society.

1.8 Germ Theory of Disease

Once spontaneous generation of microbes was disproved, microbiology exploded. It changed from an observational science to an experimental science. The way was opened to study the cause of infectious diseases. Building on Pasteur's work, a German physician, Robert Koch, proved that microorganisms (germs, as they were and are still sometimes called) cause disease. He showed further that specific microorganisms cause specific diseases. Koch also introduced higher scientific standards of rigor to microbiology, as exemplified by those called Koch's postulates.

1.8.1 Koch's postulates

In 1876 Robert Koch while studying anthrax, a disease of cattle and sheep that also affects humans established "scientific rules" to show a cause and effect relationship between a microbe and a disease known as Koch'r postulates as follows:

- The same organisms must be found in all cases of a given disease.
- The organism must be isolated and grown in pure culture.
- The isolated organism must reproduce the same disease when inoculated into a healthy susceptible animal.
- The original organism must again be isolated from the experimentally infected animal.

During his work on anthrax, Koch made another critically important contribution to microbiology. He developed a technique to obtain a pure culture of a bacterium (one that contains only a single kind of bacteria) and propagate it. In nature many kinds of bacteria are found growing together (mixed cultures). It is difficult to do valid experiments using mixed cultures because they are so complicated, although such studies are now becoming more common.

1.9 Antiseptic Surgery

Joseph Lister (1827-1912), a British surgeon was the pioneer of antiseptic surgery, who promoted the idea of sterile surgery while working at the Glasgow Royal Infirmary. Lister successfully introduced carbolic acid to sterilise surgical instruments and to clean wounds, which led to reduced post-operative infections and made surgery safer for patients.

1.10 Sterilization

In 1877, John Tyndall published his method for fractional sterilization and clarified the role of heat resistant factors (spores) in putrefaction. Tyndall's conclusion added a final footnote to the work of Pasteur and others in proving that spontaneous generation is impossible. For some of his experiments on light and gases he needed to cleanse the air of particles and so developed a completely novel way to assay the purity of air. 'The eye being kept sensitive by darkness', Tyndall

reported, 'a concentrated beam of light was found to be a most searching test for suspended matter – a test indeed indefinitely more searching than that furnished by the most powerful microscope'. Using his new piece of equipment, which relied on observing light scattered by dust particles, Tyndall quickly made a series of important observations on the properties of the 'floating matter' of air.

1.11 Milk Fermentation

In 1878, Joseph Lister published his study of lactic fermentation of milk, demonstrating the specific cause of milk souring. His research was conducted using the first method developed for isolating a pure culture of a bacterium, which he named Bacterium lactis.

1.12 Virology

Virology, the study of viruses, began in 1892, when the Russian microbiologist Dmitri Iwanowski discovered the tobacco mosaic virus. Iwanowski was studying a disease of tobacco plants called tobacco mosaic disease. To identify its cause, he forced juice from diseased plants through filters that retained the smallest bacteria. He found the filtered juice still caused disease. Because bacteria were believed to be the smallest microorganisms, Iwanowski first thought his methodology might be flawed. But repeated experimentation convinced him that minute disease-causing agents were passing through the filter. He called these tiny agents 'filterable viruses'. They could not be seen, even under the most powerful microscopes of that time. Until the electron microscope was developed in the 1930s, we knew viruses existed.

1.12.1 Bacteriophage

In 1915, the first discovery of bacteriophage was done by Frederick Twort. Twort's discovery was something of an accident. He had spent several years growing viruses and noticed that the bacteria infecting his plates became transparent. Later on in 1917 Felix d'Herrelle independently described bacterial viruses and coined the name 'bacteriophage'. In 1926, Thomas Rivers distinguished between bacteria and viruses, establishing virology as a separate area of study.

Lesson 2
FOOD, INDUSTRIAL AND ENVIRONMENTAL MICROBIOLOGY

2.1 Food Microbiology

Food microbiology encompasses the study of microorganisms which have both beneficial and deleterious effects on the quality and safety of foods. It focuses on the general biology of the microorganisms that are found in foods including: their growth characteristics, identification, and pathogenesis. Specifically, areas of interest which concern food microbiology are: food poisoning, food spoilage, , and food legislation. Pathogens in product, or harmful microorganisms, result in major public health problems worldwide and are the leading causes of illnesses and death. In the United States alone, food borne illness has been estimated to cause 5,000 deaths and 76 million illnesses per year.

2.2 Factors affecting Microbial Growth in Food

There are broadly two types of factors that affect the growth of microorganisms in food products: intrinsic and extrinsic. The intrinsic parameters are properties that exist as part of the food product itself such as pH, moisture content (water activity) ,Oxidation-reduction potential, nutrient content, antimicrobial constituents and biological structures of food. On the other hand, extrinsic parameters are those properties of the environment (processing and storage) that exist outside of the food product and, may affect both the foods and their microorganisms. These include storage temperature of food, relative humidity, presence/concentration of gases and presence/activities of other microorganisms.

2.3 Sources of Microorganisms in Food

Bacteria can be found virtually everywhere including humans and can enter food products through different routes. Some of the most common ways in which microorganisms enter food products are as follows:

- Soil, water, and in-plant environment
- Animal feeds
- Animal hides
- Gastrointestinal tract
- Food handlers
- Food Utensils
- Air and dust
- Vegetables (plant) and vegetable products
- Globalization of food supply
- Terrorist attacks

2.4 Foodborne Illness

Microorganisms can cause a variety of effects in food products including spoilage, which primarily affects product quality, and food poisoning, which is generally caused by pathogens. A foodborne

illness (or disease) is exactly what the term indicates - a disease or illness caused by the consumption of contaminated foods or beverages. Foodborne diseases are primarily of two primary types: food-borne infections and food intoxications. While the former takes place due to ingestion of microbes, followed by growth, tissue invasion, and/or release of toxins, the latter is caused as a result of ingestion of toxins in foods in which microbes have grown. More than 250 different foodborne diseases have been described. Most of these diseases are infections, caused by a variety of bacteria, viruses, and parasites. Other diseases are poisonings, caused by harmful toxins or chemicals that have contaminated the food, for example, poisonous mushrooms or heavy metal contamination.

2.5 Food Spoilage

Spoilage organisms alter food which results in changes in texture, appearance and organoleptic qualities of the food, making it unsuitable for human consumption. Spoilage is often the result of a succession. One organism creates an environment conducive to the growth of another. Common microbial food spoilage are:

I. Putrefaction- Protein + proteolytic micro organism's — amino acids + amines + ammonia + H_2S

II. Fermentation- Carbohydrates + fermenting microrganism's —acids + alcohols + gases

III. Rancidity- Fatty foods + lypolytic micro organism's — fatty acids + glycerol

2.6 Fermented Foods

These are the foods that have been subjected to the action of micro-organisms or enzymes, in order to bring about a desirable change. Numerous food products owe their production and characteristics to the fermentative activities of microorganisms. Fermented foods originated many thousands of years ago when presumably microorganism contaminated local foods. Fermentation is most common method of preservation. Furthermore, microbial fermentation can increase nutritional quality and digestibility of food while producing desirable textures and flavours (organoleptic properties). Fermentation, like spoilage, is dependent on microbial succession. The physical and chemical nature of the food determines fermentation organisms and inhibits unwanted microbes. Microbes involved are lactobacilli (lactic acid bacteria), acetic acid bacteria, yeasts and occasionally mycelial fungi. Early procedures used 'backslop' method. They retained some material from a previous fermentation and added it to a fresh batch of ingredients. Today, "starter cultures (collection of well identified and characterized microorganisms which initiate fermentation) are extensively used in the dairy industry. Some examples of fermented foods are as follows:

- Dairy Products- Buttermilk, Sour cream, Yoghurt, Cheese
- Meat Products: Many European sausages, Cured ham, Salami
- Fish products: Mainly Far East
- Vegetables and Cereal Fermentation Products: Wine, Bread, Sourdough, Tofu, Pickles, Silages

2.7 Food Preservation

It is the process of treating and handling food to stop or greatly slow down spoilage (loss of quality, edibility or nutritive value) caused or accelerated by microorganisms. Some methods, however, use benign bacteria, yeasts or fungi to add specific qualities and to preserve food (e.g., cheese, wine). Maintaining or creating nutritional value, texture and flavour is important in preserving its value as food. Preservation usually involves preventing the growth of bacteria, fungi, and other microorganisms, as well as retarding the oxidation of fats which cause rancidity. It also includes processes to inhibit natural ageing and discolouration that can occur during food preparation such as the enzymatic browning reaction in apples after they are cut. Some preservation methods require the food to be sealed after treatment to prevent recontamination with microbes; others, such as drying, allow food to be stored without any special containment for long periods.

Common methods of applying these processes include drying, spray drying, freeze drying, freezing, vacuum packing, canning, preserving in syrup, sugar crystallization, food irradiation, and adding preservatives or inert gases such as carbon dioxide. Other methods that not only help to preserve food, but also add flavour, include pickling, salting, smoking, preserving in syrup or alcohol, sugar crystallization and curing.
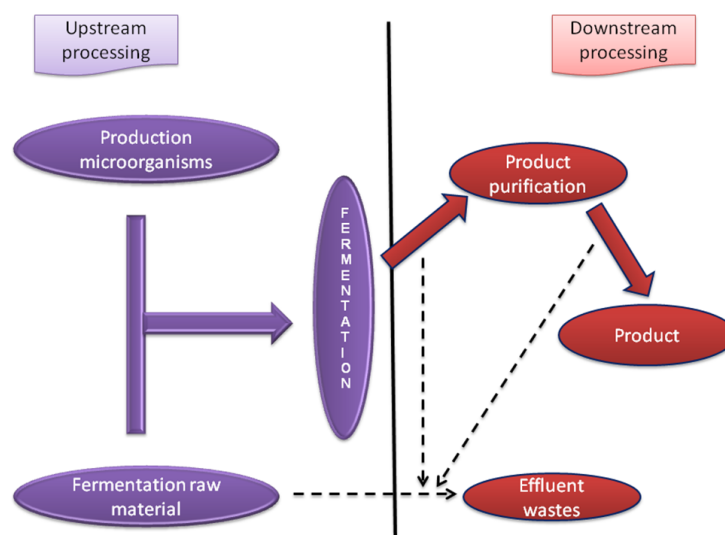
2.8 Industrial Microbiology

Humans use the versatility of microbes to make improvements in industrial production, agriculture, medicine, and environmental protection. Use of microorganisms, usually grown on a large scale, to obtain valuable commercial products by way of significant chemical transformations is called industrial microbiology. This discipline of microbiology dates back and originated with beat and wine making fermentation processes (alcoholic fermentation) and subsequently expanded in the area of production of pharmaceuticals (e.g. antibiotics), food additives (e.g. amino acids), organic acids (e.g. butyric acid and citric acid), enzymes (e.g. amylases, proteases), and vitamins. All these products are obtained by enhancing the metabolic reactions that microorganisms were already capable of carrying out in natural conditions. But, at present, in addition to this traditional industrial microbiology, a new era of microbial biotechnology is rapidly expanding in which the genes of the microorganisms responsible for such and other metabolic reactions are being manipulated to give to many new products at commercial level.

Fermentation is one of the main processes used in industrial microbiology. Fermentation is any process involving the mass culture of microorganisms, either anaerobic or aerobic. This process requires control of a series of parameters that depend on the desired final product. Throughout time, strain selection, culture media improvement, and preservation techniques have contributed to optimize the fermentation process in industry.

2.8.1 Fermentation in Industry

In industry, as well as other areas, the uses of fermentation progressed rapidly after Pasteur's discoveries. Between 1900 and 1930, ethyl alcohol and butyl alcohol were the most important industrial fermentations in the world. But by the 1960s, chemical synthesis of alcohols and other solvents were less expensive and interest in fermentations waned. Interest in microbial fermentations is experiencing a renaissance. Plant starch, cellulose from agricultural waste, and whey from cheese manufacture are abundant and renewable sources of fermentable carbohydrates. Additionally these materials, not utilized, represent solid waste that must be buried in dumps or

treated with waste water (Fig. 2.1).

Fig. 2.1 Industrial microbiology

2.8.2 Major products of industrial microbiology

The major products of industrial microbiology can be enlisted as follows:

- Food and beverage biotechnology - fermented foods, alcoholic beverages (beer, wine) and flavors
- Enzyme technology - production and application of enzymes
- Metabolites from microorganisms - amino acids, antibiotics, vaccines, biopharmaceuticals, bacterial polysaccharides and polyesters, specialty chemicals for organic synthesis (chiral synthons)
- Biological fuel generation - ethanol or methane from biomass, single cell protein, production of biomass, microbial recovery of petroleum
- Environmental biotechnology - water and wastewater treatment, composting (and landfilling) of solid waste, biodegradation/bioremediation of toxic chemicals and hazardous waste
- Agricultural biotechnology - soil fertility, microbial insecticides, plant cloning technologies
- Diagnostic tools - testing & diagnosis for clinical, food, environmental, agricultural applications, biosensors

2.9 Environmental Microbiology

Environmental microbiology is the study of the composition and physiology of microbial communities in the environment. The environment in this case means the soil, water, air and sediments covering the planet and can also include the animals and plants that inhabit these areas. Environmental microbiology also includes the study of microorganisms that exist in artificial environments such as bioreactors. Microbial life is amazingly diverse and microorganisms literally cover the planet. It is estimated that we know less than 1% of the microbial species on Earth. Microorganisms can survive in some of the most extreme environments on the planet and some can survive high temperatures, often above 100°C, as found in geysers, black smokers, and oil wells. Some are found in very cold habitats and others in highly salt / saline, acidic, or alkaline water.

Microbial life is amazingly diverse and microorganisms literally cover the planet. An average gram of soil contains approximately one billion microbes representing probably several thousand species. Microorganisms have special impact on the whole biosphere. They are the backbone of ecosystems of the zones where light cannot approach. In such zones, chemosynthetic bacteria are present which provide energy and carbon to the other organisms there. Some microbes are decomposers which have ability to recycle the nutrients. Microbes have a special role in biogeochemical cycles. Microbes, especially bacteria, are of great importance because their symbiotic relationship (either positive or negative) have special effects on the ecosystem.

2.10 Soil Microbiology

Soil is the top layer of the Earth's lithosphere, formed from weathered rock that has been transformed by living organisms. Soil is composed of mineral and organic solid particles, air, soil solution, and living organisms which occur in this edaphon. The organisms living in soil create a community called the edaphon. These are bacteria, fungi, unicellular algae, vascular plants and animals especially invertebrates that occur in the surface layer of soil. Due to the variety of their metabolic abilities the soil microorganisms ensure the permanence (continuity) of element cycles in nature. The effect of their activities is not only the mineralization of organic compounds but also the changes of mineral compounds, which have a big impact upon the development of the green plants. Edaphon constitutes about 1-10% of the dry mass of the soil organic matter. Both bacteria and fungi are the co-creators of soil's structure as they create humus - the most important component of soil that greatly influences its structure, sorption qualities and the richness in organic compounds. They have a great effect on the way of creation of crumb texture and a spongy structure of soil by producing mucous capsules, and like the filamentous bacteria and the fungi by their form of growth.

2.10 Water Microbiology

The biotopes of water microorganisms may be underground and/ or surface waters as well as bottom sediments. The underground waters (mineral and thermal springs, ground waters) - due to their oligotrophic character (nutrient - deficient) are usually inhabited by a sparse microflora that is represented by a low number of species with almost a complete lack of higher plants or animals. The surface waters such as streams, rivers, lakes and sea waters are inhabited by a diverse flora and fauna. Microorganisms in those waters are a largely varied group. Next to the typical water species, other microorganisms from soil habitats and sewage derived from living and industrial pollution occur. Bottom sediments are a transient type of habitat i.e. the soil-water habitat that is almost always typically oxygen-free in which the processes of anaerobic decomposition by microorganisms cause the release of hydrogen sulphide and methane into water. In the bottom sediment, anaerobic putrefying microflora, cellulolytic bacteria and the anaerobic chemoautotrophs develop.

Microorganisms occupy surface waters in all of the zones; they may be suspended in water (plankton), cover stationary underwater objects, plants etc (periphyton), or live in bottom sediments (benthos). Plankton can be defined as the group of organisms that passively float in water not being able to resist the movement and the flow of water mass is called plankton or bioseston.

2.11 Air Microbiology

Air is an unfavourable environment for microorganisms, in which they cannot grow or divide. It is merely a place which they temporarily occupy and use for movement. Therefore, there are no metabolic connections occurring between different microorganisms in air (such as in soil or water). As a result they form only a random collection of microorganisms. Microorganisms get into air as a consequence of wind movement, which sweeps them away from various habitats and surroundings (soil, water, waste, plant surfaces, animals, and other), or are introduced during the processes of sneezing, coughing, or sewage aeration. Air conditions are unfavorable for the microorganisms due to a lack of adequate nutrients, frequent deficit of water, threat of desiccation, and solar radiation. There are 3 main groups of microorganisms that occur in air: viruses, bacteria and fungi. Bacteria may exist as vegetative or resting forms, however fungi occur in the form of spores or fragments of mycelium.

Microorganisms in air occur in a form of colloidal system or the so-called bioaerosol. Every colloid is a system where, inside its dispersion medium, particles of dispersed phase occur, whose size is halfway between molecules and particles visible with the naked eye. In case of biological aerosols, it's the air (or other gases) that has the function of the dispersion medium, whereas microorganisms are its dispersed phase. However, it is quite rare to have microbes independently occurring in air. Usually, they are bound with dust particles or liquid droplets (water, saliva etc.), thus the particles of the bioaerosol often exceed microorganisms in size and may occur in two phases: dust phase (e.g. bacterial dust) or droplet phase (e.g. formed as the result of water-vapour condensation or during sneezing). The concentration of bioaerosol is dependent on the following factors:

- Amount of emitted microorganisms, depending on the emitter
- Distance from the source of emission
- Wind speed
- Microorganisms survival rate, depending on the factors discussed above
- Precipitation.

Lesson 3
VIROLOGY AND MEDICAL MICROBIOLOGY

3.1 Virology

Viruses are very small, infectious, obligate intracellular molecular parasites, which do not respire, move or grow. The virus genome is composed either of DNA or RNA and directs the viral replication by the synthesis of virion components within an appropriate host cell.

3.1.1 History

One of the first written records of a virus infection consists of a heiroglyph from Memphis, the capital of ancient Egypt, drawn in approximately 1400 B.C, which depicts Siptah. Judging from his mummy, he died at about 20 years of age. The body's deformed left leg suggests that Siptah suffered from a neuromuscular disease (poliomyelitis). The generally recognized beginning of virology is a paper presented to the St. Petersburg Academy of Science on the 12th February, 1892 by Dmitri Iwanowski (1864-1920), a Russian botanist. He showed that extracts from diseased tobacco plants could transmit disease to other plants after passage through ceramic filters fine enough to retain the smallest known bacteria. Six years later in Holland, Martinus Beijernick (1851-1931) confirmed Iwanowski's results on tobacco mosaic virus. He gave the term 'contagium vivum fluidum' ('soluble living germ') as the first idea of virus. Agents that pass through filters that retain bacteria came to be called ultra-filterable viruses, appropriating the term virus from the Latin for 'poison'. During the same time, the German scientists Friedrich Loeffler (1852-1915) and Paul Frosch, both former students and assistants of Robert Koch (1843-1910), observed that a similar agent was responsible for foot and mouth disease. In spite of these findings, there was resistance to the idea that these mysterious agents might have anything to do with human diseases. Bacterial viruses were first described by Frederick Twort (1915) and Felix d'Hérelle (1917). D'Hérelle named them bacteriophages because of their ability to lyse bacteria on the surface of agar plates. Following this, many scientists utilized these viruses as model systems to investigate many aspects of virology, including virus structure, genetics, and replication.

3.1.2 Virus structure

The capsid (coat) protein (Fig. 3.1) is the basic unit of structure; functions that may be fulfilled by the capsid protein are to:

- Protect viral nucleic acid
- Interact specifically with the viral nucleic acid for packaging
- Interact with vector for specific transmission
- Interact with host receptors for entry to cell
- Allow for release of nucleic acid upon entry into new cell
- Assist in processes of viral and/or host gene regulation

Nucleoprotein has two basic structure types

1. Helical: Rod shaped, varying widths and specific architectures; no theoretical limit to the amount of nucleic acid that can be packaged.

2. Cubic (Icosahedral): Spherical, amount of nucleic acid that can be packaged is limited by the particle.
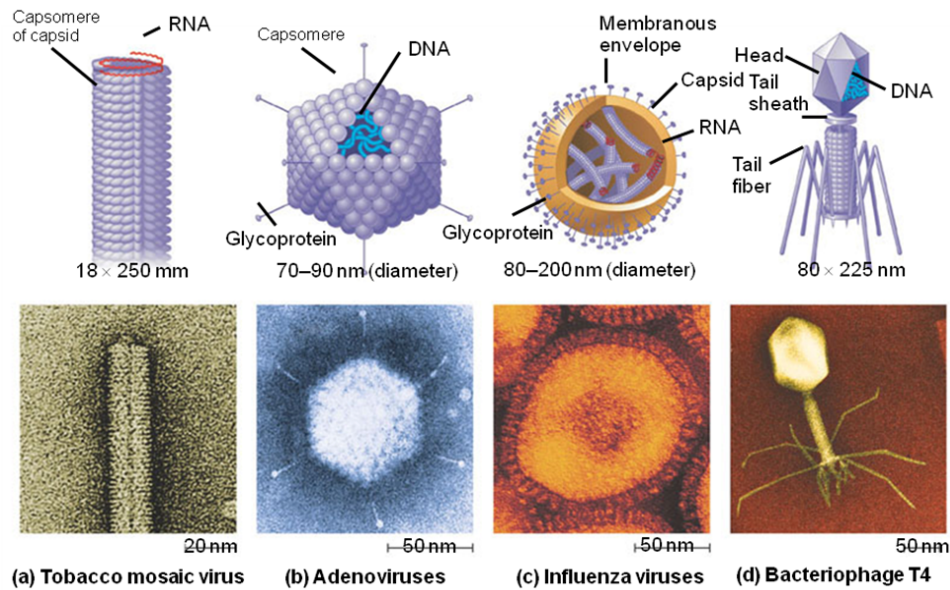
Fig. 3.1 Structure of Viruses

3.2 Medical Microbiology

Medical microbiology is the study of parasites, fungi, bacteria, and viruses that are the agents of infectious disease in humans. Modern medicine relies on the control of microorganisms to maintain human health and quality of life. The divisions of medical microbiology include bacteriology, the study of bacteria that inhabit and/or colonize the human body and cause disease; mycology, the study of fungi as causative agents of human disease; parasitology, the formal study of the human parasitic organisms (protozoans, helminths, nematodes, trematodes and arthropods); and virology, the study of viruses that cause infectious syndromes in humans. Sizes for the pathogens considered include the smallest, viruses (50-100 nm), bacteria that range from 0.1 μm (Chlamydiae) to 10μm (Bacillus rods), fungi ranging from ~8 μm (yeasts) up to 10 mm in size (filamentous fungi) and metazoan parasites that are visible to the naked eye. Medical microbiology as a discipline requires a working knowledge of human anatomy and histology, and a comprehension of the pathologies associated with the infectious disease process. The human immune response to pathogens is key to the consideration of infectious disease. Understanding the relationship between pathogens and antimicrobial pharmacology is essential as well. Microbiology places information about pathogenic organisms and their specific characteristics within the context of host disease. Developing connections between microbiology and immunology will make learning more effective in both disciplines.

3.2.1 Epidemiology

It is the study of disease patterns and trends, of the occurrence, distribution and control of disease in populations. It also deals with disease tracking and prevention. Disease transmission is the movement of the infectious agent from one host to another. The risk of infection is dependent not just upon an individual patient's susceptibility, but other factors such as on the level of disease

within the population, the extent of population mixing and 'herd immunity', the specific features of disease spread (such as communicable period, route and ease of transmission).

3.2.2 Modes of transmission

Numerous modes of transmission both direct and indirect contribute to the spread of human disease. For an infectious agent to persist within a population a cycle of transmission must be established leading from a contaminated source to a susceptible host and further propagating through the population.

Direct infection is the movement of a pathogen from human to human. Examples of organisms causing direct infection are Salmonella typhi and Shigella species, both of which infect humans only.

Indirect transmission involves human to soil, water or surface dissemination of pathogens and infection of another human through contact with contaminated materials. This idea encompasses vehicle-borne transmission, whether through infected everyday objects or surgical instruments. Vibrio cholera is an example of a pathogen that is transmitted through contaminated water, especially in brakish coastal waters.

Zoonotic transmission occurs through exposure to a nonhuman animal source of infection. The arthropod-borne agents like Borrelia and Rickettsia are included here as well as Spirillum, Brucella and Bacillus anthracis, that are contracted through animal bites or contact with animal products. Elimination is a potential outcome for a healthy host, whereby the parasite is eradicated at the end of infection cycle.

3.2.3 Routes of transmission

The direct route means physical contact between humans or between a human and an animal to cause disease. Portals of entry include the gastrointestinal tract, respiratory mucosa, genital mucosa, and direct inoculation through the skin. Mucous membranes are especially important (STDs are transmitted in this way). The airborne route or respiratory droplet transmission is very important for viral pathogens and respiratory tract infections (aerosols). Fomites are inanimate objects contaminated with microorganisms, like drinking cups, towels and computer keyboards. The water and food borne route is an especially important for enteric disease and it's an important route for fecal-oral transmission (ingestion). Vector borne transmission is critical for some viral (arbovirus) and zoonotic infections (arthropod borne parasites).

A list of pathogens and related diseases is provided in Table 3.1.

Table 3.1 Pathogens and related disease

Pathogen	Disease
Staphylococcus aureus	Staphylococcal toxic shock syndrome
Streptococcus pyogenes	Strep sore throat
Streptococcus pneumoniae	Otitis media and pneumococcal pneumonia
Neiserria gonorrhoeae	Sexually transmitted infection
Neiserria meningitidis	Meningitis
Legionella pneumophila	Legionairre's disease
Bordetella pertussis	Respiratory disease
Corynebacterium diphtheria	Diphtheria
Clostridium perfringens	Gas gangrene
Clostridium tetani	Tetanus
Salmonella typhimurium	Salmonellosis
Shigella dysenteriae	Bacillary dysentery
Vibrio cholerae	Cholera
Yersinia pestis	Bubonic plague
Treponema pallidum	Syphilis
Propionibacterium acnes	Acne

Lesson 4
LIGHT AND ELECTRON MICROSCOPY

4.1 Introduction

On the basis of source of illumination and types of lenses used, microscopes can be broadly categorized into (i) light microscopes and (ii) electron microscopes. Light or optical microscopes are characterized by light as a source of illumination and optical lenses as magnifying objects while in electron microscopes, these functions are performed by electrons and electromagnetic lenses respectively.

4.2 Light Microscopy

Microscopes using light can be categorized into following types: • Bright-field microscop

- Darkfield microscope
- Phase-contrast microscope
- Fluorescence microscopes

All these applications employ compound microscope, which essentially involve image formation by the action of two or more lenses (Fig. 4.1).
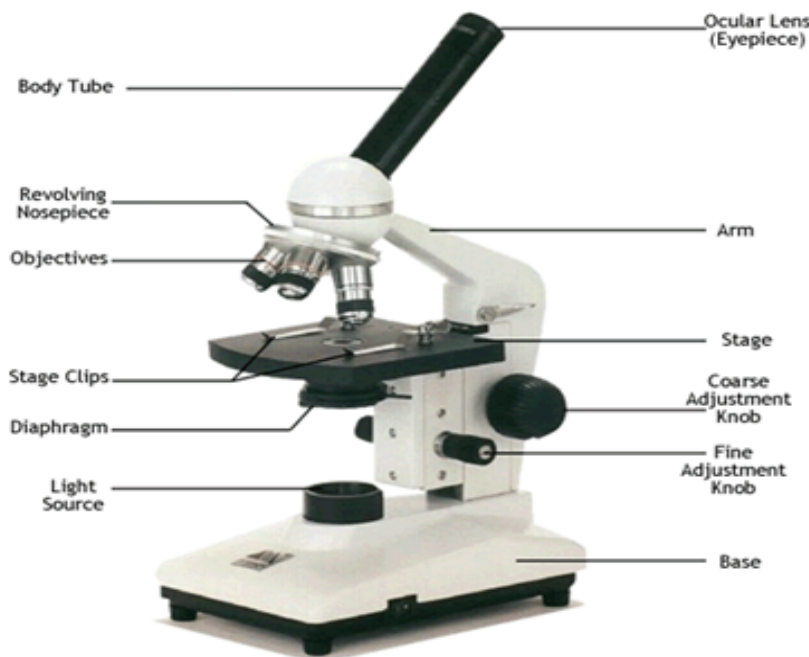
Fig. 4.1 Compound microscope

4.2.1 Bright-field microscopy

The bright-field light microscope is an instrument that magnifies images using two-lens systems. Initial magnification occurs in the objective lens. Most microscopes have at least three objective

lenses on a rotating base, and each lens may be rotated into alignment with the eyepiece or ocular lens in which the final magnification occurs. The objective lenses are identified as the low-power, high-dry, and oil immersion objectives. Each objective is also designated by other terms. These terms give either the linear magnification or the focal length. The latter is about equal to or greater than the working distance between the specimen, when in focus, and the tip of the objective lens. For example, the low-power objective is also called the 10×, or 16 millimeter (mm), objective; the high-dry is called the 40×, or 4 mm, objective; and the oil immersion is called the 90×, 100×, or 1.8 mm objective. As the magnification increases, the size of the lens at the tip of the objective becomes progressively smaller and admits less light. This is one of the reasons that changes in position of the substage condenser and iris diaphragm are required when using different objectives if the specimens viewed are to be seen distinctly. The condenser focuses the light on a small area above the stage, and the iris diaphragm controls the amount of light that enters the condenser. When the oil immersion lens is used, immersion oil fills the space between the objective and the specimen. Because immersion oil has the same refractive index as glass, the loss of light is minimized. The eyepiece, or ocular, at the top of the tube magnifies the image formed by the objective lens. As a result, the total magnification seen by the observer is obtained by multiplying the magnification of the objective lens by the magnification of the ocular, or eyepiece. For example, when using the 10X ocular and the 40X objective, total magnification is 10 × 40 = 400 times.

4.2.2 Darkfield microscopy

The compound microscope may be fitted with a darkfield condenser (Fig. 4.2) that has a numerical aperture (resolving power) greater than the objective. The condenser also contains a darkfield stop. The compound microscope now becomes a darkfield microscope. Light passing through the specimen is diffracted and enters the objective lens, whereas undiffracted light does not, resulting in a bright image against a dark background. Since light objects against a dark background are seen more clearly by the eye than the reverse, darkfield microscopy is useful in observing unstained living microorganisms, microorganisms that are difficult to stain, and spirochetes, which are poorly defined by bright-field microscopy.
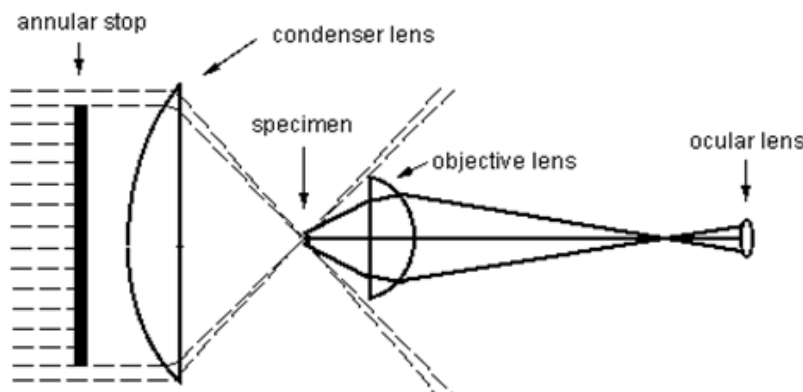

Fig. 4.2 Darkfield microscopy

A central obstruction blocks the central cone. The sample is only illuminated by the marginal rays. These marginal rays must be at angles too large for the objective lens to collect. Only light scattered by the object is collected by the lens.

4.2.3 Phase contrast microscopy

This is first microscopic method which allowed visualization of live cells in action. The Nobel prize in physics was awarded to Frits Zernike in 1953 for its discovery. Certain transparent, colorless living microorganisms and their internal organelles are often impossible to see by ordinary bright or darkfield microscopy because they do not absorb, reflect, refract, or diffract sufficient light to contrast with the surrounding environment or the rest of the microorganism. Microorganisms and their organelles are only visible when they absorb, reflect, refract, or diffract more light than their environment. The phase contrast microscope permits the observation of otherwise invisible living, unstained microorganisms. It enhances contrast in transparent and colorless objects by influencing the optical path of light. It uses the fact that light passing through the specimen travels slower than the undisturbed light beam, i.e. its phase is shifted. In figure 4.3, let S be light passing through medium surrounding sample and D light interacting with specimen. S and D typically interfere to yield P, which is what we can usually detect. P will be phase shifted compared to S, but our eyes cannot detect phase shifts. Phase contrast microscopy effectively converts this phase shift into an intensity difference we can detect.
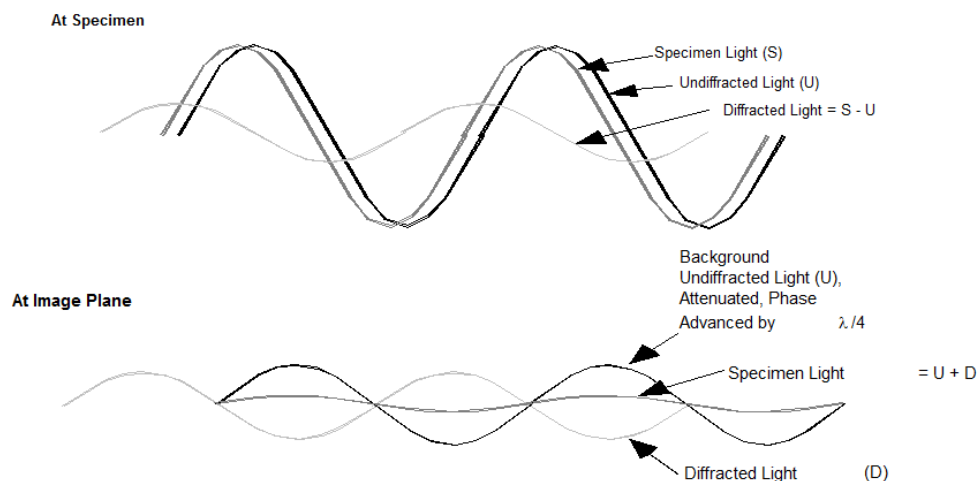
Fig. 4.3 Phase Contrast formation

In the phase contrast microscope, the condenser has an annular diaphragm, which produces a hollow cone of light; the objective has a glass disk (the phase plate) with a thin film of transparent material deposited on it, which accentuates phase changes produced in the specimen. This phase change is observed in the specimen as a difference in light intensity. Phase plates may either retard (positive phase plate) the diffracted light relative to the undiffracted light, producing dark phase contrast microscopy, or advance (negative phase plate) the undiffracted light relative to the directed light, producing bright phase contrast microscopy.

4.2.4 Fluorescence microscopy

A fluorescence microscope is much the same as a conventional light microscope with added features to enhance its capabilities.

- The conventional microscope uses visible light (400-700 nanometers) to illuminate and produce a magnified image of a sample.
- A fluorescence microscope, on the other hand, uses a much higher intensity light source which excites a fluorescent species in a sample of interest. This fluorescent species in turn emits a lower energy light of a longer wavelength that produces the magnified image instead of the original light source.

Fluorescence microscopy is based on the principle of removal of incident illumination by selective absorption, whereas light that has been absorbed by the specimen and re-emitted at an altered wavelength is transmitted. The light source must produce a light beam of appropriate wavelength. An excitation filter removes wavelengths that are not effective in exciting the fluorochrome used. The light fluoresced by the specimen is transmitted through a filter that removes the incident wavelength from the beam of light. As a result, only light that has been produced by specimen fluorescence contributes to the intensity of the image being viewed (Figure 4.4).
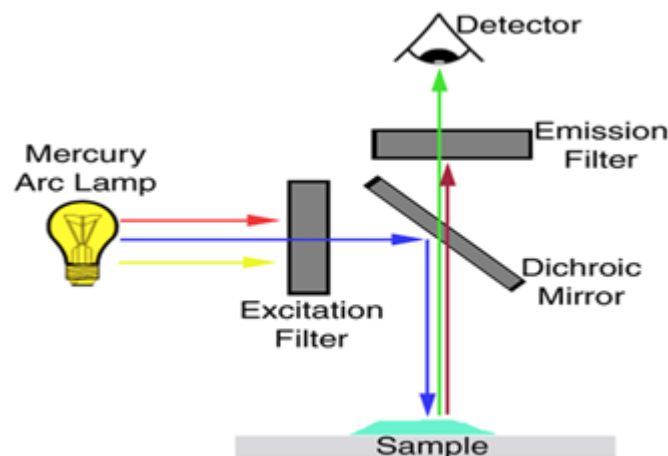
Fig. 4.4 Principle of fluorescence microscopy

4.3 Electron Microscopy

Electron microscopes were developed in order to overcome the limitations of light microscopes, which are constrained by the physics of light to 500x or 1000x magnification and a resolution of 0.2 µm (Fig. 4.5)

Click for animation (Fig. 4.5 Comparative scale of different microscopes)

In the early 1930s this theoretical limit had been reached and there was a scientific desire to see the fine details of the interior structures of organic cells (nucleus, mitochondria, etc.). This required 10,000x plus magnification which was just not possible using Light Microscopes. Electro microscopes based on their construction and application are of two types viz. transmission and scanning electron microscopes. The Transmission Electron Microscope (TEM) was the first type of EM to be developed and is patterned exactly on the Light Transmission Microscope except that a

focused beam of electrons is used instead of light to 'see through' the specimen. It was developed by Max Knoll and Ernst Ruska in Germany in 1931. The first Scanning Electron Microscope (SEM) debuted in 1942 with the first commercial instruments around 1965. Its late development was due to the electronics involved in 'scanning' the beam of electrons across the sample. Electron Microscopes (EMs) function exactly as their optical counterparts except that they use a focused beam of electrons instead of light to 'image' the specimen and gain information as to its structure and composition.
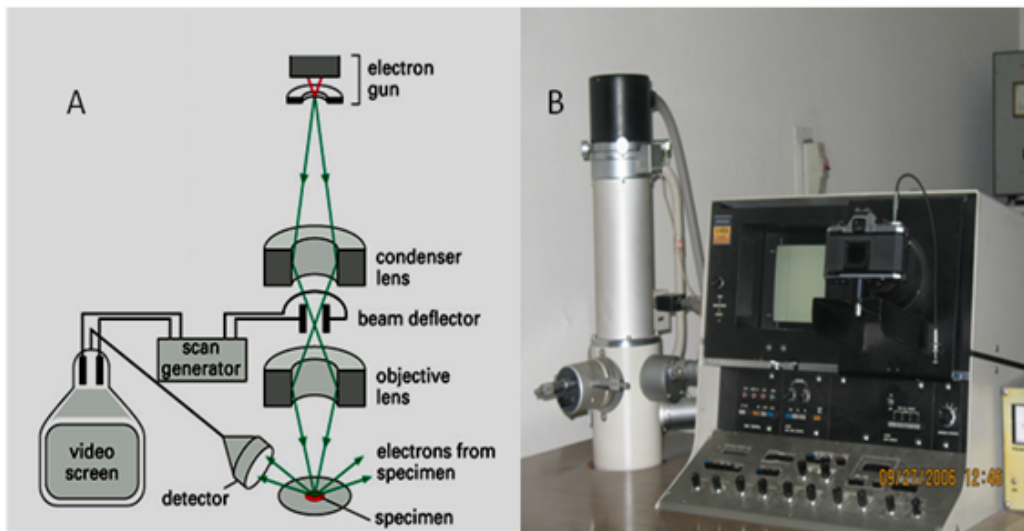
4.4 Scanning Electron Microscope

SEM is patterned after reflecting light microscopes and yield similar information.

- Topography: The surface features of an object or 'how it looks', its texture; detectable features limited to a few manometers
- Morphology: The shape, size and arrangement of the particles making up the object that are lying on the surface of the sample or have been exposed by grinding or chemical etching; detectable features limited to a few manometers
- Composition: The elements and compounds the sample is composed of and their relative ratios, in areas ~1 μm in diameter
- Crystallographic Information: The arrangement of atoms in the specimen and their degree of order; only useful on single-crystal particles >20 μm

A detailed explanation of how a typical SEM functions follows: (Fig. 4.6)

- The 'Virtual Source' at the top represents the electron gun, producing a stream of monochromatic electrons.
- The stream is condensed by the first condenser lens (usually controlled by the 'coarse probe current knob'). This lens is used to both form the beam and limit the amount of current in the beam. It works in conjunction with the condenser aperture to eliminate the high-angle electrons from the beam
- The beam is then constricted by the condenser aperture (usually not user selectable), eliminating some high-angle electrons
- The second condenser lens forms the electrons into a thin, tight, coherent beam and is usually controlled by the 'fine probe current knob'
- A user selectable objective aperture further eliminates high-angle electrons from the beam
- A set of coils then 'scan' or 'sweep' the beam in a grid fashion (like a television), dwelling on points for a period of time determined by the scan speed (usually in the microsecond range)
- The final lens, the objective, focuses the scanning beam onto the part of the specimen desired
- When the beam strikes the sample (and dwells for a few microseconds) interactions occur inside the sample and are detected with various instruments
- Before the beam moves to its next dwell point these instruments count the number of interactions and display a pixel on a CRT whose intensity is determined by this number (the more reactions the brighter the pixel)
- This process is repeated until the grid scan is finished and then repeated, the entire pattern can be scanned 30 times per second

Fig. 4.6 (A) Diagrammatic representation of pathway of electrons in SEM, (B) SEM available at Electron Microscopy Centre, NDRI, Karnal

4.5 Transmission Electron Microscope

TEM are patterned after Transmission Light Microscopes and will yield similar information.

- Morphology: The size, shape and arrangement of the particles which make up the specimen as well as their relationship to each other on the scale of atomic diameters.
- Crystallographic Information: The arrangement of atoms in the specimen and their degree of order, detection of atomic-scale defects in areas a few nanometers in diameter

TEM work the same way except that they shine a beam of electrons (like the light) through the specimen (like the slide). Whatever part is transmitted is projected onto a phosphor screen for the user to see. A more technical explanation of a typical TEM working is as follows: (Fig. 4.7)

- The 'Virtual Source' at the top represents the electron gun, producing a stream of monochromatic electrons.
- This stream is focused to a small, thin, coherent beam by the use of condenser lenses 1 and 2. The first lens (usually controlled by the 'spot size knob') largely determines the lens (usually controlled by the 'intensity or brightness knob' actually changes the size of the spot on the sample; changing it from a wide dispersed spot to a pinpoint beam.
- The beam is restricted by the condenser aperture (usually user selectable), knocking out high angle electrons (those far from the optic axis, the dotted line down the center)
- The beam strikes the specimen and parts of it are transmitted
- This transmitted portion is focused by the objective lens into an image
- Optional objective and selected area metal apertures can restrict the beam; the objective aperture enhancing contrast by blocking out high-angle diffracted electrons, the selected area aperture enabling the user to examine the periodic diffraction of electrons by ordered arrangements of atoms in the sample.
- The image is passed down the column through the intermediate and projector lenses, being enlarged all the way

- The image strikes the phosphor image screen and light is generated, allowing the user to see the image. The darker areas of the image represent those areas of the sample that fewer electrons were transmitted through (they are thicker or denser). The lighter areas of the image represent those areas of the sample that more electrons were transmitted through (these are thinner or less dense).
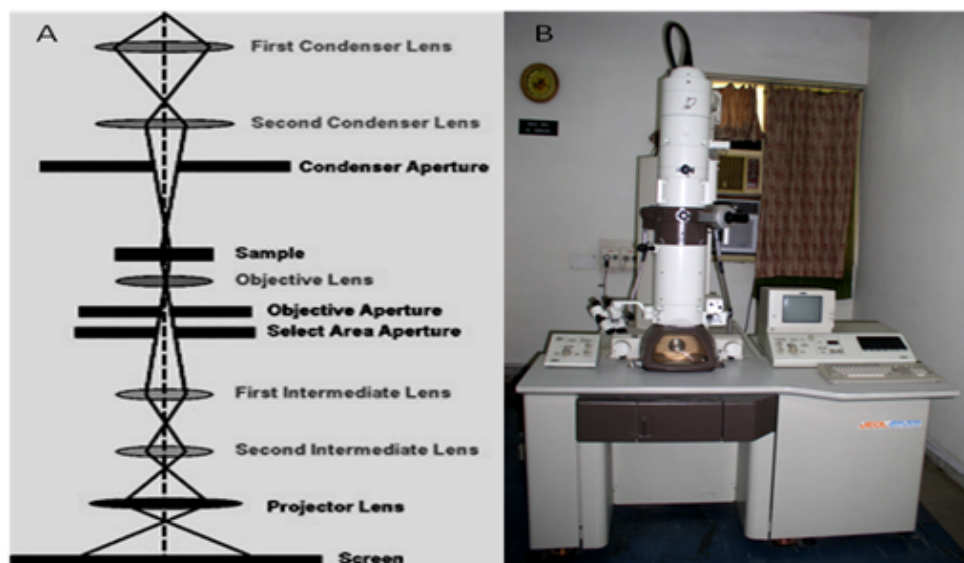
Fig. 4.7 (A) Diagrammatic representation of pathway of electrons in TEM, (B): EM available at Electron Microscopy Centre, NDRI, Karnal

Lesson 5
PREPARATION FOR LIGHT MICROSCOPY

5.1 Introduction

Microscopy is one of the most important techniques use in biological sciences. Owing to their minute size, microbial cells need to be given special treatments for their visualization under microscope. This chapter describes a number of such techniques used in light microscopy for preparation of microbial specimens.

5.2 Methods for Studying Microbes with a Compound Microscope

Two methods are generally used, 'wet method', and 'dry and fix method'.

5.2.1 Wet method

There are two primary methods generally used for studying microorganisms in wet conditions (i) wet mount method and (ii) hanging drop method.

5.2.1.1 Wet mount method

It is the most widely used method. A drop of fluid containing microorganisms to be-examined put on a glass slide and a coverslip made of thin glass is placed on it (Fig. 5.1). The fluid spreads out in a thin layer between coverslip and slide. The mount is now examined under the microscope. For higher magnifications (e.g. with 100 X objective) the oil-immersion technique is employed.
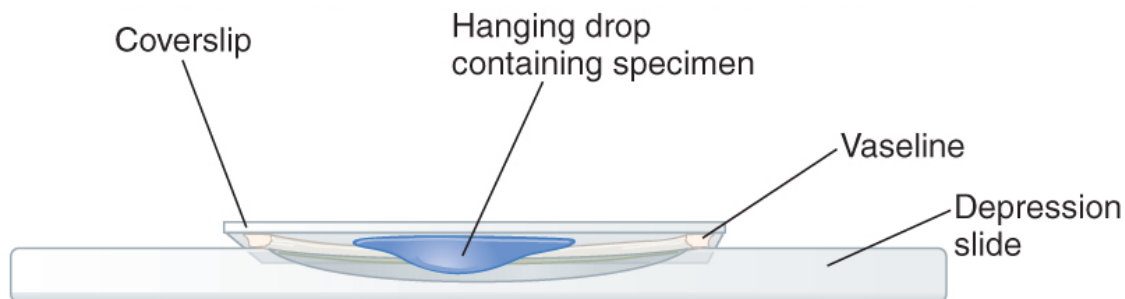
Fig. 5.1 Wet mount slide

A drop of immersion oil is put between the objective lens and cover slip before the microorganisms are examined under the microscope. The immersion oil fills the space between the specimen and the objective lens and thus replaces the air present between the specimen and the objective lens. The result is that the numerical aperture (NA) is improved and the level of magnification is increased.

A drop of immersion oil is put between the objective lens and coverslip before the microorganisms are examined under the microscope. The immersion oil fills the space between the specimen and the objective lens and thus replaces the air present between the specimen and the objective lens. The result is that the NA is improved and the level of magnification is increased (Figure 5.2).
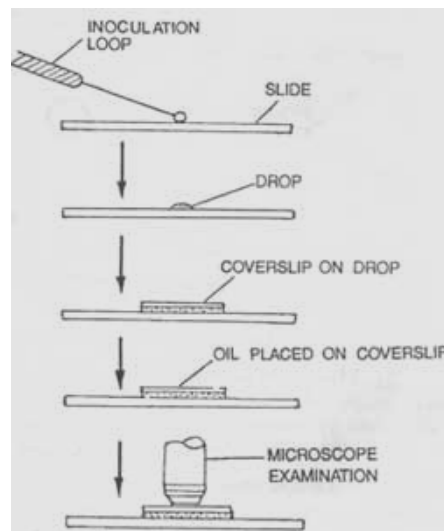
Fig. 5.2 Wet mount method

5.2.1.2 Hanging drop method

Microscopic examination of live bacteria in wet mounts reveals whether the bacteria are motile or non-motile. Motility is an inheritable phenotype and is a useful criterion for identification and classification of bacteria. Because unstained transparent cells are examined, more examination time is usually needed to visualize and locate the cells than for stained preparations. This is particularly true because 400X rather than 1,000X magnification is used to see bacterial cells in this type of preparation, and therefore examination is critical. Due to these limitations, special techniques are used in order to prevent the wet mount from drying during the time required for microscopic examination. The hanging drop technique is a method in which a drop of bacterial suspension, preferably in mid-logarithmic phase, is enclosed in an air-tight chamber prepared in a special depression slide having a concave depression in the center (Fig. 5.3). The technique is done by applying petroleum jelly to all sides of a cover glass. It is a 'hanging drop' slide because the droplet remains untouched due to the concave shape of the cover glass and it just hangs from the cover glass.

Many bacteria show no motion and are termed non-motile. However, in an aqueous environment, these same bacteria appear to be moving erratically. This erratic movement is due to Brownian movement. Brownian movement results from the random motion of the water molecules bombarding the bacteria and causing them to move. True motility (self-propulsion) has been recognized in other bacteria and involves several different mechanisms. Bacteria that possess flagella exhibit flagellar motion. Helical-shaped spirochetes have axial fibrils (modified flagella that wrap around the bacterium) that form axial filaments. These spirochetes move in a corkscrew and bending-type motion. Other bacteria simply slide over moist surfaces in a form of gliding motion. The above types of motility or non-motility can be observed over a long period in a hanging drop slide (Fig. 5.3). Hanging drop slides are also useful in observing the general shape of living bacteria and the arrangement of bacterial cells when they associate together. A ring of Vaseline around the edge of the coverslip keeps the slide from drying out.
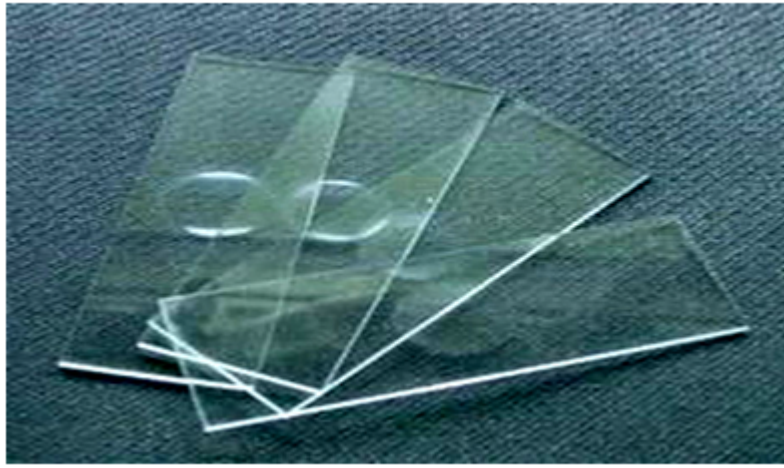
Fig. 5.3 Concave slide used in hanging drop method.

Procedure for Hanging Drop Method (Fig. 5.4)

- With a toothpick, spread a small ring of Vaseline around the concavity of a depression slide. Do not use too much Vaseline.
- After thoroughly mixing one of the cultures, use the inoculating loop to aseptically place a small drop of one of the bacterial suspensions in the center of a coverslip.
- Lower the depression slide, with the concavity facing down, onto the coverslip so that the drop protrudes into the center of the concavity of the slide. Press gently to form a seal.
- Turn the hanging drop slide over and place on the stage of the microscope so that the drop is over the light hole.
- Examine the drop by first locating its edge under low power and focusing on the drop. Switch to the high-dry objective and then, using immersion oil, to the 90 to 100× objective. In order to see the bacteria clearly, close the diaphragm as much as possible for increased contrast. Note bacterial shape, size, arrangement, and motility. Be careful to distinguish between motility and Brownian movement.
- Discard the coverslip and any contaminated slides in a container with disinfectant solution.
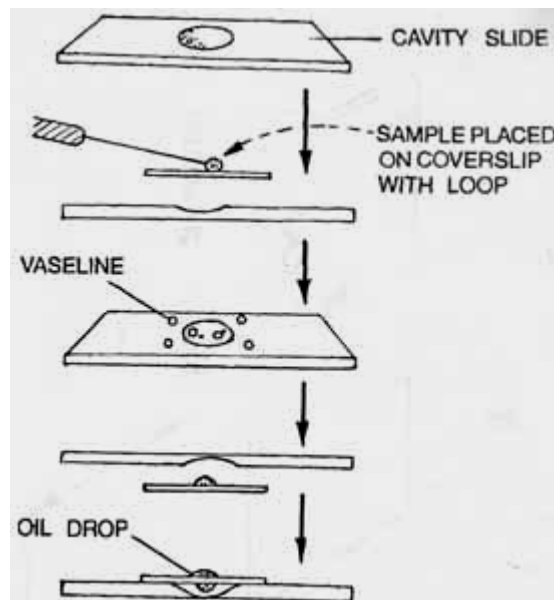
Fig. 5.4 Hanging Drop Method

5.3 Dry and Fix Method

Microorganisms, particularly bacteria, being too small need their permanent preparation be made by drying and fixing them on clean slide with or without staining. For preparing a dry mount, a drop of distilled water with a small amount or culture is spread as a thin smear on a clean slide. The smear is allowed to dry and it is then 'fixed' by passing it through a flame two to three times with the smeared slide away from the flame. If desired, this dried and fixed amount may be stained and the preparation dried again for observation under the microscope.

Lesson 6
MICROBIOLOGICAL STAINING METHODS

6.1 Introduction

Sometimes it is convenient to determine overall bacterial morphology without the use of harsh staining or heat-fixing techniques that change the shape of cells. This might be the case when the bacterium does not stain well or when it is desirable to confirm observations made on the shape and size of bacteria observed in either a wet-mount or hanging drop slide. Staining of specimens is carried out to increase visibility of specimen, accentuate specific morphological features and preserves specimens. Staining of specimen makes internal and external structures of cell more visible by increasing contrast with background.

6.2 Preparing a Smear

A properly prepared smear accomplishes two things. One, it causes bacteria to adhere to a slide so that they can be stained and observed and second, it also kills them, rendering pathogenic bacteria safe to handle. An objective in preparing smears is to learn to recognize the correct density of bacteria to place on the slide. If there are too many cells, they will overlap each other giving false positives or crowding effect and if they are too low in number, they cannot be located on the slide. A smear is also required to undergo fixation, a process by which organism is killed and firmly attached to microscope slide and internal and external structures are preserved and fixed in position. Fixation can be achieved either by heat fixation (commonly used in light microscopy) which preserves overall morphology but not internal structures or chemical fixation using chemical fixatives (used in light and electron microscopy) which protects fine cellular substructure and morphology of larger, more delicate organisms.

6.2.1 Procedure

- A circle should be marked on the under side of a slide with a glass etching tool. Several circles can be located on the same slide.
- The slide must be grease-free. A good way to clean a slide is to repeatedly breathe on it, followed by rubbing vigorously with a soft tissue or paper towel to remove the fog. When the slide de-fogs immediately after breathing on it, it is sufficiently clean.
- To prepare a smear from a dry culture, a very small drop of distilled water should be placed over the circled area. After aseptically removing material from a culture it is them mixed with the drop or placed directly on the slide if it is a dilute broth culture. It takes very little material to produce a successful smear.
- The drop is air-dried completely, which takes a short time if a small drop is prepared.
- While holding the slide with a clothes pin it is quickly passed it through a flame. Three quick passes are usually sufficient to kill the bacteria and cause them to adhere.
- After cooling the slide, the staining procedure is conducted.

6.3 Staining Methods

Staining methods can be broadly categorized in to two types, simple and differential and include including negative staining, Gram staining, endospore staining, capsule staining, flagellar staining,

acid fast staining etc.

6.3.1 Simple staining

In this type of staining bacterial cells are stained with a single reagent. Positively charged dyes such as methylene blue, crystal violet etc. is used for this purpose. These stains are taken up by the cells and bind to negatively charged cell components (cell wall, nucleic acids). Staining can be positive or negative (Fig. 6.1) depending up on the type of dyes (cationic or anionic)
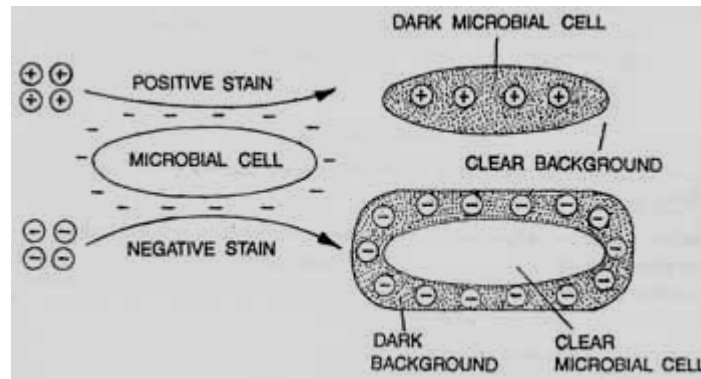
Fig. 6.1 Types of staining

6.3.1.1 Procedure

- Prepare a thin smear of bacterial culture by placing a loopful of culture on a clean slide and spreading it slowly in circular motion on the surface of slide. Allow the smear to get dried.
- Heat fix the smear by passing it quickly on the bunsen flame.
- Flood the smear with methylene blue/crystal violet for 2 or 1 min. respectively.
- Wash the smear with tap water and dry it.
- Put a drop of immersion oil and observe under 100X lens of light microscope.
- Cells will appear blue (methylene blue) or purple (crystal violet). Look for various cell shapes.

6.3.2 Negative staining

In this stain the bacterial cells do not take up the cell but the background gets stained and the cell appears as unstained transparent entity. Acidic dyes such as nigrosin are used. Negative staining is advantageous because the bacteria which do not take up the stain can be observed in this way. Another advantage of this procedure is that it does not require heat fixing of smear so cell distortion due to heat do not take place.

6.3.2.1 Procedure (Fig. 6.2)

- Place a drop of nigrosin close to one end of a clean slide.
- Mix a loopful of culture in it.
- Prepare a smear of this mixture using the edge of another slide.
- Air-dry it and observe under oil immersion lens.

Unstained bacterial cells will appear in contrast to a dark background.

a. Place a second slide on top of the first as shown.

b. While maintaining contact with the bottom slide, move the top slide to contact the drop. It will spread by capillary action.

c. Maintain contact with the bottom slide and move the top slide in one motion to produce the smear.
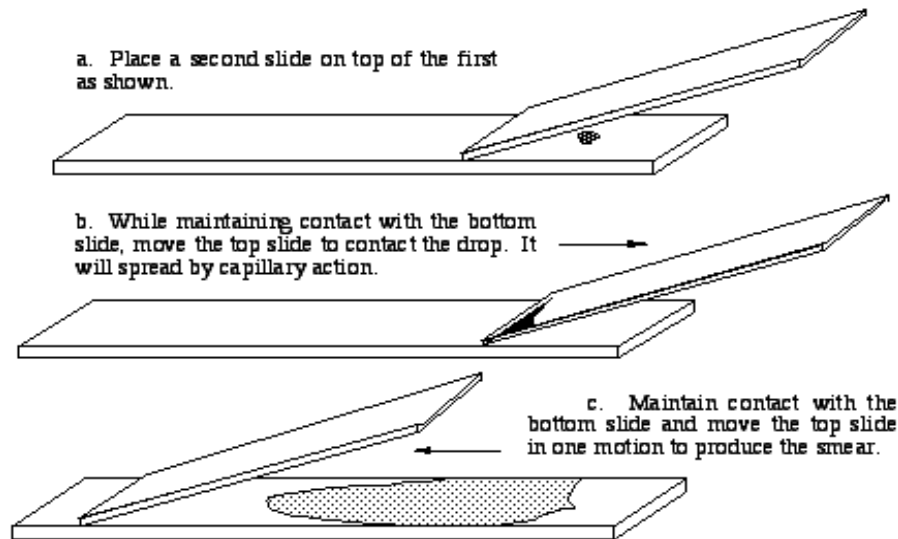
Fig. 6.2 Procedure for negative staining

6.4 Differential Staining

Simple staining depends on the fact that bacteria differ chemically from their surroundings and thus can be stained to contrast with their environment. Bacteria also differ from one another chemically and physically and may react differently to a given staining procedure. This is the principle of differential staining. Differential staining can distinguish between types of bacteria.

6.4.1 Gram staining

The Gram stain (named after Christian Gram, Danish scientist and physician, 1853–1938) is the most useful and widely employed differential stain in bacteriology. It divides bacteria into two groups, Gram negative and Gram positive. The first step in the procedure involves staining with the basic dye crystal violet. This is the primary stain. It is followed by treatment with an iodine solution, which functions as a mordant; that is, it increases the interaction between the bacterial cell and the dye so that the dye is more tightly bound or the cell is more strongly stained. The smear is then decolorized by washing with an agent such as 95% ethanol or isopropanol-acetone. Gram positive bacteria retain the crystal violet-iodine complex when washed with the decolorizer, whereas Gram negative bacteria lose their crystal violet-iodine complex and become colorless. Finally, the smear is counterstained with a basic dye, different in color than crystal violet. This counter stain is usually safranin. The safranin will stain the colorless, Gram negative bacteria pink but does not alter the dark purple color of the Gram positive bacteria. The end result is that Gram positive bacteria are deep purple in color and Gram negative bacteria are pinkish to red in color (Fig. 6.3). The Gram stain does not always yield clear results. Species will differ from one another in regard to the ease with which the crystal violet-iodine complex is removed by ethanol. Gram positive cultures may often turn Gram negative if they get too old. Thus, it is always best to Gram stain young, vigorous cultures rather than older ones. Furthermore, some bacterial species are Gram variable. That is, some cells in the same culture will be Gram positive and some, Gram negative. Therefore, one should always ensure to run Gram stains on several cultures under carefully controlled conditions in

order to make certain that a given bacterial 'strain' is truly Gram positive or Gram negative. Indistinct Gram-stain results can be confirmed by a simple test using KOH. Place a drop of 10% KOH on a clean glass slide and mix with a loopful of bacterial paste. Wait for 30 seconds and then pull the loop slowly through the suspension and up and away from the slide. A Gram negative organism will produce a mucoid string; a Gram positive organism remains fluid.
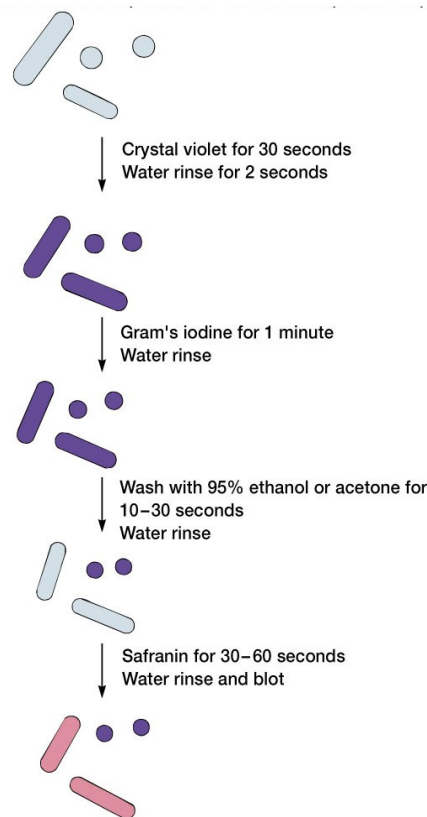
Fig. 6.3 Gram staining method

6.4.2 Acid-fast staining (Ziehl-Neelsen and Kinyoun)

A few species of bacteria in the genera Mycobacterium and Nocardia, and the parasite Cryptosporidium do not readily stain with simple stains. However, these microorganisms can be stained by heating them with carbolfuchsin. The heat drives the stain into the cells. Once the microorganisms have taken up the carbolfuchsin, they are not easily decolorized by acid-alcohol, and hence are termed acid-fast. This acid-fastness is due to the high lipid content (mycolic acid) in the cell wall of these microorganisms. The Ziehl-Neelsen acid-fast staining procedure (developed by Franz Ziehl, a German bacteriologist, and Friedrich Neelsen, a German pathologist, in the late 1800s) is a very useful differential staining technique that makes use of this difference in retention of carbolfuchsin. Acid-fast microorganisms will retain this dye and appear red (Fig. 6.4). Microorganisms that are not acid-fast, termed non-acid-fast, will appear blue or brown due to the counterstaining with methylene blue after they have been decolorized by the acid-alcohol. A modification of this procedure that employs a wetting agent (Tergitol No. 7) rather than heat to ensure stain penetration is known as the Kinyoun staining procedure (developed by Joseph Kinyoun, German bacteriologist, in the early 1900s).
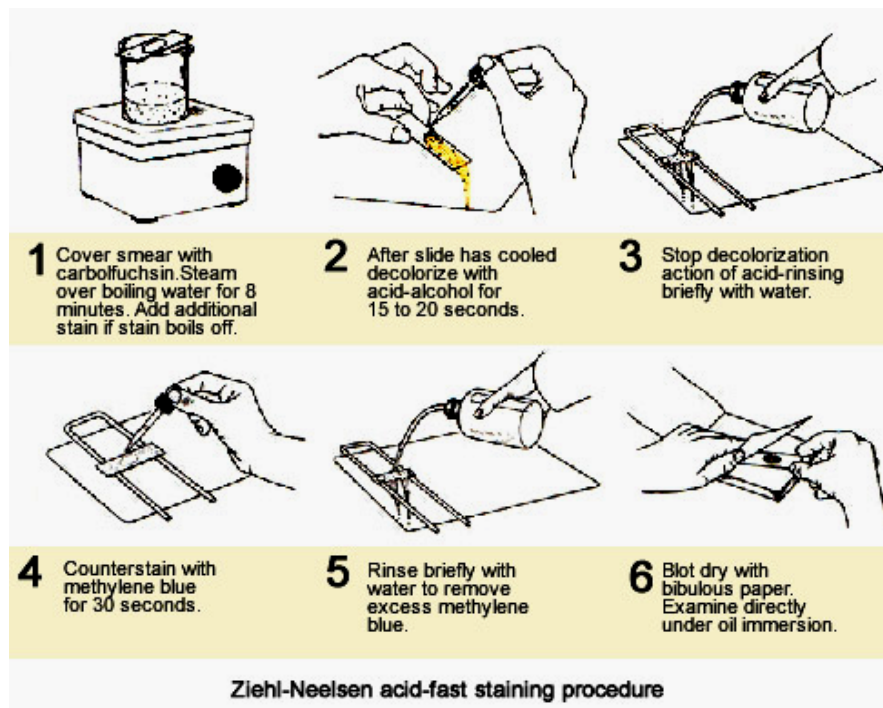
Fig. 6.4 Acid fast staining method

6.4.3 Endospore staining

Bacteria in genera such as Bacillus and Clostridium produce quite a resistant structure capable of surviving for long periods in an unfavorable environment and then giving rise to a new bacterial cell. This structure is called an endospore since it develops within the bacterial cell. Endospores are spherical to elliptical in shape and may be either smaller or larger than the parent bacterial cell. Endospore position within the cell is characteristic and may be central, subterminal, or terminal. Endospores do not stain easily, but, once stained, they strongly resist decolorization. This property is the basis of the Schaeffer-Fulton (Alice B. Schaeffer and MacDonald Fulton were microbiologists at Middlebury College, Vermont, in the 1930s) or Wirtz-Conklin method (Robert Wirtz and Marie E. Conklin were bacteriologists in the early 1900s) of staining endospores. The endospores are stained with malachite green. Heat is used to provide stain penetration. The rest of the cell is then decolorized and counterstained a light red with safranin.

6.4.3.1 Procedure (Fig. 6.5)

- With a wax pencil, place the names of the respective bacteria on the edge of four clean glass slides.
- Aseptically transfer one species of bacterium with an inoculating loop to each of the respective slides, air dry (or use a slide warmer), and heat-fix.
- Place the slide to be stained on a hot plate or boiling water bath equipped with a staining loop or rack. Cover the smear with paper toweling that has been cut the same size as the microscope slide.
- Soak the paper with the malachite green staining solution. Gently heat on the hot plate (just until the stain steams) for 5 to 6 min after the malachite green solution begins to steam. Replace the malachite green solution as it evaporates so that the paper remains saturated during heating. Do not allow the slide to become dry.

- Remove the paper using forceps, allow the slide to cool, and rinse the slide with water for 30 sec.
- Counterstain with safranin for 60 to 90 sec.
- Rinse the slide with water for 30 sec.
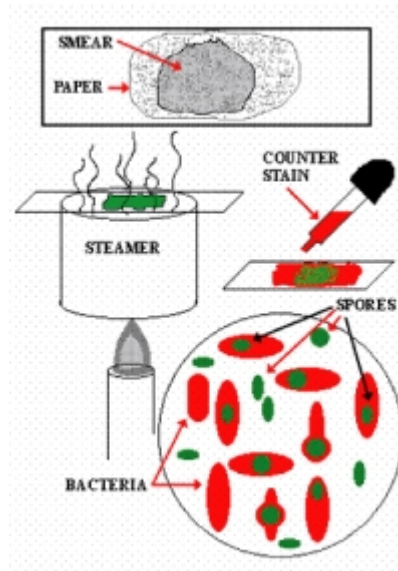
Fig. 6.5 Endospore staining

Lesson 7
CLASSIFICATION, NOMENCLATURE AND IDENTIFICATION

7.1 Introduction

Taxonomy (Greek taxis, arrangement or order, and nomos, law, or nemein, to distribute or govern) is defined as the science of biological classification. In a broader sense it consists of three separate but interrelated parts: classification, nomenclature, and identification.

7.1.1 Identification

Identification is "the practical side of taxonomy, the process of determining that a particular (organism) belongs to a recognized taxon."

7.1.2 Classification

Classification is "the arrangement of organisms into groups or taxa."

7.1.3 Nomenclature

Nomenclature is "the branch of taxonomy concerned with the assignment of names to taxonomic groups in agreement with published rules.

7.2 History of Taxonomy

Earlier Concept: The ancient Greek philosopher Aristotle apparently began the discussion on taxonomy. Aristotle divided organisms into plants and animals. He subdivided them by their habitat - land, sea, or air dwellers.

John Ray (1627–1705): British naturalist John Ray is credited with revising the concept of naming and describing organisms. He was the first to use Latin for naming. The names given by him were very long descriptions telling everything about the plant. He was an English naturalist, sometimes referred to as the father of English natural history. He published important works on plants, animals, and natural theology. His classification of plants in his Historia Plantarum, was an important step towards modern taxonomy. He coined the term species.

Carolus Linnaeus (1707–1778), Linnaeus, 18th century taxonomist, classified organisms by their structure. He is credited with developing the modern system of naming known as binomial nomenclature and is called the 'Father of Taxonomy'.

Two-word name (Genus and species)

- – Genus species
- – Latin or Greek
- – Italicized in print
- – Capitalize genus, but NOT species

– Underline when writing

Carolus Linnaeus distinguished two kingdoms of living things: Animalia for animals and Vegetabilia for plants (Linnaeus also included minerals, placing them in a third Kingdom, Mineralia). He divided each kingdom into classes, later grouped into phyla for animals and divisions for plants.

Edouard Chatton (1883-1947), a French biologist, contributed to our knowledge of single-celled protoctists, especially ciliates and dinoflagellates, free-living and/or symbiotic, in relation to the marine invertebrate animals in which they reside. More than the description of many new families, genera and species, and of their life cycles, he anticipated several major concepts of cell biology, including the fundamental difference between prokaryote and eukaryote protists, long time before the advent of electron microscopy. It gradually became apparent how important the prokaryote/eukaryote distinction is, and Stanier and van Niel popularized Chatton's proposal in the 1960s to divide them.

Ernst Heinrich Philipp August Haeckel (1834–1919), was an eminent German biologist, naturalist, philosopher, physician, professor and artist who discovered, described and named thousands of new species, mapped a genealogical tree relating all life forms, coined many terms in biology, including phylum, phylogeny, ecology and the kingdom Protista. In 1866, Ernst Haeckel divided animals, plants, and microorganisms into 3 kingdoms namely Animalia, Plantae and Protista.

Robert Harding Whittaker (1920–1980), recognized an additional kingdom for the Fungi. The resulting five-kingdom system, proposed in 1969, has become a popular standard and with some refinement is still used in many works and forms the basis for newer multi-kingdom systems. It is based mainly on differences in nutrition; his Plantae were mostly multicellular autotrophs, his Animalia multicellular heterotrophs, and his Fungi multicellular saprotrophs. The remaining two kingdoms, Protista and Monera, included unicellular and simple cellular colonies.

In biological taxonomy, kingdom and/or regnum is a taxonomic rank in either (historically) the highest rank, or (in the new three-domain system) the rank below domain. Each kingdom is divided into smaller groups called phyla (or in some contexts these are called 'divisions'). Currently, many textbooks from the United States use a system of six kingdoms (Animalia, Plantae, Fungi, Protista, Archaea, Bacteria) while British and Australian textbooks may describe five kingdoms (Animalia, Plantae, Fungi, Protista, and Prokaryota or Monera). The classifications of taxonomy are life, domain, kingdom, phylum, class, order, family, genus, and species.

7.3 Classification Systems

Hierarchical classification: In classification taxonomist follow a hierarchy of designations; means in ascending sequence. The full description of a given organism's place among all the world's organisms does not end with its binomial designation. There exists a hierarchy of designations only the last of which describe genera and species denomination. A category in any rank unites groups in the level below it based on shared properties. The major designations, listed in terms of increasing specificity, include

- Domain (empire/super-kingdom)

- Kingdom
- Phylum
- Class
- Order
- Family
- Genus
- Species

Example

Genus: *Escherichia*

Species: *coli*

Family: Enterobacteriaceae

Class: Scotobacteria

Division: Gracilicutes

Kingdom: Procaryotae

7.3.1 Five kingdoms of life

Living organisms as suggested by are subdivided into 5 major kingdoms, including the Monera, the Protista (Protoctista), the Fungi, the Plantae, and the Animalia (Figure 7.1). Each kingdom is further subdivided into separate phyla or divisions. Generally 'animals' are subdivided into phyla, while 'plants' are subdivided into divisions. These subdivisions are analogous to subdirectories or folders on your hard drive. The five kingdom system of classification for living organisms, including the prokaryotic Monera and the eukaryotic Protista, Fungi, Plantae and Animalia is complicated by the discovery of archaebacteria. The prokaryotic Monera include three major divisions: The regular bacteria or eubacteria; the cyanobacteria (also called blue-green algae); and the archaebacteria. Lipids of archaebacterial cell membranes differ considerably from those of both prokaryotic and eukaryotic cells, as do the composition of their cell walls and the sequence of their ribosomal RNA subunits. In addition, recent studies have shown that archaebacterial RNA polymerases resemble the eukaryotic enzymes, not the eubacterial RNA polymerase.

Click for animation (Fig. 7.1 Five Kingdom System)

7.3.2 Six kingdoms

Around 1980, there was an emphasis on phylogeny and redefining the kingdoms to be monophyletic groups, groups made up of relatively closely related organisms. The Animalia, Plantae, and Fungi were generally reduced to core groups of closely related forms, and the others placed into the Protista. Based on RNA studies, Carl Woese divided the prokaryotes (Kingdom Monera) into 2 kingdoms -Eubacteria and Archaebacteria. Carl Woese attempted to establish a 3 Primary Kingdom system in which Plants, Animals, Protista, and Fungi were lumped into one primary kingdom of all

eukaryotes. The Eubacteria and Archaebacteria made up the other two kingdoms. The initial use of 'six kingdom system' represents a blending of the classic five kingdom system and Woese's three domain system (Fig. 7.2). Such six kingdom system has become standard in many works. A variety of new eukaryotic kingdoms were also proposed, but most were quickly invalidated, ranked down to phyla or classes, or abandoned. The only one which is still in common use is the kingdom Chromista proposed by Cavalier-Smith, including organisms such as kelp, diatoms, and water moulds. Thus the eukaryotes are divided into three primarily heterotrophic groups, the Animalia, Fungi, and Protozoa, and two primarily photosynthetic groups, the Plantae (including red and green algae) and Chromista. However, it has not become widely used because of uncertainty over the monophyly of the latter two kingdoms.

Click for animation (Fig. 7.2 Six Kingdom System)

7.3.3 Three domain system

In 1970, Carl Woese, by analyzing RNA, developed the 3 domain classification system (Fig.7.3)

- Archaebacteria
- Bacteria
- Eucarya

Click for animation (Fig. 7.3 Three Domain System)

Woese stresses genetic similarity over outward appearances and behaviour, relying on comparisons of ribosomal RNA genes at the molecular level to sort out classification categories. A plant does not look like an animal, but at the cellular level, both groups are eukaryotes, having similar subcellular organization, including cell nuclei, which the Eubacteria and Archaebacteria do not have. More importantly, plants, animals, fungi, and protists are more similar to each other in their genetic makeup at the molecular level, based on RNA studies, than they are to either the Eubacteria or Archaebacteria. Woese also found that all of the eukaryotes, lumped together as one group, are more closely related, genetically, to the Archaebacteria than they are to the Eubacteria. This means that the Eubacteria and Archaebacteria are separate groups even when compared to the eukaryotes. Therefore, Woese established the three domain system, clarifying that all the Eukaryotes are more closely genetically related compared to their genetic relationship to either the bacteria or the archaebacteria, without having to replace the 'six kingdom system' with a three kingdom system. The three domain system is a 'six kingdom system' that unites the eukaryotic kingdoms into the Eukarya Domain based on their relative genetic similarity when compared to the Bacteria Domain and the Archaea Domain. Woese also recognized that the Protista kingdom is not a monophyletic group and might be further divided at the level of kingdom. Others have divided the Protista kingdom into the Protozoa and the Chromista, for instance.

<center>**Lesson 8**
MAJOR CHARACTERISTICS USED IN MICROBIAL TAXONOMY</center>

8.1 Introduction

Major characteristics used in microbial taxonomy are morphology, Gram reaction, nutritional classification, cell wall, lipid, cell inclusions and storage products, pigments, carbon source utilization, nitrogen source utilization, sulfur source utilization, fermentation products, gaseous needs, temperature range, pH range, pathogenicity, symbiotic relationships, habitat, etc.

Often Guanine to Cytosine (GC) ratio of deoxyribonucleic acid (DNA) is also used. If two organisms that are thought to be closely related, based on phenotypic criteria, do not have similar GC values, then they are not in fact closely related.

8.2 Dichotomous Key

It is a means of assigning an organism to a specific taxonomic category. It typically involves the use of specific criteria that may be posed as questions (e.g. what does the organism look like?). Relevant criteria may be arranged as a dichotomous key. In a dichotomous key, questions are arranged hierarchically (just as taxonomic categories are) with more general questions (i.e. those arranging organisms into large categories) being asked first, and increasingly specific questions (better suited to arranging organisms into more specific taxa) subsequently. In addition, questions are dichotomous, meaning that each of them have two possible answers, with each answer distinguishing the organisms as well as the path to the next question.

8.3 Strain Differentiation Methods

Very closely related organisms, i.e. members of the same species, are typically sufficiently similar that there exist additional methods that are able to distinguish the small differences seen between them. These methods include protein profiling, immunological reactions, phage typing etc. These methods compare phenotypes and that, though useful, are not as precise as genetic homologies in determining evolutionary relationships.

8.3.1 Protein profile

Various techniques exist for isolating (separating) and then visualizing the proteins from cells. By distinguishing proteins in terms of their size and/or charges one can construct reproducible patterns that are typical of a given organism. Similar organisms display similar protein patterns. The size and other differences between proteins among different organisms may be determined very easily employing methods of protein separation based on the technique known as gel electrophoresis.

8.3.2 Immunological reactions

The ability of antibodies to bind to and/or inactivate microorganisms can be employed to determine evolutionary relationships. The basic premise is that antibodies are highly selective in terms of the proteins to which they bind. Thus, antibodies are able to distinguish the proteins coming from

different bacterial species. Two organisms that a single antibody (or antibody preparation) binds to are considered to be more closely related than a third organism to which the antibody preparation does not bind. Various methods based on this principle are agglutination tests, enzyme-linked immunosorbent assays (ELISA) and Western blots.

8.3.3 Flow cytometry

Flow cytometry is a technique employing serological methods that analyze cells suspended in a liquid medium by light, electrical conductivity, or fluorescence as the cells individually pass through a small orifice.

8.3.4 Phage typing

Bacteriophages (or phages) are viruses that infect bacteria. Phage can be very specific in what bacteria they infect and the pattern of infection by many phages may be employed in phage typing to distinguish bacterial species and strains.

8.4 Types of Strains

8.4.1 Type strain

It is the first strain isolated or best characterized. In classifying an organism, it is helpful to have some standard to compare it to. Such standards for a given strain are termed type strains. Often the type strain is the first example of a species or strain. Type strains are kept preserved by the culture collection centres such as American Type Culture Collection (ATCC).

8.4.2 Serovar

A serovar is a strain differentiated by serological means. Individual strains of Salmonella spp. are often distinguished and distinguishable by serological means.

8.4.3 Biovar (biotype)

Biovars are strains that are differentiated by biochemical or other non-serological means.

8.4.4 Morphovar (morphotype)

A morphovar is a strain which is differentiated on the basis of morphological distinctions.

8.4.5 Isolate

An isolate is a pure culture derived from a heterogeneous, wild population of microorganisms. The term isolate is also applicable to eucaryotic microorganisms as well as to viruses.

Lesson 9
METHODS OF CLASSIFICATION

9.1 Introduction

Two common approaches used in biological world are referred to as phenotypic and phylogenetic classification.

9.1.1 Phenotypic (phenetic) classification

Phenotypic classification is concerned with grouping individual species into phenotypic categories (taxons) based on how organisms 'look'. In the recent past, taxonomists were not equipped to classify beyond the level of phenotypic groupings except via inference from phenotypic similarities. Phenotypic similarity and evolutionary relationship do not always map one to one upon each other. This approach is guided by a set of morphological and biochemical tests, constituting the cornerstone of 'Determinative Microbiology'.

Limitations: From the standpoint of many areas of microbiology, a determinative classification is sufficient. For example, in clinical microbiology the identification of organisms permits the physician to assess pathogenicity and to select a treatment. In this context, the purely determinative nature of a classification is not crucial. If the organism has previously been described and hence is already in the classification, then it can be identified and treated. However, from a biological point of view, the lack of a natural system does not permit the projection of properties of previously described organisms onto new ones that might be closely related, but not identical, to those known before. In addition, it does not help us understand an organism that we have been unable to cultivate in the laboratory. Finally, it does not permit studies of the origin and evolution of cellular functions (e.g. drug resistance, aerobiosis or photosynthesis), because there is no evolutionary (historical) framework.

9.1.2 Phylogenetic (phyletic) classification

Phylogenetic classification is concerned with grouping individual species into evolutionary categories. Since the early 1980s, phylogenetic classfication has been made much more facile by the invention of molecular taxonomy. The evolutionary classification of organisms is based on the nucleotide sequence divergence at individual loci (genes).

9.2 Phylogeny from Phenotype

These two approaches (phenotypic and phylogenetic classification) often fully match. This is because there is usually a correlation between evolutionary relatedness and phenotypic relatedness. However, such things as convergent evolution can create confusion between the two classification philosophies since convergent evolution, by definition, produces phenotypic similarity in the absence of close evolutionary relatedness. The trick to solving these discrepancies is to concentrate on true homologies and ignore convergence. Conflicts between phenotypic and phylogenetic classification are at the root of the various monophyly-paraphyly debates.

9.2.1 Molecular phylogeny

A homology is a similarity between two organisms that exists because the two organisms are closely evolutionarily related (that is, the feature in question existed in the common ancestor to the two organisms). The similarity of the DNA (or RNA) of organisms may be determined by a number of means including determinations of base composition, nucleotide sequence, or DNA hybridization rates. Typically these means include very powerful ways by which organisms may be classified, either in terms of distinctions between organisms (i.e. the organisms may be classified as representing two or more species) or similarities (i.e. it may be concluded from evidence of genotypic similarity that the organisms are closely related, i.e. evolutionarily related); the latter similarities we would classify as a genetic homology. The downside of genetic homology is that the acquisition of data often requires a laboratory and at least a little effort. The upside is that genetic homology describes evolutionary relationships with only minimal interference from phenotype.

9.3 Numerical Taxonomy

It is a classification system in biological systematics which deals with the grouping by numerical methods of taxonomic units based on their character states. It aims to create a taxonomy using numeric algorithms like cluster analysis. The concept was first developed by Robert R. Sokal and Peter H. A. Sneath in 1963. Phenetics is a closely related discipline and draws heavily from the methods of numerical taxonomy.

Although intended as an objective classification method, in practice the choice and weighing of morphological characteristics is often guided by available methods and research interests. Furthermore, the general consensus has become that the taxonomic classification should reflect evolutionary (phylogenetic) processes. Some connections between phylogenetic trees and the spectral decomposition of the variance-covariance matrix of quantitative traits subject to Brownian motion over time have been established, providing a theoretical link between phylogenetic methods and numerical taxonomy. The specific phenetic algorithms proposed in numerical taxonomy, however, often fail to properly reconstruct the phylogenetic history of organisms.

9.4 All Species Inventory

In 2001 an international project was launched to identify and record every species on earth in the next 25 years. It is a very challenging undertaking considering that to date 1.5 million organisms have been named. It is estimated that anywhere from 7 – 100 million living species exist.

9.5 Discovery of New Microorganisms in the Stratosphere

On March 18th, 2009, three new species of bacteria, which are not found on earth and which are highly resistant to ultra-violet radiation, were discovered in the upper stratosphere by Indian scientists. The new species have been named as:

- *Janibacter hoylei*, after the distinguished astrophysicist Fred Hoyle
- *Bacillus isronensis* recognizing the contribution of Indian Space Research Organization (ISRO) in the balloon experiments which led to its discovery

- *Bacillus aryabhata* after India's celebrated ancient astronomer Aryabhata and also the first satellite of ISRO.

Lesson 10
SIZE, SHAPE AND ARRANGEMENT OF PROKARYOTIC CELLS

10.1 Prokaryotic Cell

Antony van Leeuwenhoek invented the microscope in the late 1600s, which first showed that all living things are composed of cells. Also, he was the first to see microorganisms. Light microscopes have a limited resolution; magnification of more than about 2000-fold does not improve what one can see. Electron microscopes use electrons instead of light. The short wavelength of electrons allows magnifications much better than visible light. Prokaryotes are much more diverse in both habitat and metabolism than the eukaryotes. However, prokaryotes are not very diverse in body shape or size. Much of their classification into different species is done by examining their internal biochemistry and their DNA. Nearly all prokaryotes are single-celled. Differentiation into different cell types almost never occurs in prokaryotes.

Prokaryotes are simple cells. The DNA is loose in the cytoplasm; there is no separate nucleus. The ribosomes are also in the cytoplasm. In prokaryotes, transcription (synthesis of RNA) and translation (synthesis of proteins) occurs simultaneously. The cell is surrounded by a membrane, but there are no internal membranes. Outside the membrane is a cell wall and sometimes an outer capsule which can have structures projecting form it. Bacteria move using flagella; whip-like hairs similar to the flagellum of a sperm cell.

10.2 Shapes of Bacteria

Prokaryotes display following shapes (Fig. 10.1)

- Spherical, rod like and spiral but variation abound
- Spherical cells called cocci (Greek = berry)
- Cylinders called rods or bacilli (Latin bacillus = walking stick)
- Coma shaped: Vibrio
- Spiral: Spirilli (Greek sprillum = little coil)
- Corkscrew shaped: Spirochete
- Others: Star, Square (discovered on the shores of Red Sea in 1981; 2-4 μm on a side and sometimes aggregated in waffle like sheets)

Click for Animation (Fig.10.1. Shape and Arrangement of Bacteria)

Bacterial shapes, depending on the organism, can change subtly when cells are growing or existing under different conditions, e.g. a shortening of rods as nutrient concentrations are used up and therefore as growth rates decline. Most bacteria are monomorphic - they maintain the same shape. Nevertheless, some bacteria do not display a constant shape even during growth in an otherwise unchanging, homogeneous environment. Such bacteria are termed pleomorphic to indicate that they do not possess a relatively constant standard shape even under relatively constant, standard conditions shape (e.g. Rhizobium and Corynebacterium).

10.3 Arrangement

Cells are found in distinctive arrangements of group of cells; Occur when cell divide without separating. Cocci can divide in one or more planes leading to different arrangements of cells as follows (Fig. 10.2)

- Division in one plane in pairs (Diplococci) or in chains (Streptococci)
- Division in two planes: Tetrads (4 cells in a cube)
- Division in 3 planes: Sarcinae (8 cells arranged in a cube)
- Random division: Grape like clusters (Staphylococci)
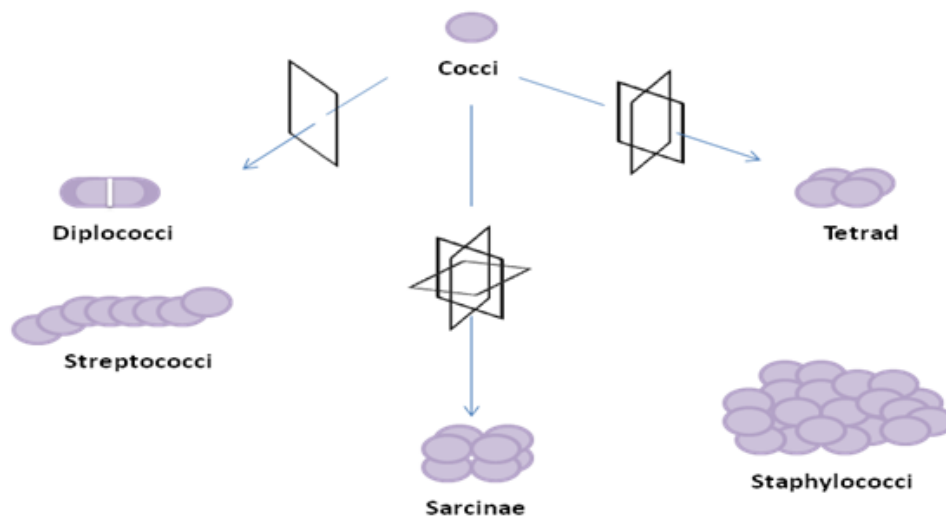
Fig. 10.2 Arrangement of cocci

However, bacilli divide in one plane (Exception: Bacilli arranged in a rosette attached by stalks to a substrate e.g. Caulobacter and spiral not generally grouped together). Like cocci these can be termed as diplo and streptobacilli (Fig. 10.3).
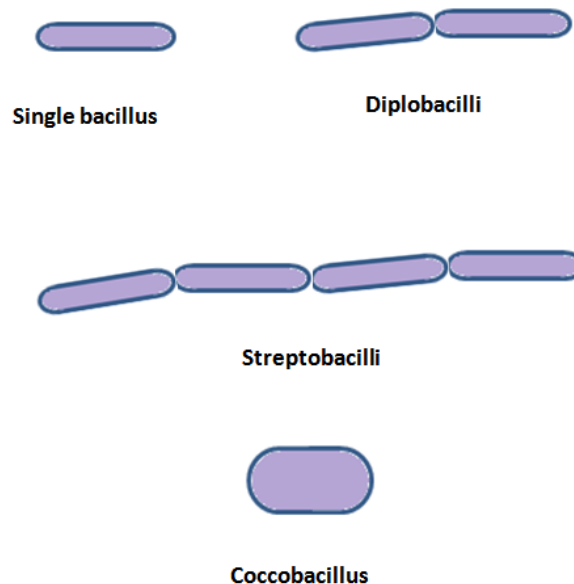
Fig. 10.3 Arrangement of rod shaped bacteria

10.4 Size of Prokaryotic Cells

Prokaryotes are smallest of all living cells (1-5 µm long and 1-2 µm in dia). Some spiral has much larger 'diameter' and some cyanobacteria are 60 µm long. The dot of typed 'i' can accommodate about 500 bacteria of 1 µm size if placed from end to end. The largest bacterium isolated from sediments of the coast of Namibia (1 mm in dia) *Thiomargarita nambiensis*, visible to naked eyes, in 1998.

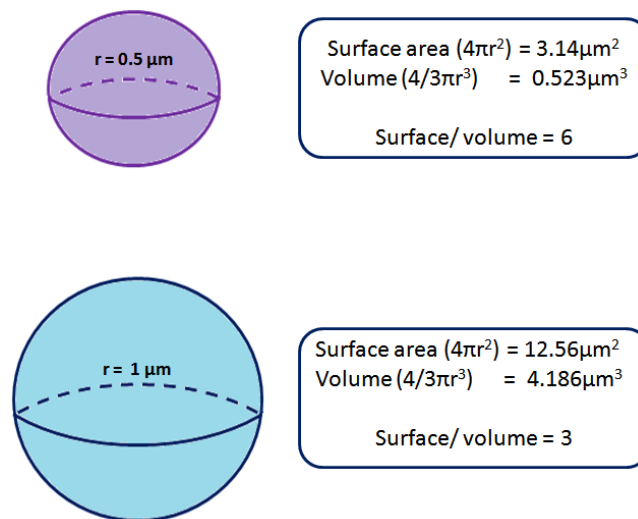Small is better: Average bacteria 0.5 - 2.0 µm in diameter with a surface area ~12 µm2 and volume (Vol) ~4 µm. Thus surface area (SA) to volume is 3:1 and can reach to 6:1 in case of smaller bacteria (Fig. 10.4). On the other hand typical eukaryote cell SA/Vol is 0.3:1 as eukaroytes need structures and organelles.

r = 0.5 µm

Surface area $(4\pi r^2)$ = 3.14µm^2
Volume $(4/3\pi r^3)$ = 0.523µm^3

Surface/ volume = 6

r = 1 µm

Surface area $(4\pi r^2)$ = 12.56µm^2
Volume $(4/3\pi r^3)$ = 4.186µm^3

Surface/ volume = 3

Fig. 10.4 Smaller the size; larger the surface area/volume ratio

Large surface area/volume ratio in tiny prokaryotic cells gives a number of advantages as follows:

- Cocci: More resistant to drying
- Rods: More surface area and easily takes in dilute nutrients from the environment
- Spiral: Corkscrew motion and therefore less resistant to movement
- Square: Assists in dealing with extreme salinities

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Lesson 11
CELL WALL, CYTOPLASMIC MEMBRANE, MEMBRANE TRANSPORT SYSTEMS

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11.1 Bacterial Cell Wall

The bacterial cell wall is a unique structure which surrounds the cell membrane. Although not present in every bacterial species, the cell wall is very important as a cellular component. Structurally, the wall is necessary for:

- Maintaining the cell's characteristic shape- the rigid wall compensates for the flexibility of the phospholipid membrane and keeps the cell from assuming a spherical shape
- Countering the effects of osmotic pressure- the strength of the wall is responsible for keeping the cell from bursting when the intracellular osmolarity is much greater than the extracellular one
- Providing attachment sites for bacteriophages - teichoic acids attached to the outer surface of the wall are like landing pads for viruses that infect bacteria
- Providing a rigid platform for surface appendages - flagella, fimbriae, and pili all emanate from the wall and extend beyond it

All the members of domain Bacteria, with the exception of the genera Mycoplasma, Ureaplasma, Spiroplasma, and Anaeroplasma contain cell walls.

11.2 Chemical Composition of Bacterial Cell wall

Cell walls are chemically peptidoglycans i.e. peptides (short amino acids chains) and glycans (sugars); peptidoglycans are also known as mureins, mucopeptide (Fig. 11.1).

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(Click for Animation Fig.11.1 Synthesis of Cellular Structures)

</div>

11.2.1 Gram Positive Bacterial Cell Wall

The cell wall of Gram positive bacteria is as thick as 20-80 nm and chemically contains mostly peptidoglycan (>50%). Proteins and other molecules diffuse freely into and rough the peptidoglycan (Fig. 11.2)

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(Click for Animation Fig. 11.2 Cell wall of Gram Positive Bacteria)

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11.2.1.1 Glycans

These are modified sugars viz. N-acetyl muramic acid (NAM or M) and N-acetly glucose amine (NAG or G). M and G are linked to each other by a beta 1, 4 glycosidic bond and alternate to form the wall backbone. Lysozyme (an enzyme produced by organisms that consume bacteria, and normal body secretions such as tears, saliva, and egg white = protect against would-be pathogenic bacteria) digests beta 1, 4 glycosidic bonds. It growing or non growing cells but cell wall-less microbes are not affected high osmotic pressure in high solute concentrations prevents lysis of Gram positive and Gram negative cells when treated with lysozyme. As such, it has mild

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antibacterial action and indeed was one of the first antibiotics studied by Sir Fleming, the discoverer of penicillin.

11.2.1.2 Peptides

Short peptides (4 amino acids, tetrapeptides) are attached to M. Some of the amino acids are only found in cell walls and not in other cellular proteins (D- amino acids, eg D-alanine and diaminopimelic acid, DAP). Tetrapeptides chains are crosslinked (interlinked) by a peptide bridge (the carboxyl group of one tetrapeptide with an amino group of an adjacent (direct interbridge) or a different tetrapeptide chain (indirect interbridge). Transpeptidase enzyme builds peptide bridges in actively dividing cells; penicillin binds to it, stopping cell wall synthesis. Autolysins restructure and reshape cell walls by breaking specific bonds in the peptidoglycan in actively growing cells. Cell wall synthesis stops but cell degrading enzymes still function resulting in weakened cell walls and ultimately death. Glycans and peptides therefore form a single, large and strong cross- linked molecule in a form of a multilayered sheet, (sacculus, Latin = little sac) that surrounds the entire bacterial cell.

11.2.1.3 Teichoic acid

It consists of glycerol, phosphates and sugar alcohol, ribitol; occurs in polymers of 30 units long; extends beyond the cell wall and its functions include:

- Attachment sites for phages
- Binds protons and thus maintain cell wall at low pH which prevents autolysis by autolysins
- Teichuronic acid: formed when phosphate concentration low; help conserve phosphate essential for ATP, DNA and other cellular components
- Are phosphate chains of uronic acid and NAG

11.2.1.4 Synthesis of peptidoglycan

(Click for Animation Fig 11.3 Synthesis of Peptidoglycan)

The peptidoglycan monomers are synthesized in the cytosol of the bacterium where they attach to a membrane carrier molecule called bactoprenol (Fig. 11.3). Bacterial enzymes called autolysins break both the glycosidic bonds at the point of growth along the existing peptidoglycan, as well as the peptide cross-bridges that link the rows of sugars together. Bactoprenol and transglycosidase enzymes then insert the new peptidoglycan monomers into the breaks in the peptidoglycan. Transglycosidase enzymes catalyze the formation of glycosidic bonds between the NAM and NAG of the peptidoglycan momomers and the NAG and NAM of the existing peptidoglycan. Finally, transpeptidase enzymes reform the peptide cross-links between the rows and layers of peptidoglycan to make the wall strong. During normal bacterial growth, bacterial enzymes called autolysins put breaks in the peptidoglycan in order to allow for insertion of peptidoglycan building blocks (monomers of NAG-NAM-peptide). These monomers are then attached to the growing end of the bacterial cell wall with transglycosidase enzymes. Finally, transpeptidase enzymes join the peptide of one monomer with that of another in order to provide strength to the cell wall. Penicillins and cephalosporins bind to the transpeptidase enzyme and block the formation of the peptide cross-

links. This results in a weak cell wall and osmotic lysis of the bacterium.

11.2.2 Gram negative bacterial cell wall

The cell wall of Gram negative bacteria contains relatively thin (~10 nm) layer of peptidoglycan comprising only10 – 20 % of wall (Fig. 11.4).

(Click for Animation Fig.11.4. Cell Wall of Gram Negative Bacteria)

Outside the peptidoglycan layer exists bilayered outer membrane which contains Phospholipids, protein,lipoprotein and Lipoplysaccharides. Its inner layer consists of phospholipid while outer layer is made of lipopolysaccharide (LPS or endotoxin). Functionally, outer membrane is a coarse molecular sieve and permeability to nutrients is partly due to Omp, called porins, which form cross-membrane channels through which some molecules can diffuse. Molecules with molecular weight 800 kDa, for E. coli and higher (3000-10000 kDa) for Pseudomonas neisseria can pass through outer membrane. Pores in porin don't allow molecules as large as proteins to cross. The outer membrane excludes external protein from the periplasm and keeps proteins secreted by cell to periplasm . It also protects peptidoglycan from Lysozyme and antibiotic by keeping them out. Bacterial lipopolysaccharides are toxic to animals. When injected in small amounts endotoxins activate macrophages to produce pyrogens, activate the complement cascade causing inflammation, and activate blood factors resulting in intravascular coagulation and hemorrhage. Endotoxins may play a role in infection by any Gram negative bacterium. The toxic component of endotoxin is lipid A. The O-specific polysaccharide may provide ligands for bacterial attachment and confer some resistance to phagocytosis. Variations in the exact sugar content of the O polysaccharide (also referred to as the O antigen) accounts for multiple antigenic types (serotypes) among Gram negative bacterial pathogens. Therefore, even though lipid A is the toxic component in lipopolysaccharide, it contributes to virulence of Gram negative bacteria.

A bacterial surface component plays an indispensable role in the pathogenesis of infectious disease. Bacterial surface structures may act as:

- Permeability barriers that allow selective passage of nutrients and exclusion of harmful substances (e.g. antimicrobial agents);
- Adhesins used to attach or adhere to specific surfaces or tissues;
- Enzymes to mediate specific reactions on the cell surface important in the survival of the organism;
- Protective structures against phagocytic engulfment or killing;
- Antigenic disguises;
- Sensing proteins that can respond to temperature, osmolarity, salinity, light, oxygen, nutrients, etc., resulting in a molecular signal to the genome of the cell that will cause expression of some determinant of virulence (e.g. an exotoxin).

In medical situations, the surface components of bacterial cells are major determinants of virulence for many pathogens. Pathogens can colonize tissues, resist phagocytosis and the immune response, and induce inflammation, complement activation and immune responses in animals by means of various structural components.

Table 11.1 Functions of the outer membrane components of Escherichia coli.

Component	Function
Lipopolysaccharide (LPS)	Acts as permeability barrier
Mg++ bridges	Stabilizes LPS and is essential for permeability characteristics
Braun Lipoprotein	Anchors outer membrane to peptidoglycan
Omp C and Omp F porins	Proteins that form pores or channels through outer membrane for passage of hydrophilic molecules
Omp A protein	Provides receptor for some viruses and bacteriocins; stabilizes mating cells during conjugation

11.2.3 Archaeal cell walls

Archaeal cells have more variations in their cell wall chemistries, and some do not contain cell walls (e.g. Thermoplasma). Methanobacterium sp. contains glycans (sugars) and peptides in their cell walls. Glycans are modified sugars viz. N-acetyl talosaminouronic acid (NAT or T) and N-acetly glucose amine (NAG or G). T and G are linked to each other by a beta 1, 3 glycosidic bond and alternate to form the cell wall backbone. Lysozyme (an enzyme produced by organisms that consume bacteria, and normal body secretions such as tears, saliva, and egg white; protect against would-be pathogenic bacteria) cannot digest beta 1, 3 glycosidic bonds. Peptides are short peptides attached to T. The amino acids are only of the L-type. Penicillin is ineffective in inhibiting the cell wall peptide bridge formation. Some examples of unique cell wall composition are given below:

- Methanosarcina sp. cell walls contain non-sulfated polysaccharides
- Halococcus sp. contain sulfated polysaccharides similar to Methanosarcina sp.
- Halobacterium sp. contain negatively charged acidic amino acids in their cell walls which counteract the positive charges of the high Na+ environment. Therefore, cells lyse in NACl concentrations below 15%
- Methanomicrobium sp. and Methanococcus sp. cell walls are exclusively made up of proteins subunits.

11.3 Periplasmic Space

It is the region between cell membrane and outer membrane/peptidoglycan, an area where diverse reaction occurs - osmoregulation, solute transport, protein secretion and hydrolytic activities. The term gel implies that peptidoglycan may actually fill the region between cell membrane and outer membrane. Several proteins are found e.g. binding proteins (transport), chemoreceptors (chemotaxis), hydrolytic enzymes (transportation of small products). It also stores enzymes like alkaline phosphatase which degrade incoming DNA from other bacteria. It contains oligosaccharides that help in adjustment with change in osmolarity of medium. The periplasmic space is 20 to 40% of total volume of cells grown under typical conditions.

Entry of sugars into periplasmic space: The outer membrane is penetrated by water-filled pores. Each pore consists of a trimer of porin and has a diameter of 1.5-2.0 nm. Substances of molecular weight less than 650 may diffuse rapidly through the pore (if hydrophilic). Substances of molecular

weight greater than 900-1000 are excluded. The porins are arranged on the outer surface of the mucopeptide in a regular array and are mostly beta-sheet. They are tightly, but non-covalently, bound to the mucopeptide. Vesicles, reconstituted from lipid, LPS and porins show the same diffusion properties as an intact cell. E. coli K-12 has two porins, coded by genes ompC and ompF. (omp = Outer Membrane Protein). A regulatory gene ompB controls the expression of the ompC and F proteins, for example, when osmotic pressure increases, ompF decreases and ompC increases. Somehow, ompB detects changes in osmotic pressure. Both ompC and ompF act as receptors for certain bacteriophages.

11.4 Plasma Membrane

Plasma membrane (cytoplasmic membrane) is a biological membrane that separates the interior of all cells from the outside environment. The cell membrane is selectively-permeable to ions and organic molecules and controls the movement of substances in and out of cells.

11.4.1 Functions of plasma membrane

The plasma membrane retains the cytoplasm, particularly in cells without cell walls, and separates (the cell) from the surroundings. The plasma membrane of prokaryotes and eukaryotes are functionally equivalent, though the prokaryote plasma membrane additionally serves in roles that eukaryotes reserve for internal membranes. Various functions of the prokaryotic plasma membrane are as follows:

- Osmotic or permeability barrier
- Location of transport systems for specific solutes (nutrients and ions)
- Energy generating functions, involving respiratory and photosynthetic electron transport systems, establishment of proton motive force, and transmembranous, ATP-synthesizing ATPase
- Synthesis of membrane lipids (including lipopolysaccharide in Gram-negative cells)
- Synthesis of murein (cell wall peptidoglycan)
- Assembly and secretion of extracytoplasmic proteins
- Coordination of DNA replication and segregation with septum formation and cell division
- Chemotaxis (both motility per se and sensing functions)
- Location of specialized enzyme system

11.4.2 Structure of Plasma Membrane

The plasma membrane is approximately 7.5 nm (0.0075 μm) thick, forms the limiting boundary of the cell and is made up of phospholipids (about 20-30%) and proteins (about 60-70%) as illustrated in Fig. 11.5.

(Click for Animation Fig. 11.5 Structure of Plasma Membrane)

Several models have been proposed to explain the ultra-structure of the plasma membrane; the most widely accepted one is Fluid Mosaic Model introduced by Singer and Nicolson (1974) as depicted in Fig.11.6.

(Click for Animation Fig.11.6 Fluid Mosaic Model of Plasma Membrane)

According to this model the membrane is a bi-layer of phospholipids and the two opposing layers of phospholipids overlap slightly; each phospholipid molecule consisting of a phosphate group and a lipid. Each phospholipid is structurally asymmetric with polar and nonpolar ends and is called amphipathic (Fig. 11.7). The polar ends interact with water and are hydrophilic; the nonpolar ends do not interact with water (i.e. insoluble in water) and are hydrophobic. The hydrophilic ends occur towards the outer surface of the membrane whereas the hydrophobic ends are burried in the interior away from the surrounding water. The arrangement of hydrophilic heads and hydrophobic tails of the lipid bilayer prevent polar solutes (e.g. amino acids, nucleic acids, carbohydrates, proteins, and ions) from diffusing across the membrane, but generally allows for the passive diffusion of hydrophobic molecules. This affords the cell the ability to control the movement of these substances via transmembrane protein complexes such as pores and gates.
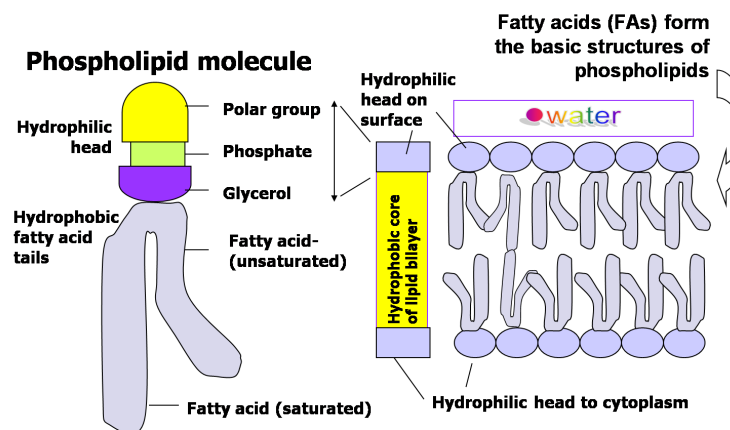
Fig. 11.7 Structure of lipid bilayer and phospholipids

Unsaturated fatty acids remain liquid at low temperatures and become denatured as the temperatures increase, however saturated fatty acids are more stable than unsaturated fatty acids at high temperatures, e.g. butter is solid at room temperature. The membranes of psychrophilic (cold-loving) bacteria have high content of polyunsaturated fatty acids. Thermophilic bacteria use mainly saturated fatty acids, otherwise their membrane would be too soft to maintain cell structure and function at high temperature.

The bi-layer phospholipid is interrupted by proteins which are distributed in a mosaic-like pattern (Fig. 11.4). Some of the proteins are confined to the outer surface of bilipid layer (extrinsic or peripheral proteins) and others are partially or totally buried within it (intrinsic or integral proteins). The integral proteins, like membrane lipids, are amphipathic. Their hydrophobic regions are buried in the lipid while the hydrophilic regions project out from the plasma membrane surface.

The cell membrane plays host to a large amount of protein that is responsible for its various activities. The amount of protein differs between species and according to function, however the typical amount in a cell membrane is 50%. Proteins are in dynamic state and distribution is according to the fluid mosaic model. On the basis of their location and interaction in plasma

membrane these proteins can be broadly termed as integral, lipid anchored and peripheral proteins and can be categorized in following six groups on the basis of their functions:

- Transport proteins
- Receptor proteins
- Enzymatic proteins
- Cell recognition proteins
- Attachment proteins
- Intercellular junction proteins

Sterols are not present in bacteria but are present in cell wall less bacteria (Mycoplasma, Ureaplasma, Spiroplasma, Anaeroplasma) - required for growth, provide stability e.g. sterols. Poylene antibiotics (e.g. nystatin, candicidin) inhibit growth by interacting with sterols and destabilizing eukaryotic and cell wall-less bacterial membranes (but do not inhibit growth of cell wall containing bacteria). Often carbohydrates are attached to the outer surface of plasma membrane proteins and seem to perform important functions. Both proteins and lipids move within the phospholipid matrix of the membrane. However, many bacterial plasma membranes do contain pentacyclic sterol-like molecules called hopanoids which are synthesized from the same precursors as steroids. Like steroids in eukaryotic cells, hopanoids are thought to provide stability to bacterial plasma membrane.

11.5 Differences with Eukaryotic Plasma Membrane

Although the bacterial plasma membrane resembles its counterpart of eukaryotic cells, it differs from the latter in two distinctive features:

1. Sterols (such as cholesterols) that occur in eukaryotic cell membranes are absent in bacteria (except in the mycoplasmas that do not have cell wall). These substances help stabilize the phospholipids in eukaryotic membrane and make it more rigid.
2. The proportion of protein to phospholipid is high (typically 2:1 in prokaryotes, and 1:1 or less in eukaryotes).

11.6 Membrane Transport Systems

There are four basic types of transport systems (Fig.11.8).

(Click for Animation Fig.11.8. Membrane Transport Systems)

11.6.1 Passive diffusion

Passive diffusion is the net movement of gases or small uncharge polar molecules across a phospholipid bilayer membrane from an area of higher concentration to an area of lower concentration. Examples of gases that cross membranes by passive diffusion include N_2, O_2, and CO_2; examples of small polar molecules include ethanol, H_2O, and urea.

11.6.2 Facilitated diffusion

The rate of diffusion across selectively permeable membranes is greatly increased by the use of carrier proteins, sometimes called permeases, which are embedded in the plasma membrane. Since the diffusion process is aided by a carrier, it is called facilitated diffusion. The rate of facilitated diffusion increases with the concentration gradient much more rapidly and at lower concentrations of the diffusing molecule than that of passive diffusion.

11.6.3 Active transport

Active transport is the transport of solute molecules to higher concentrations, or against a concentration gradient, with the use of metabolic energy input. In active transport the target is not altered and a significant accumulation occurs in the cytoplasm with the inside concentration reaching many times its external concentration. Active transport proteins are molecular pumps that pump their substrates against a concentration gradient. As in all pumps, fuel is necessary and in the case of cells, this fuel comes in two forms, ATP or the proton motive force (PMF). Ion driven transport systems (IDT) and binding-protein dependent transport systems (BPDT) are active transport systems that are used for transport of most solutes by bacterial cells. The former is driven by PMF while ATP is used to drive later system. IDT is used for accumulation of many ions and amino acids; BPDT is frequently used for sugars and amino acids.

11.7 Group Translocation

In this system, a protein specifically binds the target molecule and during transport a chemical modification takes place. No actual concentration of the transported substance takes place, because as it enters the cell, it becomes chemically different. The best known group translocation system is the phosphoenolpyruvate: sugar phosphotransferase system (PTS), which transports a variety of sugars into prokaryotic cells, while simultaneously phosphorylating them using phosphoenolpyruvate (PEP) as the phosphate donor. Table 11.2 enlists the distinct features of various prokaryotic transport systems.

Table 11.2 Distinguishing characteristics of bacterial transport systems

Characteristics	Passive diffusion	Facilitated diffusion	Ion-driven	Binding protein dependent	Group translocation
Carrier mediated	No	Yes	Yes	Yes	Yes
Conc. against gradient	No	No	Yes	Yes	No
Specificity	No	Yes	Yes	Yes	Yes
Energy expended	No	No	PMF	ATP	PEP
Solute modified during transport		No	No	No	Yes

Lesson 12
CYTOPLASM, CYTOPLASMIC INCLUSIONS AND VACUOLES, CYTOSKELETON

12.1 Cytoplasm

The term cytoplasm refers to everything between the cell membrane and the nuclear envelope. The cytoplasm consists of water, proteins including enzymes, vitamins, ions, nucleic acids and their precursors, amino acids and their precursors, sugars, carbohydrates and their derivatives, fatty acids and their derivatives. It serves as a fluid container for cell organelles and other cell substances. It is relatively featureless by electron microscope - although small granules can be seen. This is the area in which all of the work of the cell is done and contains all chemicals and structures to do that work. Things occurring within this area are protein synthesis, DNA and ribonucleic acid (RNA) synthesis, energy transfer, and preparation for cell division. Prokaryotic cell consists mainly of cytoplasm as it has only a few clearly defined structures

The cytoplasm of Prokaryotic cell is about 80% water and 20% dissolved substances which include enzymes for energy production and synthesis of cellular components such as peptidogoglycan, lipids, inorganic ions. The eukaryotic cell's cytoplasm makes up a relatively smaller portion of cell as it contains nucleus, other membrane bound organelles, and cytoskeleton. It carries out a characteristic movement known as 'streaming'.

12.2 Ribosomes

Ribosomes are cytoplasmic organelles found in prokaryotes and eukaryotes. Ribosomes are small, but complex structures, roughly 20 to 30 nm in diameter, consisting of two unequally sized subunits, referred to as large and small which fit closely together. A subunit is composed of a complex between RNA molecules and proteins; each subunit contains at least one ribosomal RNA (rRNA) subunit and a large quantity of ribosomal proteins. Small prokaryotic and eukaryotic ribosomal subunits have a head and a base with an arm like platform extending from one side (Fig. 12.1). Eukaryotic ribosomes are larger and more complex than prokaryotic ribosomes. The main function of ribosomes is to serve as the site of mRNA translation (protein synthesis, the assembly of amino acids into proteins); once the two (large and small) subunits are joined by the mRNA from the nucleus, the ribosome translates the mRNA into a specific sequence of amino acids, or a polypeptide chain (Fig. 12.2).

(Click for Animation Fig.12.1 Structure of Prokaryotic Ribosome)

(Click for Animation Fig.12.2. Protein Synthesis)

12.3 Cytoplasmic Inclusions

Often contained in the cytoplasm of procaryotic cells is one or another of some type of inclusion granule (Table 12.1). Inclusions are distinct granules that may occupy a substantial part of the cytoplasm. Inclusion granules are usually reserve materials of some sort. For example, carbon and energy reserves may be stored as glycogen (a polymer of glucose) or as polybetahydroxybutyric acid (a type of fat) granules. Some inclusion bodies are actually membranous vesicles or intrusions

into the cytoplasm which contain photosynthetic pigments or enzymes.

12.3.1 Poly-beta-hydroxyalkanoate (PHA)

The PHA, one of the more common storage inclusions is in fact a long polymer of repeating hydrophobic units that can have various carbon chains attached to them. The most common form of this class of polymers is poly-beta-hydroxybutyrate that has a methyl group as the side chain to the molecule. Some PHA polymers have plastic like qualities and there is some interest in exploiting them as a form of biodegradable plastic. The function of PHA in bacteria is as a carbon and energy storage product. Just as we store fat, bacteria store PHA.

12.3.2 Glycogen

Glycogen is another common carbon and energy storage product. Humans also synthesize and utilize glycogen. Glycogen is a polymer of repeating glucose units.

12.3.3 Phosphate and sulfur globules

Many organisms will accumulate granules of polyphosphate, since this is a limiting nutrient in the environment. The globules are long chains of phosphate. Photosynthetic bacteria that do not evolve oxygen will often use sulfides as their source of electrons. Some of them accumulate sulfur globules. These globules may later be further oxidized and disappear if the sulfide pool dries up.

Table12.1 Cytoplasmic Inclusions ptresent in Bacteria

Cytoplasmic inclusions	Where found	Composition	Function
Glycogen	Many bacteria e.g. *E. coli*	Polyglucose	Reserve carbon and energy source
Polybetahydroxybutyric acid (PHB)	Many bacteria e.g. *Pseudomonas*	Polymerized hydroxy butyrate	Reserve carbon and energy source
Polyphosphate (volutin granules)	Many bacteria e.g. *Corynebacterium*	Linear or cyclical polymers of PO4	Reserve phosphate; possibly a reserve of high energy phosphate
Sulfur globules	Phototrophic purple and green sulfur bacteria and lithotrophic colorless sulfur bacteria	Elemental sulfur	Reserve of electrons (reducing source) in phototrophs; reserve energy source in lithotrophs
Gas vesicles	Aquatic bacteria especially cyanobacteria	Protein hulls or shells inflated with gases	Buoyancy (floatation) in the vertical water column
Parasporal crystals	Endospore-forming bacilli (genus *Bacillus*)	Protein	Unknown but toxic to certain insects
Magnetosomes	Certain aquatic bacteria	Magnetite (iron oxide) Fe_3O_4	Orienting and migrating along geo-magnetic field lines
Carboxysomes	Many autotrophic bacteria	Enzymes for autotrophic CO_2 fixation	Site of CO_2 fixation
Phycobilisomes	Cyanobacteria	Phycobiliproteins	Light-harvesting pigments
Chlorosomes	Green bacteria	Lipid and protein and bacteriochlorophyll	Light-harvesting pigments and antennae

12.4 Cytoskeleton

The cytoskeleton is a cellular 'scaffolding' or 'skeleton' contained within the cytoplasm and is made out of protein. The cytoskeleton is present in all cells; it was once thought to be unique to eukaryotes (Fig. 12.3), but recent research has identified the prokaryotic cytoskeleton as well. It is a dynamic structure that maintains cell shape, protects the cell, enables cellular motion (using structures such as flagella, cilia and lamellipodia), and plays important roles in both intracellular transport (the movement of vesicles and organelles, for example) and cellular division.
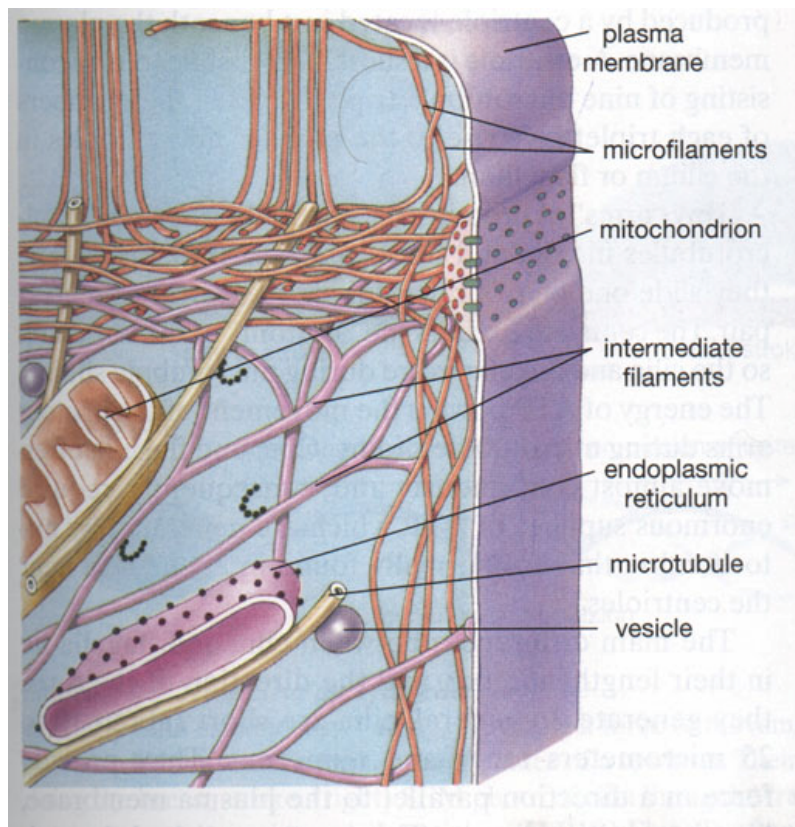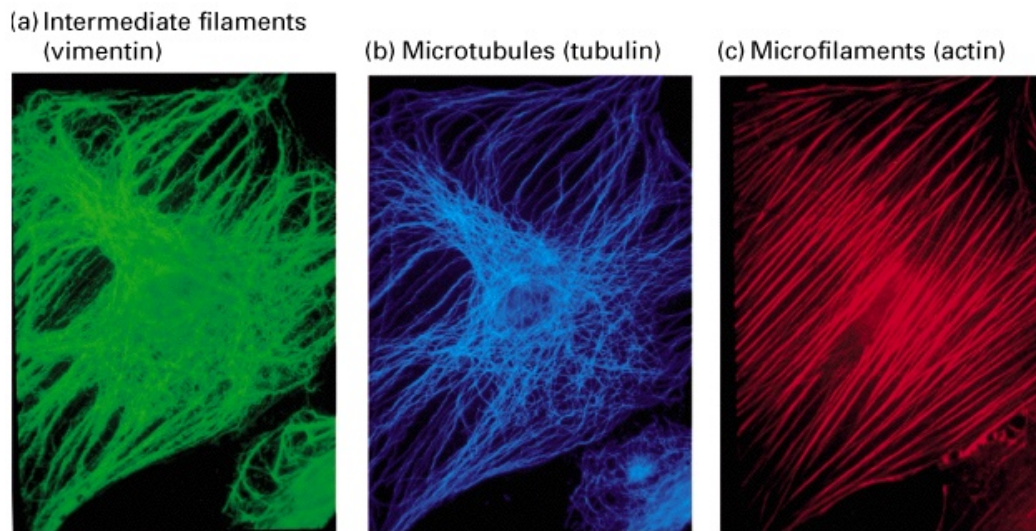
Fig. 12.3 The eukaryotic cytoskeleton

12.4.1 The eukaryotic cytoskeleton

Eukaryotic cells contain three main kinds of cytoskeletal filaments, which are microfilaments, intermediate filaments, and microtubules (Fig. 12.4). The cytoskeleton provides the cell with structure and shape, and by excluding macromolecules from some of the cytosol. Cytoskeletal elements interact extensively and intimately with cellular membranes.

12.4.1.1 Actin filaments / microfilaments

Around 6 nm in diameter, this filament type is composed of two intertwined chains. Microfilaments are most concentrated just beneath the cell membrane, and are responsible for resisting tension and maintaining cellular shape, forming cytoplasmatic protuberances (like pseudopodia and microvilli), and participation in some cell-to-cell or cell-to-matrix junctions. In association with these latter roles, microfilaments are essential to transduction. They are also important for cytokinesis (specifically, formation of the cleavage furrow) and, along with myosin, muscular contraction. Actin/myosininteractions also help produce cytoplasmic streaming in most cells. Microtubules serve as conveyor belts moving other organelles through the cytoplasm, and are the major components of cilia and flagella, and participate in the formation of spindle fibers during cell division (mitosis).

Fig. 12.4 Cytoskeletal filaments of eukaryotic cytoskeleton

12.4.1.2 Intermediate filaments

These filaments, around 10 nm in diameter, are more stable (strongly bound) than actin filaments, and heterogeneous constituents of the cytoskeleton. Although little work has been done on intermediate filaments in plants, there is some evidence that cytosolic intermediate filaments might be present, and plant nuclear filaments have been detected. Like actin filaments, they function in the maintenance of cell-shape by bearing tension (microtubules, by contrast, resist compression). It may be useful to think of micro and intermediate filaments as cables, and of microtubules as cellular support beams. Intermediate filaments organize the internal tridimensional structure of the cell, anchoring organelles and serving as structural components of the nuclear lamina and sarcomeres. They also participate in some cell-cell and cell-matrix junctions.

12.4.1.3 Microtubules

Microtubules are hollow cylinders about 23 nm in diameter (lumen = approximately 15 nm in dia), most commonly comprising 13 protofilaments which, in turn, are polymers of alpha and beta tubulin. They have a very dynamic behaviour, binding GTP for polymerization. They are commonly organized by the centrosome. In nine triplet sets (star-shaped), they form the centrioles, and in nine doublets oriented about two additional microtubules (wheel-shaped) they form cilia and flagella. The latter formation is commonly referred to as a '9+2' arrangement, wherein each doublet is connected to another by the protein dynein. As both flagella and cilia are structural components of the cell, and are maintained by microtubules, they can be considered part of the cytoskeleton.

12.4.2 The prokaryotic cytoskeleton

The cytoskeleton was previously thought to be a feature only of eukaryotic cells, but homologues to all the major proteins of the eukaryotic cytoskeleton have recently been found in prokaryotes. Although the evolutionary relationships are so distant that they are not obvious from protein sequence comparisons alone, the similarity of their three-dimensional structures and similar functions in maintaining cell shape and polarity provides strong evidence that the eukaryotic and

prokaryotic cytoskeletons are truly homologous. However, some structures in the bacterial cytoskeleton may have yet to be identified (Fig. 12.5).

12.4.2.1 FtsZ

FtsZ was the first protein of the prokaryotic cytoskeleton to be identified. Like tubulin, FtsZ forms filaments in the presence of GTP, but these filaments do not group into tubules. During cell division, FtsZ is the first protein to move to the division site, and is essential for recruiting other proteins that synthesize the new cell wall between the dividing cells.

12.4.2.2 MreB and ParM

Prokaryotic actin-like proteins, such as MreB, are involved in the maintenance of cell shape. All non-spherical bacteria have genes encoding actin-like proteins, and these proteins form a helical network beneath the cell membrane that guides the proteins involved in cell wall biosynthesis. Some plasmids encode a partitioning system that involves an actin-like protein ParM. Filaments of ParM exhibit dynamic instability, and may partition plasmid DNA into the dividing daughter cells by a mechanism analogous to that used by microtubules during eukaryotic mitosis.

12.4.2.3. Crescentin

The bacterium Caulobacter crescentus contains a third protein, crescentin, which is related to the intermediate filaments of eukaryotic cells. Crescentin is also involved in maintaining cell shape, such as helical and vibrioid forms of bacteria, but the mechanism by which it does this is currently unclear.
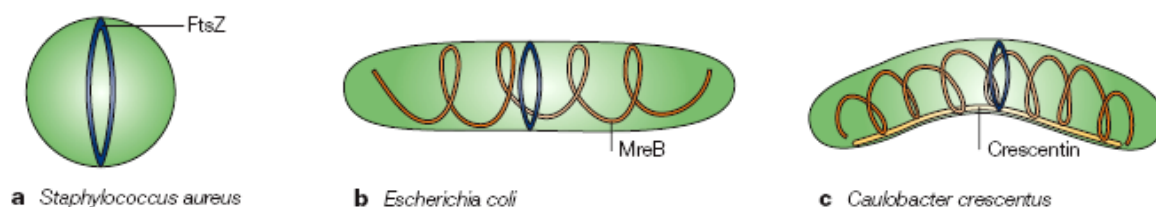
Fig. 12.5 Division proteins of bacterial cells

a) Cells such as Staphylococcus aureus contain the tubulin-like division protein FtsZ, which is present in virtually all eubacteria. Whereas FtsZ forms a ring-shaped structure (blue) during cell division that is required for the division process, it seems to impart no shape to non-dividing cells. Therefore, most cells containing FtsZ as the sole cytoskeletal element are spherical.

b) When actin-like MreB homologues are present, cells can take on a rod shaped morphology like that seen in Escherichia coli. MreB and its homologues often appear as intracellular helical structures (red) when viewed with fluorescence microscopy.

c) Caulobacter crescentus cells contain crescentin (yellow) in addition to FtsZ and MreB, and shows a crescent-shaped cell morphology. In C. crescentus cells, MreB localizes to apparent helices during cell elongation and to the division plane with FtsZ during cell division (Adapted from "Matthew T.

Cabeen and Christine Jacobs-Wagner.2005. Bacterial Cell. Nature Reviews Microbiology, 3 : 601-10.").

12.5. Flagellum

Flagella (singular-flagellum) are filamentous protein structures attached to the cell surface that provide the swimming movement for most motile prokaryotes. Prokaryotic flagella are much thinner than eukaryotic flagella, and they lack the typical 9+2 arrangement of microtubules. The diameter of a prokaryotic flagellum is about 20 nm, well-below the resolving power of the light microscope. About half of the bacilli and all of the spiral and curved bacteria are motile by means of flagella. In bacteria (Fig.12.6) flagella may be variously distributed over the surface of bacterial cells in distinguishing patterns, but basically flagella are either polar (one or more flagella arising from one or both poles of the cell) or peritrichous (lateral flagella distributed over the entire cell surface).
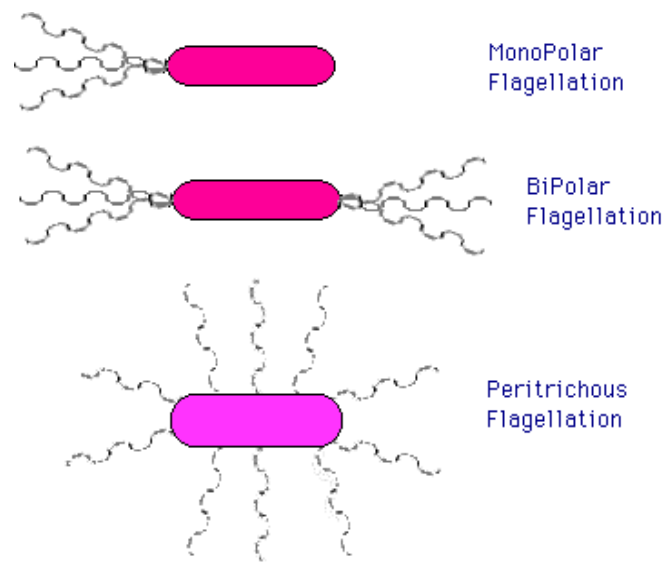
Fig.12.6. Arrangement of flagella in bacteria

A bacterial flagellum has 3 basic parts: a filament, a hook, and a basal body. The filament is the rigid, helical structure that extends from the cell surface. The hook is a flexible coupling between the filament and the basal body. The basal body consists of a rod and a series of rings that anchor the flagellum to the cell wall and the cytoplasmic membrane. The basal body acts as a molecular motor, enabling the flagellum to rotate and propel the bacterium through the surrounding fluid (Fig. 12.7).

(Click for Animation Fig.12.7 Movement of bacterial flagellum)

Lesson 13
STRUCTURE AND GERMINATION OF ENDOSPORE, MICROBIAL LOCOMOTION

13.1 Introduction

The spore (i.e. endospore), discovered in 1876 by Cohn, Koch and Tyndall independently, is one of the hardiest dormant life forms on earth formed during the resting stage in the life cycle of spore-forming genera. Endospores are produced within cells and are refractile - light cannot penetrate them so that they are very easy to see in the phase microscope. They are resting structures, meaning that there is little or no metabolism inside the spore and it is a real form of suspended animation. Spores can survive for a very long time, and then re-germinate. Spores that were dormant for thousands of years in the great tomes of the Egyption Pharohs were able to germinate and grow when placed in appropriate medium. There are even claims of spores that are over 250 million years old being able to germinate when placed in appropriate medium.

Bacterial endospores are complex structures whose basic architecture is conserved across species. Endospore enables an organism to resist extreme environmental conditions such as

- Temperature
- Drying
- UV radiation
- Strong acids and bases
- Oxidizing agents
- Extremes of both vacuum and ultrahigh hydrostatic pressure

The resistance of spore is attributed to following factors:

- The presence of high content of calcium-dipicolinate which stabilizes and protect the DNA
- DNA-binding proteins protect DNA from heat, drying, chemicals, and radiation
- Dehydration that results in resistance to heat and radiation
- DNA repair enzymes are able to repair damaged DNA during germination

These resistant structures survive heating to 150°C although mostly killed at 121°C in moist medium. They can remain dormant for immense periods of time, perhaps even millions of years.

13.2 Endospore Forming Bacteria

Spore formers generally found in soils as their ability to form spores as advantageous for soil microorganisms. Spore formers are distinguished on the basis of cell morphology, shape and cellular position of endospore (Fig. 13.1). Key spore forming genera are*Bacillus, Clostridium*, *Sporosarcina*, *Heliobacterium*. *Sporosarcinae*are unique among endospore formers because cells are cocci instead of rods and are strictly aerobic, spherical cells. Other spore forming genera are *Paenibacillus*, *Sporolactobacillus*, *Desulfotomaculum*,*Thermoanarobacter*, *Sporomusa*, *Sporohalobacter*, *Amphibacillus*,*Heliophylium*, *Heliorestis*, *S*
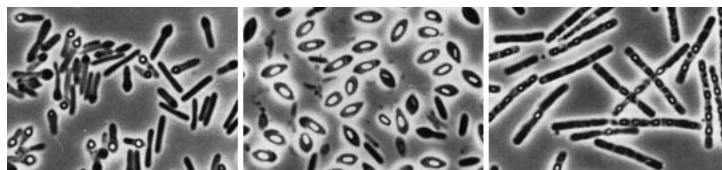

Fig. 13.1 Endospore forming bacteria

13.3 Endospore Structure

It is more complex than of vegetative cell. Inside spore there is a core (Spore protoplast), containing cytoplasm, nucleoid and ribosomes (Fig. 13.2). The core of the mature endospore has only 10-25 % of the vegetative cell water content what increases its resistance to heat and chemicals. The pH of core is one unit lower than that of vegetative cell cytoplasm and the core contains a high level of small acid-soluble proteins, SASPs, able to bind DNA and to protect it from potential damage. The coat contains 50-78% of total spore protein (Cot) which can be divided into two groups - alkali soluble and alkali insoluble.
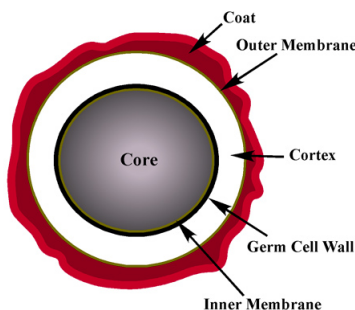

Fig. 13.2 Structure of Endospore

13.4 Endospore Formation

The formation of a spore is an expensive and complex process for the bacterial cell. Spores are only made under conditions where cell survival is threatened such as starvation for certain nutrients or accumulation of toxic wastes. Regulation of sporulation is tight and the first few steps are reversible. This helps the cell conserve energy and only sporulate when necessary. Sporulation is a seven step process (Fig. 13.3). When a bacterium detects environmental conditions are becoming unfavourable it may start the process of endosporulation, which takes about eight hours. The first stages of sporulation are involved in forming a separate compartment for the spore in the mother cell. Once this occurs, sporulation is irreversible. The DNA is replicated and a membrane wall known as a spore septum begins to form between it and the rest of the cell. The plasma membrane of the cell surrounds this wall and pinches off to leave a double membrane around the DNA, and the developing structure is now known as a forespore. Calcium dipicolinate is incorporated into the forespore during this time. The next stages involve laying down the various layers of the spore. The peptidoglycan cortex forms between the two layers and the bacterium adds a spore coat to the outside of the forespore. Both, the spore and the mother cell, play a role in this process. In the final stages, the spore dehydrates its cytoplasm and is released from the cell. Research on sporulation in Bacillus subtilis is intense. It is a wonderful model system for development, since the microbe is genetically tractible, allowing the isolation of many mutants in each stage of sporulation.

(Click for Animation Fig. 13.3 Various stages in Endospore Formation)

Endospores are resistant to most agents that would normally kill the vegetative cells they formed from. Household cleaning products generally have no effect, nor do most alcohols, quaternary ammonium compounds or detergents. Alkylating agents however, such as ethylene oxide, are effective against endospores. While resistant to extreme heat and radiation, endospores can be destroyed by burning or by autoclaving. Endospores are able to survive boiling at 100°C for hours, although the longer the number of hours the fewer that will survive. An indirect way to destroy them is to place them in an environment that reactivates them to their vegetative state. They will germinate within a day or two with the right environmental conditions, and then the vegetative cells can be straightforwardly destroyed. This indirect method is called Tyndallization. It was the usual method for a while in the late 19th century before the advent of inexpensive autoclaves. Prolonged exposure to high energy radiation, such as x-rays and gamma rays, will also kill most endospores.

13.5 Reactivation/Germination

Reactivation of the endospore occurs when conditions are more favourable and involves activation, germination, and outgrowth. Even if an endospore is located in plentiful nutrients, it may fail to germinate unless activation has taken place. This may be triggered by heating the endospore. Germination involves the dormant endospore starting metabolic activity and thus breaking hibernation. It is commonly characterised by rupture or absorption of the spore coat, swelling of the endospore, an increase in metabolic activity, and loss of resistance to environmental stress. Outgrowth follows germination and involves the core of the endospore manufacturing new chemical components and exiting the old spore coat to develop into a fully functional vegetative bacterial cell, which can divide to produce more cells.

13.6 Significance

Endospore formation can be considered a primitive system of cell differentiation and has become a paradigm for the study of this phenomenon in prokaryotes. As a simplified model for cellular differentiation, the molecular details of endospore formation have been extensively studied, specifically in the model organism Bacillus subtilis. These studies have contributed much to our understanding of the regulation of gene expression, transcription factors, and the sigma factor subunits of RNA polymerase.

Endospores of the bacterium Bacillus anthracis were used in the 2001 anthrax attacks. The powder found in contaminated postal letters was composed of extracellular anthrax endospores. Inhalation, ingestion or skin contamination of these endospores, which were technically incorrectly labelled as 'spores', led to a number of deaths.

The presence of endospore formers can be problematic as they can not be inactivated by heat treatments normally applied in food industry and cause food poisoning (Bacillus cereus, Clostridium botulinum) and spoilage of even heat treated foods (B. subtilis, B. coagulans).

Geobacillus stearothermophilus endospores are used as biological indicators when an autoclave is used in sterilization procedures.

On positive note, Endospore preparations derived from Bacillus thuringiensis and Paenibacillus popillae are commercially available as biological agents for control of insects. Popularly known by one of its trade names, Botox or Dysport, a preparation prepared from Botulinum toxin is used for various cosmetic and medical procedures.

Lesson 14
AN OVERVIEW OF DIFFERENCE BETWEEN PROKARYOTES AND EUKARYOTES

14.1 Introduction

Cells in our world come in two basic types, prokaryotic and eukaryotic. 'Karyose' comes from a Greek word which means 'kernel', as in a kernel of grain. In biology, we use this word root to refer to the nucleus of a cell. 'Pro' means 'before' and 'eu' means 'true', or 'good'. So 'Prokaryotic' means 'before a nucleus' and 'eukaryotic' means 'possessing a true nucleus'. This is a big hint about one of the differences between these two cell types. Prokaryotic cells have no nuclei, while eukaryotic cells do have true nuclei. This is far from the only difference between these two cell types.

Click for Animation (Fig.14.1. Structure of Prokaryotic Cell)

Click for Animation (Fig.14.2. Structure of Eukaryotic Cell)

14.2 What Prokaryotic and Eukaryotic Cells Have in Common

- Both have DNA as their genetic material.
- Both are covered by a cell membrane.
- Both contain RNA.
- Both are made from the same basic chemicals: carbohydrates, proteins, nucleic acid, minerals, fats and vitamins.
- Both have ribosomes (the structures on which proteins are made).
- Both regulate the flow of the nutrients and wastes that enter and leave them.
- Both have similar basic metabolism (life processes) like photosynthesis and reproduction.
- Both require a supply of energy.
- Both are highly regulated by elaborate sensing systems ('chemical noses') that make them aware of the reactions within them and the environment around them.

That's what prokaryotic and eukaryotic cells have in common. But there are significant differences between them too.

14.3 Prokaryotic and Eukaryotic Differences

If we take a closer look at the comparison of these cells, we see the following differences:

- Eukaryotic cells have a true nucleus, bound by a double membrane. Prokaryotic cells have no nucleus. The purpose of the nucleus is to sequester the DNA-related functions of the big eukaryotic cell into a smaller chamber, for the purpose of increased efficiency. This function is unnecessary for the prokaryotic cell, because it is much smaller size means that all materials within the cell are relatively close together. Of course, prokaryotic cells do have DNA and DNA functions. Biologists describe the central region of the cell as its 'nucleoid' (-oid = similar or imitating), because it's pretty much where the DNA is located. But note that the nucleoid is essentially an imaginary 'structure'. There is no physical boundary enclosing the nucleoid.

- Eukaryotic DNA is linear; prokaryotic DNA is circular (it has no ends).
- Eukaryotic DNA is complexed with proteins called 'histones', and is organized into chromosomes; prokaryotic DNA is 'naked', meaning that it has no histones associated with it, and it is not formed into chromosomes. Though many are sloppy about it, the term 'chromosome' does not technically apply to anything in a prokaryotic cell. A eukaryotic cell contains a number of chromosomes; a prokaryotic cell contains only one circular DNA molecule and a varied assortment of much smaller circlets of DNA called 'plasmids'. The smaller, simpler prokaryotic cell requires far fewer genes to operate than the eukaryotic cell.
- Both cell types have many, many ribosomes, but the ribosomes of the eukaryotic cells are larger and more complex than those of the prokaryotic cell. Ribosomes are made out of a special class of RNA molecules (ribosomal RNA, or rRNA) and a specific collection of different proteins. A eukaryotic ribosome is composed of five kinds of rRNA and about eighty kinds of proteins. Prokaryotic ribosomes are composed of only three kinds of rRNA and about fifty kinds of protein.
- The cytoplasm of eukaryotic cells is filled with a large, complex collection of organelles, many of them enclosed in their own membranes; the prokaryotic cell contains no membrane-bound organelles which are independent of the plasma membrane. This is a very significant difference, and the source of the vast majority of the greater complexity of the eukaryotic cell. There is much more space within a eukaryotic cell than within a prokaryotic cell, and many of these structures, like the nucleus, increase the efficiency of functions by confining them within smaller spaces within the huge cell, or with communication and movement within the cell.

Examination of these differences is interesting. As mentioned above, they are all associated with larger size and greater complexity. This leads to an important observation. Yes, these cells are different from each other. However, they are clearly more alike than different, and they are clearly evolutionarily related to each other. Biologists have no significant doubts about the connection between them. The eukaryotic cell is clearly developed from the prokaryotic cell.

One aspect of that evolutionary connection is particularly interesting. Within eukaryotic cells you find a really fascinating organelle called a mitochondrion. And in plant cells, you'd find an additional family of organelles called plastids, the most famous of which is the renowned chloroplast. Mitochondria (the plural of mitochondrion) and chloroplasts almost certainly have a similar evolutionary origin. Both are pretty clearly the descendants of independent prokaryotic cells, which have taken up permanent residence within other cells through a well-known and very common phenomenon called endosymbiosis.

The salient differences among Bacteria, Archaea and Eukarya are listed in Table14.1.

Table 14.1 Differences among Bacteria, Archaea and Eukarya

| Characteristic	Bacteria	Archaea	Eukarya
Prokaryotic cell structure	Present	Present	Absent
DNA in covalently closed and circular form	Present	Present	Absent
Histone proteins	Absent	Present	Present
Membrane-enclosed nucleus	Absent	Absent	Present
Cell wall	Muramic acid present	Muramic acid present	Muramic acid present
Sensitivity to chloramphenicol, streptomycin, and kanamycin	Present	Absent	Absent
Plasmids	Present	Present	Rare
Membrane lipids	Easter-linked	Easter-linked	Easter-linked
Ribosomes (mass)	70S	70S	70S
Ribosome sensitivity to diphtheria toxin	Absent	Present	Present
Initiator tRNA	Formyl methionine	Methionine	Methionine
Introns in most genes	Absent	Absent	Present
Operons	Present	Present	Absent
Capping and poly-A tailing of mRNA	Absent	Absent	Present
RNA polymerases	One (4- subunits)	Several (8-12 subunits each)	Three (12-14 subunits each)
Transcription factors required	Absent	Present	Present
Promoter structure	-10 and -35 sequences (Pribnow box)	TATA box	TATA box
Methanogenesis	Absent	Present	Absent
Dissimilative reduction of sulphur or SO_4^{2-} to H_2S, or Fe^{3+} to Fe^{2+}	Present	Present	Absent
Nitrification	Present	Absent	Absent
Denitrification	Present	Present	Absent
Nitrogen fixation	Present	Present	Absent
Chlorophyll-based photosynthesis	Present	Absent	Present (in chloroplasts)
Rhodopsin-based energy metabolism	Present	Present	Absent
Chemilithotrophy (Fe, S, H_2)	Present	Present	Absent
Gas vesicles	Present		Absent
Synthesis of carbon storage granules	Present	Present	Absent
Composed of poly-b-hydroxyalkanoates	Present	Present	Absent
Growth above 80°C	Absent	Present	Absent

Lesson 15
MODES OF CELL DIVISION

15.1 Binary Cell Division

Prokaryotes are much simpler in their organization than are eukaryotes. There are a great many more organelles in eukaryotes, also more chromosomes. The usual method of prokaryote cell division is termed binary fission. The prokaryotic chromosome is a single DNA molecule that first replicates, then attaches each copy to a different part of the cell membrane. When the cell begins to pull apart, the replicate and original chromosomes are separated. Following cell splitting (cytokinesis), there are then two cells of identical genetic composition (except for the rare chance of a spontaneous mutation). The prokaryote chromosome is much easier to manipulate than the eukaryotic one. We thus know much more about the location of genes and their control in prokaryotes. One consequence of this asexual method of reproduction is that all organisms in a colony are genetic equals. When treating a bacterial disease, a drug that kills one bacteria (of a specific type) will also kill all other members of that clone (colony) it comes in contact with. Binary fission begins with DNA replication. DNA replication starts from an origin of replication, which opens up into a replication bubble (note: prokaryotic DNA replication usually has only 1 origin of replication, whereas eukaryotes have multiple origins of replication). The replication bubble separates the DNA double strand, each strand acts as template for synthesis of a daughter strand by semi conservative replication, until the entire prokaryotic DNA is duplicated. (Fig. 15.1)
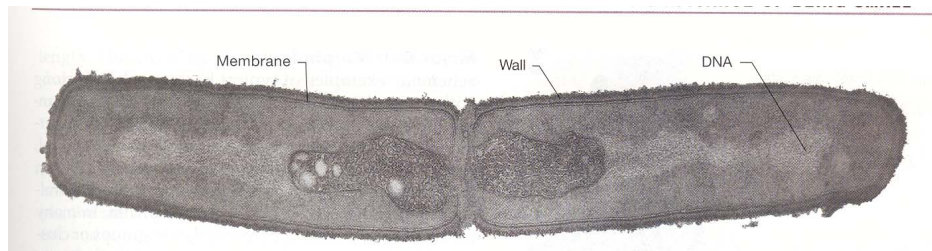

Fig. 15.1 Binary cell division in bacterial cell

After this replication process, cell growth occurs. Each circular DNA strand then attaches to the cell membrane. The cell elongates, causing the two chromosomes to separate.

Cell division in bacteria is controlled by the FtsZ, a collection of about a dozen proteins that collect around the site of division. There, they direct assembly of the division septum. The cell wall and plasma membrane starts growing transversely from near the middle of the dividing cell. This separates the parent cell into two nearly equal daughter cells, each having a nuclear body. The cell membrane then invaginates (grows inwards) and splits the cell into two daughter cells, separated by a newly grown cell plate. (Fig. 15.2)
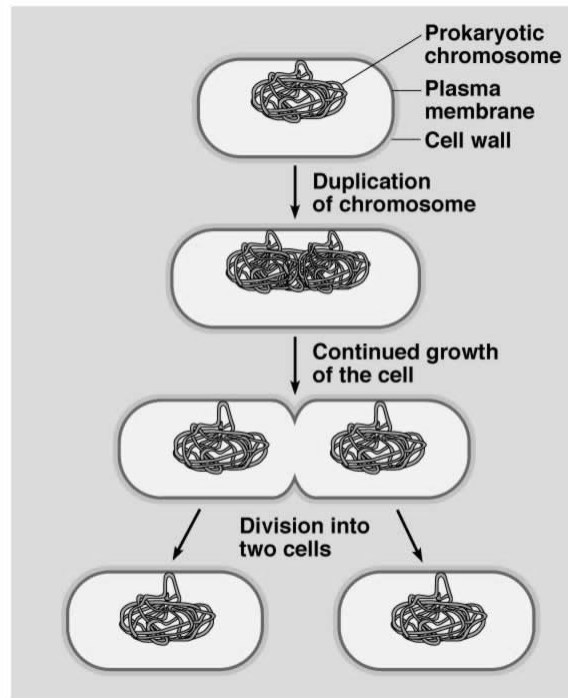
Fig. 15.2 Binary fission in bacteria

15.2 Microbial Growth

When bacteria are inoculated into a liquid growth medium, we can plot of the number of cells in the population over time. (Fig. 15.3)

15.2.1 Four phases of bacterial growth

15.2.1.1 Lag Phase

Immediately after inoculation of the cells into fresh medium, the population remains temporarily unchanged. Although there is no apparent cell division occurring, the cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, etc., and increasing in metabolic activity. The length of the lag phase is apparently dependent on a wide variety of factors including the size of the inoculum; time necessary to recover from physical damage or shock in the transfer; time required for synthesis of essential coenzymes or division factors; and time required for synthesis of new (inducible) enzymes that are necessary to metabolize the substrates present in the medium. This is the period of adjustment to new conditions. Little or no cell division occurs, population size doesn't increase. This is the phase of intense metabolic activity, in which individual organisms grow in size. It may last from one hour to several days.

15.2.1.2 Log Phase

Cells begin to divide and generation time reaches a constant minimum. This is the period of most rapid growth. The numbers of cells produced are more than the number of cells dying. Cells are at highest metabolic activity. Cells are most susceptible to adverse environmental factors such as antibiotic and radiation at this stage. The exponential phase of growth is a pattern of balanced

growth wherein all the cells are dividing regularly by binary fission, and are growing by geometric progression. The cells divide at a constant rate depending upon the composition of the growth medium and the conditions of incubation. The rate of exponential growth of a bacterial culture is expressed as generation time, also the doubling time of the bacterial population. Generation time (G) is defined as the time (t) per generation (n = number of generations). Hence, G=t/n is the equation from which calculations of generation time (below) derive.

15.2.1.3 Stationary Phase

By this stage, population size begins to stabilize. Number of cells produced is equal to number of cells dying. Overall cell number does not increase. Cell division begins to slow down. Factors that slow down microbial growth:

- Exhaustion of available nutrients;
- Accumulation of toxic waste materials
- Exhaustion of biological space
- Acidic pH of media
- Insufficient oxygen supply
- Cell functions necessary for growth will cease, but different functions necessary for survival are turned on.

The length of this phase is dependent upon the particular microorganism and the conditions of the medium (habitat) Stationary phase can last for long periods of time and especially when the microbes in nutrient-poor environment. During the stationary phase, if viable cells are being counted, it cannot be determined whether some cells are dying and an equal number of cells are dividing, or the population of cells has simply stopped growing and dividing. The stationary phase, like the lag phase, is not necessarily a period of quiescence. Bacteria that produce secondary metabolites, such as antibiotics, do so during the stationary phase of the growth cycle (Secondary metabolites are defined as metabolites produced after the active stage of growth). It is during the stationary phase that spore-forming bacteria have to induce or unmask the activity of dozens of genes that may be involved in sporulation process.

15.2.1.4 Death or Decline Phase

If incubation prolongs beyond stationary phase, a death phase ensues, in which the viable cell population begins to decline. Population size begins to decrease. This is the stage where number of cells dying starts exceeding number of cells produced. During the death phase, the number of viable cells decreases geometrically (exponentially), essentially the reverse of growth during the log phase.
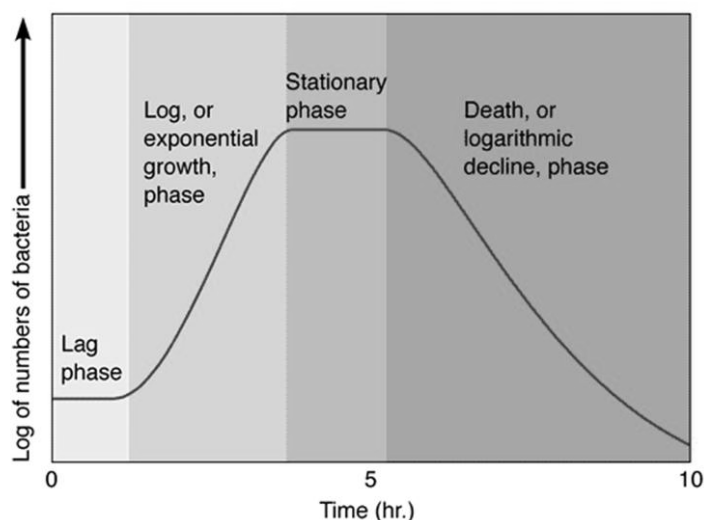
Fig. 15.3 Bacterial Growth Curve

15.3 Growth Rate and Generation Time

As mentioned above, bacterial growth rates during the phase of exponential growth, under standard nutritional conditions (culture medium, temperature, pH, etc.), define the bacterium's generation time. Generation times for bacteria vary from about 12 min to 24 h or more. The generation time for E. coli in the laboratory is 15-20 min, but in the intestinal tract, the coliform's generation time is estimated to be 12-24 h. For most known bacteria that can be cultured, generation times range from about 15 min to 1 h. Symbionts such as Rhizobium tend to have longer generation times. Many lithotrophs, such as the nitrifying bacteria, also have long generation times. Some bacteria that are pathogens, such as Mycobacterium tuberculosis and Treponema pallidum, have especially long generation times, and this is thought to be an advantage in their virulence. Generation times for a few bacteria are shown in Table (15.1)

Table 15.1 Generation times for some common bacteria under optimal conditions of growth.

Bacterium	Medium	Generation Time (minutes)
Escherichia coli	Glucose-salts	17
Bacillus megaterium	Sucrose-salts	25
Streptococcus lactis	Milk	26
Streptococcus lactis	Lactose broth	48
Staphylococcus aureus	Heart infusion broth	27-30
Lactobacillus acidophilus	Milk	66-87
Rhizobium japonicum	Mannitol-salts-yeast extract	344-461
Mycobacterium tuberculosis	Synthetic	792-932
Treponema pallidum	Rabbit testes	1980

15.3.1 Calculation of generation time

When growing exponentially by binary fission, the increase in a bacterial population is by geometric progression. If we start with one cell, when it divides, there are 2 cells in the first generation, 4 cells in the second generation, 8 cells in the third generation, and so on. The generation time is the time interval required for the cells (or population) to divide.

G (generation time) = (time, in minutes or hours)/n(number of generations)

$G = t/n$

t = time interval in hours or minutes

B = number of bacteria at the beginning of a time interval

b = number of bacteria at the end of the time interval

n = number of generations (number of times the cell population doubles during the time interval)

b = B x 2n (This equation is an expression of growth by binary fission)

Solve for n:

$\log b = \log B + n \log 2$

$$n = \frac{\log b - \log B}{\log 2}$$

$$n = \frac{\log b - \log B}{.301}$$

n = 3.3 logb/B

$G = t/n$

Solve for G:

$$G = \frac{t}{3.3 \log b/B}$$

Example: Taking example of Lactococcus lactis. Suppose in milk medium initial count of is 10,000 and final count is 10,000,000 and time interval is 4.3 h then what should be the generation time of L. lactis in milk.

$$G = \frac{t}{3.3 \log b/B}$$

Milk

$$G = \frac{260 \text{ min}}{3.3 \log 10^7 / 10^4}$$

$$G = \frac{260 \text{ min}}{3.3 \times 3}$$

$$G = 26.26 \text{ min}$$

Lactose broth

$$G = \frac{478 \text{ min}}{3.3 \log 10^7 / 10^4}$$

$$G = \frac{478 \text{ min}}{3.3 \times 3}$$

$$G = 47.95 \text{ min}$$

Similarly in milk medium initial count of milk is 10,000 and final count is 10,000,000 and time interval is 7.9 h then what should be the generation time of L. lactis in lactose broth

15.4 Synchronous Growth

A synchronous or synchronized culture is a microbiological culture or a cell culture that contains cells that are all in the same growth stage. Studying the growth of bacterial populations in batch or continuous cultures does not permit any conclusions about the growth behavior of individual cells, because the distribution of cell size (and hence cell age) among the members of the population is completely random. Information about the growth behavior of individual bacteria can, however, be obtained by the study of synchronous cultures. Synchronized cultures must be composed of cells which are all at the same stage of the bacterial cell cycle. Measurements made on synchronized cultures are equivalent to measurements made on individual cells.

Since numerous factors influence the cell cycle, some of them stochastic (random), normal, non-synchronous cultures have cells in all stages of the cell cycle. Obtaining a culture with a unified cell-cycle stage is very useful for biological research. Since cells are too small for certain research techniques, a synchronous culture can be treated as a single cell; the number of cells in the culture can be easily estimated, and quantitative experimental results can simply be divided in the number of cells to obtain values that apply to a single cell. Synchronous cultures have been extensively used to address questions regarding cell cycle and growth, and the effects of various factors on these.

A number of clever techniques have been devised to obtain bacterial populations at the same stage in the cell cycle. Some techniques involve manipulation of environmental parameters which induces the population to start or stop growth at the same point in the cell cycle, while others are physical methods for selection of cells that have just completed the process of binary fission. Theoretically, the smallest cells in a bacterial population are those that have just completed the process of cell division. Synchronous cultures rapidly lose synchrony because not all cells in the population divide at exactly the same size, age or time. Synchronous cultures can be obtained in several ways

- External conditions can be changed, so as to arrest growth of all cells in the culture, and then changed again to resume growth. The newly growing cells are now all starting to grow at the same stage, and they are synchronized. For example, for photosynthetic cells light can be eliminated for several hours and then re-introduced. Another method is to eliminate an essential nutrient from the growth medium and later to re-introduce it.
- Cell growth can also be arrested using chemical growth inhibitors. After growth has completely stopped for all cells, the inhibitor can be easily removed from the culture and the

cells then begin to grow synchronously. Nocodazole, for example, is often used in biological research for this purpose.

- Cells in different growth stages have different physical properties. Cells in a culture can thus be physically separated based on their density or size, for instance. This can be achieved using centrifugation (for density) or filtration (for size).
- In the Helmstetter-Cumming technique, a bacterial culture is filtered through a membrane. Most bacteria pass through, but some remain bound to the membrane. Fresh medium is then applied to the membrane and the bound bacteria start to grow. Newborn bacteria that detach from the membrane are now all at the same stage of growth; they are collected in a flask that now harbors a synchronous culture.

15.5 Continuous Culture

The cultures so far discussed for growth of bacterial populations are called batch cultures. Since the nutrients are not renewed, exponential growth is limited to a few generations. Bacterial cultures can be maintained in a state of exponential growth over long periods of time using a system of continuous culture, designed to relieve the conditions that stop exponential growth in batch cultures. Continuous culture, in a device called a chemostat, can be used to maintain a bacterial population at a constant density, a situation that is, in many ways, more similar to bacterial growth in natural environments.

In a chemostat, the growth chamber is connected to a reservoir of sterile medium. Once growth is initiated, fresh medium is continuously supplied from the reservoir. The volume of fluid in the growth chamber is maintained at a constant level by some sort of overflow drain. Fresh medium is allowed to enter into the growth chamber at a rate that limits the growth of the bacteria. The bacteria grow (cells are formed) at the same rate that bacterial cells (and spent medium) are removed by the overflow. The rate of addition of the fresh medium determines the rate of growth because the fresh medium always contains a limiting amount of an essential nutrient. Thus, the chemostat relieves the insufficiency of nutrients, the accumulation of toxic substances, and the accumulation of excess cells in the culture, which are the parameters that initiate the stationary phase of the growth cycle. The bacterial culture can be grown and maintained at relatively constant conditions, depending on the flow rate of the nutrients.

A microbial population of can be maintained in the exponential growth phase and at a constant biomass concentration for extended periods. Continuous Culture It is an open system where fresh medium is added and spent medium and cells are removed. Open systems are more complex due to the need to aseptically add and remove medium.

Two common major types of continuous culture systems:

15.5.1.1 Chemostat

A chemostat (from Chemical environment is static) is a bioreactor to which fresh medium is continuously added, while culture liquid is continuously removed to keep the culture volume constant. By changing the rate with which medium is added to the bioreactor the growth rate of the microorganism can be easily controlled. One of the most important features of chemostat is that micro-organisms can be grown in a physiological steady state. In steady state, growth occurs at a

constant rate and all culture parameters remain constant (culture volume, dissolved oxygen concentration, nutrient and product concentrations, pH, cell density, etc.). In addition environmental conditions can be controlled by the experimenter. Micro-organisms grown in chemostat naturally strive to steady state: if a low amount of cells are present in the bioreactor, the cells can grow at growth rates higher than the dilution rate, as growth isn't limited by the addition of the limiting nutrient. The limiting nutrient is a nutrient essential for growth, present in the media at a limiting concentration (all other nutrients are usually supplied in surplus). However, if the cell concentration becomes too high, the amount of cells that are removed from the reactor cannot be replenished by growth as the addition of the limiting nutrient is insufficient. This results in an equilibrium situation (steady state), where the rate of cell growth is equal to the rate of cell removal.
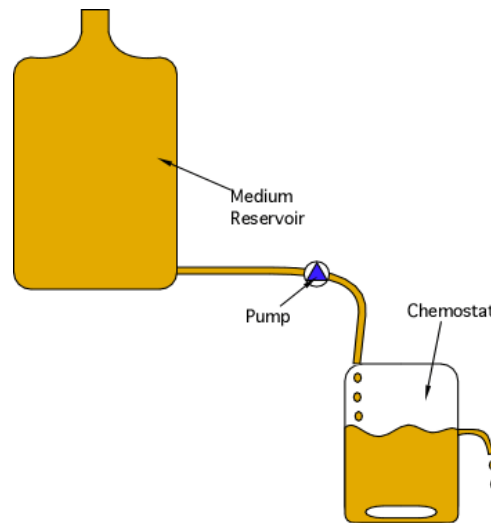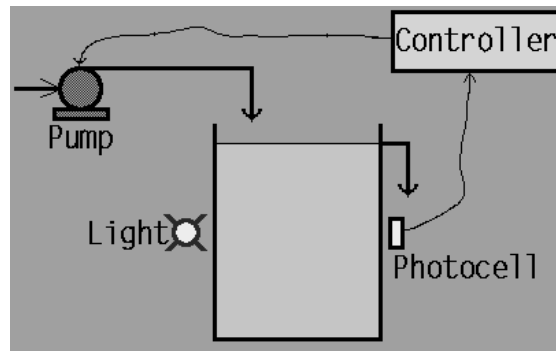

Fig. 15.4 Chemostat continuous culture system

15.1.1.2 Turbidostat

A turbidostat is a continuous culture device, similar to a chemostat or an auxostat, which has feedback between the turbidity of the culture vessel and the dilution rate. The theoretical relationship between growth in a chemostat and growth in a turbidostat is somewhat complex, in part because it is similar. A chemostat technically has a fixed volume and flow rate - thus a fixed dilution rate. When the cells are uniform and at equilibrium, operation of a chemostat and turbidostat should be identical. It is only when classical chemostat assumptions are violated (for instance, out of equilibrium; or the cells are mutating) that a turbidostat is functionally different. One case may be while cells are growing at their maximum growth rate, in which case it is difficult to set a chemostat to the appropriate constant dilution rate.

While most turbidostats use a spectrophotometer/turbidometer to measure the optical density for control purposes, there exist other options, such as dielectric permittivity.

Fig. 15.5 Turbidostat continuous culture system

15.6 Diauxic growth

Diauxie is a French word coined by Jacques Monod to mean two growth phases. The word is used to describe the growth phases of a microorganism in batch culture as it metabolizes a mixture of two sugars. Rather than metabolizing the two available sugars simultaneously, microbial cells commonly consume them in a sequential pattern, resulting in two separate growth phases. During the first phase, cells preferentially metabolize the sugar on which it can grow faster (often glucose but not always). Only after the first sugar has been exhausted do the cells switch to the second. At the time of the "diauxic shift", there is often a lag period during which cells produce the enzymes needed to metabolize the second sugar.
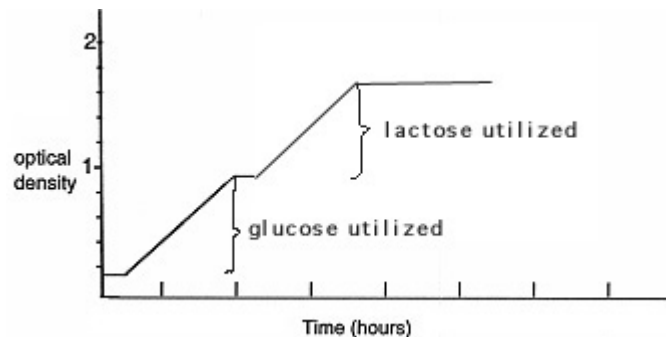
Fig 15.6 The Diauxic Growth Curve of E. coli grown in limiting concentrations of a mixture of glucose and lactose

Lesson 16
QUANTITATIVE MEASUREMENT OF GROWTH

16.1 Introduction

As stated in Lesson 15, growth in a biological system is an orderly increase in the quantity of cellular constituents and which depends upon the ability of the cell to form new protoplasm from nutrients available in the environment. In most bacteria, growth involves increase in cell mass and number of ribosomes, duplication of the bacterial chromosome, synthesis of new cell wall and plasma membrane, partitioning of the two chromosomes, septum formation, and cell division (binary fission).

Methods for measurement of the cell mass involve both direct and indirect techniques: (Table 16.1)

- Direct physical measurement of dry weight, wet weight, or volume of cells after centrifugation.
- Direct chemical measurement of some chemical component of the cells such as total N, total protein, or total DNA content.
- Indirect measurement of chemical activity such as rate of O_2production or consumption, CO_2 production or consumption, etc.
- Turbidity measurements employ a variety of instruments to determine the amount of light scattered by a suspension of cells.

Particulate objects such as bacteria scatter light in proportion to their numbers. The turbidity or optical density of a suspension of cells is directly related to cell mass or cell number, after construction and calibration of a standard curve. The method is simple and nondestructive, but the sensitivity is limited to about 107 cells per ml for most bacteria. Measuring techniques involve direct counts, visually or instrumentally, and indirect viable cell counts.

16.2 Methods for Measurement of Cell Mass
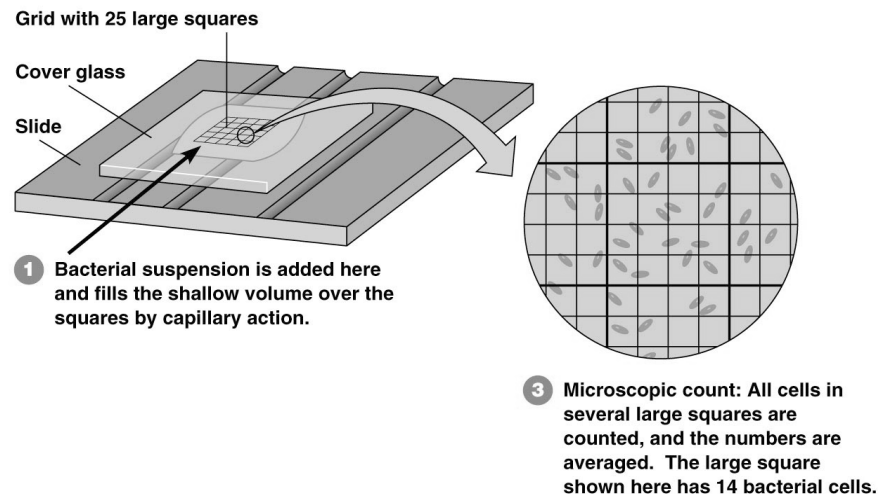
16.2.1 Direct microscopic count

In the direct microscopic count, a counting chamber consisting of a ruled slide and a coverslip is employed. It is constructed in such a manner that the coverslip, slide, and ruled lines delimit a known volume. The number of bacteria in a small known volume is directly counted microscopically and the number of bacteria in the larger original sample is determined by extrapolation.

The Petroff-Hausser counting chamber (commonly used in dairy industry) for example, (Figure 16. 1) has small etched squares 1/20 of a millimeter (mm) by 1/20 of a mm and is 1/50 of a mm deep. The volume of one small square therefore is 1/20,000 of a cubic mm or 1/20,000,000 of a cubic centimeter (cc). There are 16 small squares in the large double-lined squares that are actually counted, making the volume of a large double-lined square 1/1,250,000 cc. The normal procedure is to count the number of bacteria in five large double-lined squares and divide by five to get the average number of bacteria per large square. This number is then multiplied by 1,250,000 since the

square holds a volume of 1/1,250,000 cc, to find the total number of organisms per cc in the original sample. If the bacteria are diluted, such as by mixing the bacteria with dye before being placed in the counting chamber, then this dilution must also be considered in the final calculations.

The formula used for the direct microscopic count is

The number of bacteria per cc = The average number of bacteria per large double-lined square X The dilution factor of the large square (1,250,000) X The dilution factor of any dilutions made prior to placing the sample in the counting chamber, e.g., mixing

**Fig. 16.1 Direct microscopic count method**

A variation of the direct microscopic count has been used to observe and measure growth of bacteria in natural environments. In order to detect and prove that thermophilic bacteria were growing in boiling hot springs, T.D. Brock immersed microscope slides in the springs and withdrew them periodically for microscopic observation. The bacteria in the boiling water attached to the glass slides naturally and grew as microcolonies on the surface.

16.2.2 Electronic enumeration of cells

A Coulter counter (Fig.16.2) is an apparatus for counting and sizing particles and cells. It is used, for example, for bacteria and air quality particle size distributions. The counter detects change in electrical conductance of a small aperture as fluid containing cells is drawn through. Cells, being non-conducting particles, alter the effective cross-section of the conductive channel.

It was an American inventor named Wallace H. Coulter who was responsible for the theory and design of the Coulter Counter. He first devised the theory behind its operation in 1947 while experimenting with electronics. Coulter determined that electrical charge could be used to determine the size and number of microscopic particles in a solution. This phenomenon is now known as the Coulter Principle. A typical Coulter counter has one or more microchannels that separate two chambers containing electrolyte solutions. When a particle flows through one of the microchannels, it results in the electrical resistance change of the liquid filled microchannel. This resistance change

can be recorded as electric current or voltage pulses, which can be correlated to size, mobility, surface charge and concentration of the particles.
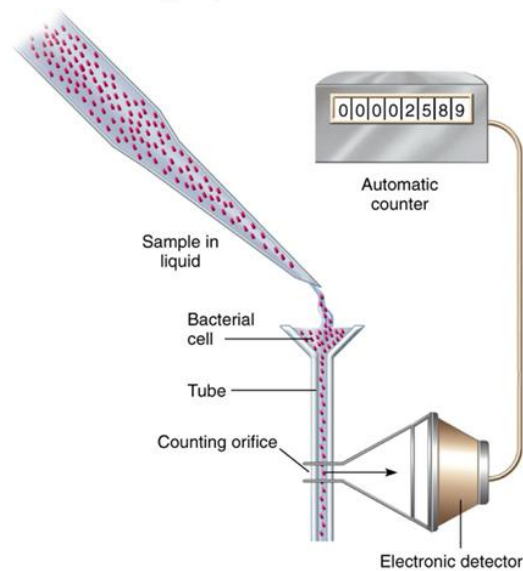
Fig. 16.2 Coulter counter

16.2.3 Plate count method

Standard Plate Count (SPC) is a techniques under this category which is commonly employed in microbiological laboratories for enumeration of bacteria .The SPC is the number of bacterial colonies that develop on a medium in a petri dish seeded with a known amount of inoculum. The number of bacteria in a given sample is usually too great to be counted directly. However, if the sample is serially diluted (Fig.16.3) and then plated out on an agar surface in such a manner that single isolated bacteria form visible isolated colonies , the number of colonies can be used as a measure of the number of viable (living) cells in that known dilution. However, keep in mind that if the organism normally forms multiple cell arrangements, such as chains, the colony-forming unit may consist of a chain of bacteria rather than a single bacterium. In addition, some of the bacteria may be clumped together. Therefore, when doing the plate count technique, we generally say we are determining the number of Colony-Forming Units (CFUs) in that known dilution. By extrapolation, this number can in turn be used to calculate the number of CFUs in the original sample.

Normally, the bacterial sample is diluted by factors of 10 and plated on agar either by pour plate or spread plate technique. After incubation, the number of colonies on a dilution plate showing between 30 and 300 colonies (Fig. 16.4 and Fig. 16.5) is determined. A plate having 30-300 colonies is chosen because this range is considered statistically significant. If there are less than 30 colonies on the plate, small errors in dilution technique or the presence of a few contaminants will have a drastic effect on the final count. Likewise, if there are more than 300 colonies on the plate, there will be poor isolation and colonies will have grown together.

Generally, one wants to determine the number of CFUs per milliliter (ml) of sample. To find this, the number of colonies (on a plate having 30-300 colonies) is multiplied by the number of times the original ml of bacteria was diluted (the dilution factor of the plate counted). For example, if a plate

containing a 1/1,000 dilution of the original ml of sample shows 159 colonies, then 159 represents 1/1,000 the number of CFUs present in the original ml. Therefore the number of CFUs per ml in the original sample is found by multiplying 159×10^3 (or preferably represented as 1.59×10^5) as shown in the formula below:

Click for Animation (Serial Dilution Method)
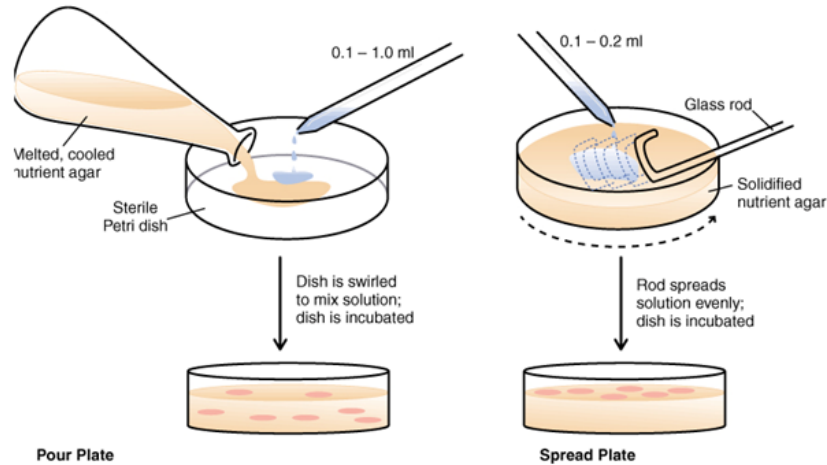
Fig 16.4 Pour plate and spread plate techniques
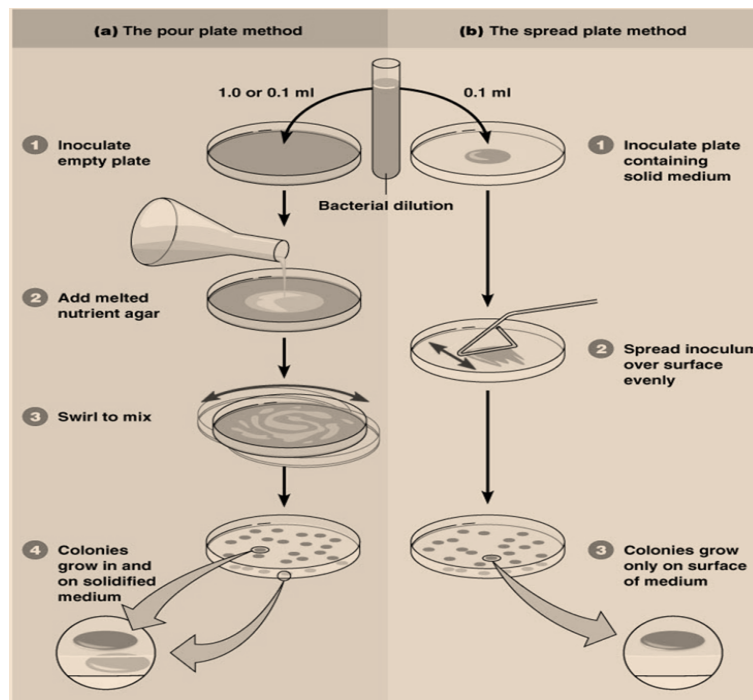
Fig.16.5 Pour plate and spread plate methods

Advantages of the technique are its sensitivity (theoretically, a single cell can be detected), and it allows for inspection and positive identification of the organism counted. Disadvantages are

- Only living cells develop colonies that are counted;
- Clumps or chains of cells develop into a single colony;

- Colonies develop only from those organisms for which the cultural conditions are suitable for growth.

The latter makes the technique virtually useless to characterize or count the total number of bacteria in complex microbial ecosystems such as soil or the animal rumen or gastrointestinal tract. Genetic probes can be used to demonstrate the diversity and relative abundance of procaryotes in such an environment, but many species identified by genetic techniques have so far proven unculturable.

**Table 16.1 Comparison of various methods of measurement of bacterial growth**

Method	Application	Comments
Direct microscopic count	Enumeration of bacteria in milk or cellular vaccines	Cannot distinguish living from nonliving cells
Viable cell count (colony counts)	Enumeration of bacteria in milk, foods, soil, water, laboratory cultures, etc.	Very sensitive if plating conditions are optimal
Turbidity measurement	Estimations of large numbers of bacteria in clear liquid media and broths	Fast and nondestructive, but cannot detect cell densities less than 10^7 cells per ml
Measurement of total N or protein	Measurement of total cell yield from very dense cultures	only practical application is in the research laboratory
Measurement of Biochemical activity e.g. O_2 uptake CO_2 production, ATP production, etc.	Microbiological assays	Requires a fixed standard to relate chemical activity to cell mass and/or cell numbers
Measurement of dry weight or wet weight of cells or volume of cells after centrifugation	Measurement of total cell yield in cultures	probably more sensitive than total N or total protein measurements

16.2.4 Membrane filter count method

This method is suitable for liquid or semi-liquid samples (e.g. water) and commonly used for enumeration of Coliform and Staphylococcus spp. Membrane filtration method is used with relatively low numbers. A known volume of liquid passed through membrane filter. Filter pore size retains organism. It filters microorganism of size more than 0.45 micrometer (Fig.16.6). Filter is placed on appropriate growth medium and incubated and cells are counted (Fig. 16.7).
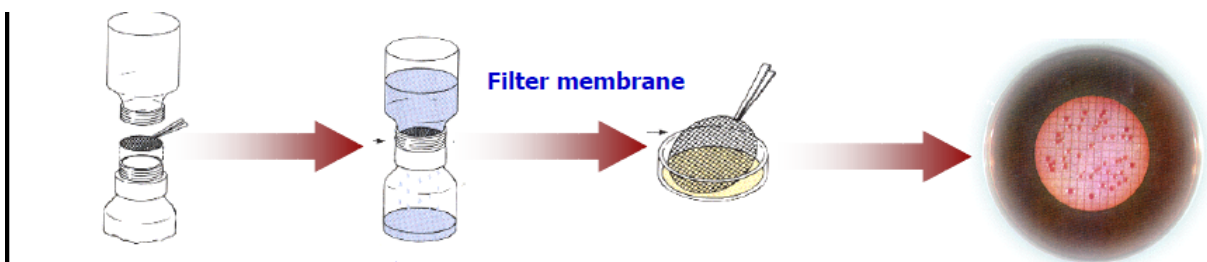

Filter membrane

Fig. 16.6 Membrane filtration method
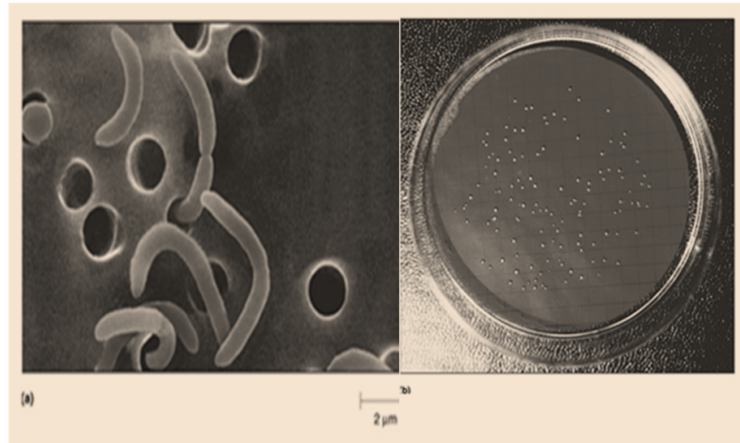
**Fig. 16.7 Membrane filter enumeration method
(A. Cells on filter as viewed by scanning electron microscopy; b. Colonies on filter)**

16.2.5 Turbidity measurement methods

When bacteria growing in a liquid medium are mixed, the culture appears turbid. This is because a bacterial culture acts as a colloidal suspension that blocks and reflects light passing through the culture. Within limits, the light absorbed by the bacterial suspension will be directly proportional to the concentration of cells in the culture. By measuring the amount of light absorbed by a bacterial suspension, one can estimate and compare the number of bacteria present. The instrument used to measure turbidity is a spectrophotometer (Fig. 16.8). It consists of a light source, a filter which allows only a single wavelength of light to pass through, the sample tube containing the bacterial suspension, and a photocell that compares the amount of light coming through the tube with the total light entering the tube. The ability of the culture to block the light can be expressed as either percent of light transmitted through the tube or the amount of light absorbed in the tube. The percent of light transmitted is inversely proportional to the bacterial concentration. The absorbance (or optical density) is directly proportional to the cell concentration.
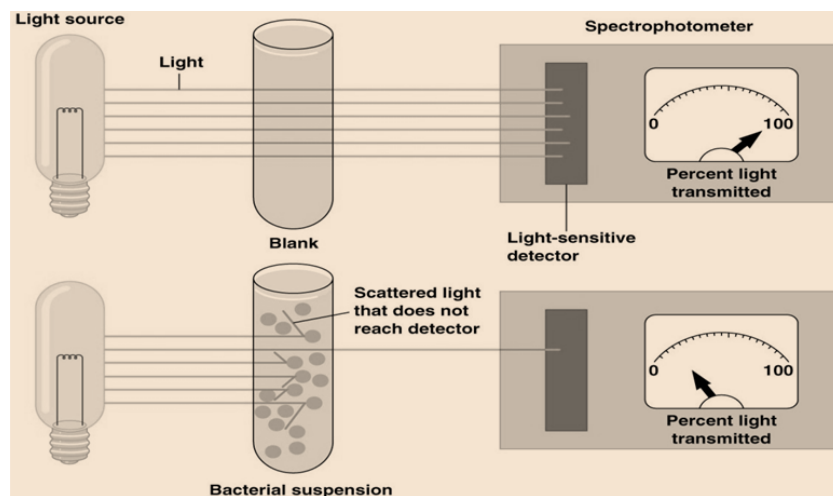
Fig. 16.8 Turbidity measurement using spectrophotometer

Turbidimetric measurement is often correlated with some other method of cell count, such as the direct microscopic method or the plate count. In this way, turbidity can be used as an indirect measurement of the cell count. For example:

- Several dilutions can be made of a bacterial stock.
- A Petroff-Hausser counter can then be used to perform a direct microscopic count on each dilution.
- Then a spectrophotometer can be used to measure the absorbance of each dilution tube.
- A standard curve comparing absorbance to the number of bacteria can be made by plotting absorbance versus the number of bacteria per cc.
- Once the standard curve is completed, any dilution tube of that organism can be placed in a spectrophotometer and its absorbance read. Once the absorbance is determined, the standard curve can be used to determine the corresponding number of bacteria per cc.

16.2.6 Determination of Nitrogen content

The major constituent of cell material is protein, and since nitrogen is a characteristic part of proteins, one can measure a bacterial population or cell crop in terms of bacterial nitrogen. Bacteria average approximately 14 percent nitrogen on a dry weight basis, although this figure is subject to some variation introduced by changes in culture conditions or differences between species. To measure growth by this technique, you must first harvest the cells and wash them free of medium and then perform a quantitative chemical analysis for nitrogen. Bacterial nitrogen determinations are somewhat laborious and can be performed only on specimens free of all other sources of nitrogen. Furthermore, the method is applicable only for concentrated populations. For these and other reasons, this procedure is used primarily in research.

16.2.7 Determination of dry weight

This is one of the simplest indirect methods in situations where determining the number of microorganisms is difficult or undesirable for other reasons. These methods measure some quantifiable cell property that increases as a direct result of microbial growth. The simplest technique of this sort is to measure the weight of cells in a sample. Portions of a culture can be taken at particular intervals and centrifuged at high speed to sediment bacterial cells to the bottom of a vessel. The sedimented cells (called a cell pellet) are then washed to remove contaminating salt, and dried in an oven at 100-105°C to remove all water, leaving only the mass of components that make up the population of cells. An increase in the dry weight of the cells correlates closely with cell growth. However, this method will count dead as well as living cells. There might also be conditions where the dry weight per cell changes over time or under different conditions. For example, some bacteria that excrete polysaccharides will have a much higher dry weight per cell when growing on high sugar levels (when polysaccharides are produced) than on low. If the species under study forms large clumps of cells such as those that grow filamentously, dry weight is a better measurement of the cell population than is a viable plate count.

It is also possible to follow the change in the amount of a cellular component instead of the entire mass of the cell. This method may be chosen because determining dry weights is difficult or when the total weight of the cell is not giving an accurate picture of the number of individuals in a population. In this case, only one component of the cell is followed such as total protein or total

DNA. This has some of the same advantages and disadvantages listed above for dry weight. Additionally, the measurement of a cellular component is more labor-intensive than previously mentioned methods since the component of interest has to be partially purified and then subjected to an analysis designed to measure the desired molecule. The assumption in choosing a single component such as DNA is that that component will be relatively constant per cell. This assumption has a problem when growth rates are different because cells growing at high rates actually have more DNA per cell because of multiple initiations of replication.

16.2.8 Measurement of specific chemical changes

The bacterial growth can be indirectly estimated by detecting specific changes caused in growth medium as a result of activity and multiplication of bacterial cells. It includes detecting activity cell products such as acid and gas production. The dye reduction tests such as methylene blue and resazurin reduction tests is based on the fact that the color imparted to milk by the addition of a dye such as methylene blue will disappear more or less quickly. The removal of the oxygen from milk and the formation of reducing substances during bacterial metabolism cause the color to disappear. The agencies responsible for the oxygen consumption are the bacteria. Though certain species of bacteria have considerably more influence than others, it is generally assumed that the greater the number of bacteria in milk, the quicker will the oxygen be consumed, and in turn the sooner will the color disappear. Thus, the time of reduction is taken as a measure of the number of organisms in milk although actually it is likely that it is more truly a measure of the total metabolic reactions proceeding at the cell surface of the bacteria. Gas production by bacteria is another major activity which can be taken up as an index of bacterial growth. Detection of gas production using Durham tube and change in color of the growth medium due to reduction of pH sensitive ingredients present in medium are commonly used for detection of acid and gas producing coliforms and yeasts. An apparatus for measuring CO_2 production is depicted in (Fig.16.9)
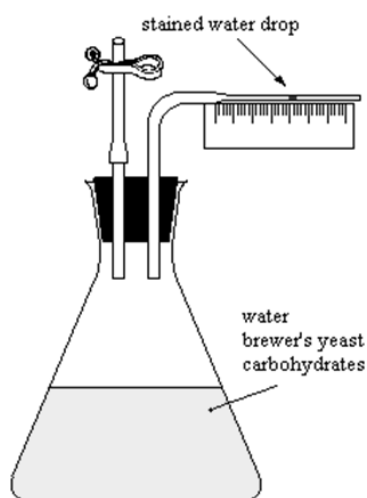
Fig. 16.9 Apparatus for measuring the carbon di oxide during fermentation

Lesson 17
BACTERIAL NUTRITION

17.1 Introduction

The survival and sustenance bacteria in the laboratory, as well as in nature, depend on their ability to grow under certain chemical and physical conditions. An understanding of these conditions is a prerequisite for us to isolate, identify and characterize different types of bacteria. Such knowledge can also be applied to control the growth of microorganisms in practical situations.

17. 2 Common Nutrient Requirements and Nutritional Types of Microorganisms (Fig.17.1)

Click for Animation (Fig. 17.1 Cellular Metabolism)

Bacteria are composed of different elements and molecules, with water (70%) and proteins (15%) being the most abundant. Microorganisms are often categorized according to their energy source and their source of carbon.

17.2.1 Energy source

- Phototrophs use radiant energy (light) as their primary energy source.
- Chemotrophs use the oxidation and reduction of chemical compounds as their primary energy source.

17.2.2 Carbon source

Carbon is the structural backbone of the organic compounds that make up a living cell. Based on their source of carbon bacteria can be classified as autotrophs or heterotrophs.

17.2.2.1 Autotrophs

Require only carbon dioxide as a carbon source. An autotroph can synthesize organic molecules from inorganic nutrients.

17.2.2.2 Heterotrophs

Require organic forms of carbon. A heterotroph cannot synthesize organic molecules from inorganic nutrients.Combining their nutritional patterns, all organisms in nature can be placed into one of four separate groups which are

a) Photoautotrophs

Use light as an energy source and carbon dioxide as their main carbon source. They include photosynthetic bacteria (green sulfur bacteria, purple sulfur bacteria, and cyanobacteria), algae, and green plants. Photoautotrophs transform carbon dioxide and water into carbohydrates and oxygen gas through photosynthesis. Cyanobacteria, as well as algae and green plants, use hydrogen atoms

from water to reduce carbon dioxide to form carbohydrates, and during this process oxygen gas is given off (an oxygenic process). Other photosynthetic bacteria (the green sulfur bacteria and purple sulfur bacteria) carry out an anoxygenic process, using sulfur, sulfur compounds or hydrogen gas to reduce carbon dioxide and form organic compounds.

b) Photoheterotrophs

Use light as an energy source but cannot convert carbon dioxide into energy. Instead they use organic compounds as a carbon source. They include the green nonsulfur bacteria and the purple nonsulfur bacteria.

c) Chemolithoautotrophs

Use inorganic compounds such as hydrogen sulfide, sulfur, ammonia, nitrites, hydrogen gas, or iron as an energy source and carbon dioxide as their main carbon source.

d) Chemoorganoheterotrophs

Use organic compounds as both an energy source and a carbon source. Saprophytes live on dead organic matter while parasites get their nutrients from a living host. Most bacteria, and all protozoans, fungi, and animals are chemoorganoheterotrophs.

17.2.3 Nitrogen source

Nitrogen is needed for the synthesis of such molecules as amino acids, DNA, RNA and ATP. Depending on the organism, nitrogen, nitrates, ammonia, or organic nitrogen compounds may be used as a nitrogen source.

17.2.4 Minerals

- Sulfur is needed to synthesisize sulfur-containing amino acids and certain vitamins. Depending on the organism, sulfates, hydrogen sulfide, or sulfur- containing amino acids may be used as a sulfur source.
- Phosphorus is needed to synthesize phospholipids, DNA, RNA, and ATP. Phosphate ions are the primary source of phosphorus.
- Potassium, magnesium, and calcium. These are required for certain enzymes to function as well as additional functions.
- Iron is a part of certain enzymes.
- Trace elements are elements required in very minute amounts, and like potassium, magnesium, calcium, and iron, they usually function as cofactors in enzyme reactions. They include sodium, zinc, copper, molybdenum, manganese, and cobalt ions. Cofactors usually function as electron donors or electron acceptors during enzyme reactions.

17.2.5 Water

17.2.6 Growth factors

Growth factors are organic compounds such as amino acids, purines , pyrimidines, and vitamins that a cell must have for growth but cannot synthesize itself. Organisms having complex nutritional requirements and needing many growth factors are said to be fastidious (Table 17.1).

Table 17.1 Nutritional types of microorganisms

Major nutritional type	Sources of energy, hydrogen/electrons, and carbon	Representative microorganisms
Photoautotroph (Photolithotroph)	Light energy, inorganic hydrogen/electron(H/e^-) donor, CO_2 carbon source	Algae, Purple and green bacteria, Cyanobacteria
Photoheterotroph (Photoorganotroph)	Light energy, inorganic H/e^- donor, Organic carbon source	Purple nonsulfur bacteria, Green sulfur bacteria
Chemoautotroph (Chemolithotroph)	Chemical energy source (inorganic), Inorganic H/e^- donor, CO_2 carbon source	Sulfur-oxdizing bacteria, Hydrogen bacteria, Nitrifying bacteria
Chemoheterotroph (Chenoorganotroph)	Chemical energy source (organic), Organic H/e^- donor, Organic carbon source	Most bacteria, fungi, protozoa

17.3 Types and Composition of Microbiological Media

Culture media are needed to grow microorganisms in the laboratory and to carry out specialized procedures like microbial identification, water and food analysis, and the isolation of particular microorganisms. A wide variety of media is available for these and other purposes. Media can be classified on the basis of physical state, chemical composition and functional type (Table 17.2).

Table 17.2 Types of Microbiological Media

Criteria for classification		
Physical State	Chemical Composition	Functional Type
Liquid	Synthetic (Chemically defined)	General Enriched
Solid	Non Synthetic (Complex; Not chemically defined)	Selective
Semi Solid (Liquefiable		Differential
Semisolid (Not liquefiable)		Maintenance Anaerobic growth Assay Enumeration

17.3.1 Classification by physical state

17.3.1.1 Liquid Media

In these media Growth seen as cloudiness or particulates and examples are Broths, milks, or infusions (Fig.18.2)

17.3.1.2 Semisolid media

They have clot like consistency at room temperature and are used to determine motility and to localize reactions at a specific site. Example: SIM for hydrogen sulfide production and indole reaction

17.3.1.3 Solid media

A firm surface on which cells can form discrete colonies. These can be liquefiable and non-liquefiable useful for isolating and culturing bacteria and fungi example: gelatin and agar (liquefiable); rice grains, cooked meat media, potato slices (non-liquefiable)
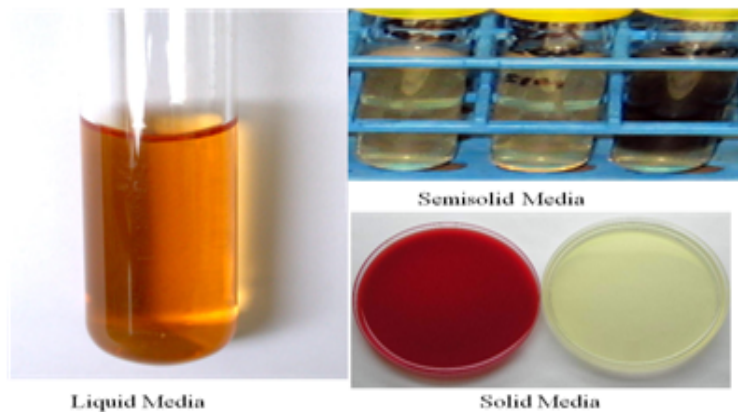
Fig. 17.2 Classification of media on the basis of physical state

17.3.2 Classification by chemical content

17.3.2.1 Synthetic media

Their compositions are precisely chemically defined, contain pure organic and inorganic compounds ex. glucose salts medium contains glucose plus inorganic salts in known amounts plus

17.3.2.2 Complex (non-synthetic) media

Contains at least one ingredient that is not chemically definable (extracts from plants and animals), no exact formula / tend to be general and grow a wide variety of organisms. For example nutrient broth which contains beef extract, yeast extract, peptone, NaCl. The exact composition of these is not known.

17.3.3 Classification by function

17.3.3.1 General purpose media

Such media are used to grow as broad a spectrum of microbes as possible and are usually non-synthetic; contain a mixture of nutrients to support a variety of microbes. Examples: nutrient agar and broth, brain-heart infusion, trypticase soy agar (Fig. 17.3).

17.3.3.2 Enriched Media

Contain complex organic substances (for example blood, serum, growth factors) to support the growth of fastidious bacteria e.g. blood agar and Thayer-Martin medium (chocolate agar) which contains heated blood which turns a chocolate brown color.

17.3.3.3 Selective and differential media

These contain one or more agents that inhibit the growth of certain microbes but not others e.g. Mannitol salt agar (MSA), MacConkey agar, Hektoen enteric (HE) agar.

17.3.3.4 Differential media

Allow multiple types of microorganisms to grow but display visible differences among those microorganisms. MacConkey agar can also be used as a differential medium.

17.3.3.5 Miscellaneous media

- Reducing media: absorbs oxygen or slows its penetration in the medium, used for growing anaerobes or for determining oxygen requirements
- Carbohydrate fermentation media: contain sugars that can be fermented and a pH indicator; useful for identification of microorganisms
- Transport media: used to maintain and preserve specimens that need to be held for a period of time.
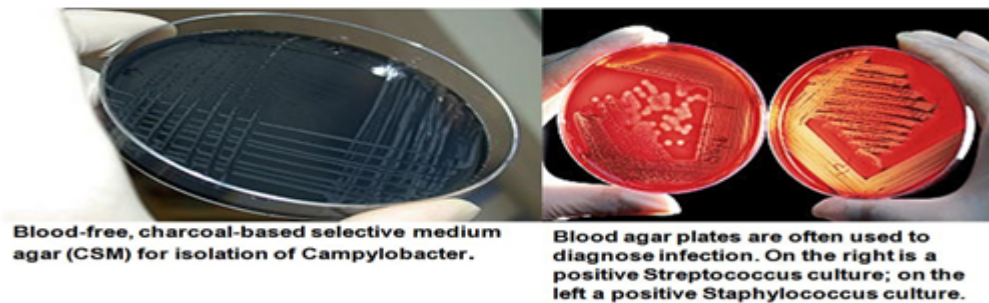

Blood-free, charcoal-based selective medium agar (CSM) for isolation of Campylobacter.

Blood agar plates are often used to diagnose infection. On the right is a positive Streptococcus culture; on the left a positive Staphylococcus culture.

Fig. 17.3 Enrichment and selective media

17.4 Use of Culture Media

Different media are used for cultivation of bacteria under laboratory conditions and bacteria from their natural niche or mixed population can be isolated and identified with appropriate selection of media (Fig. 17.4).
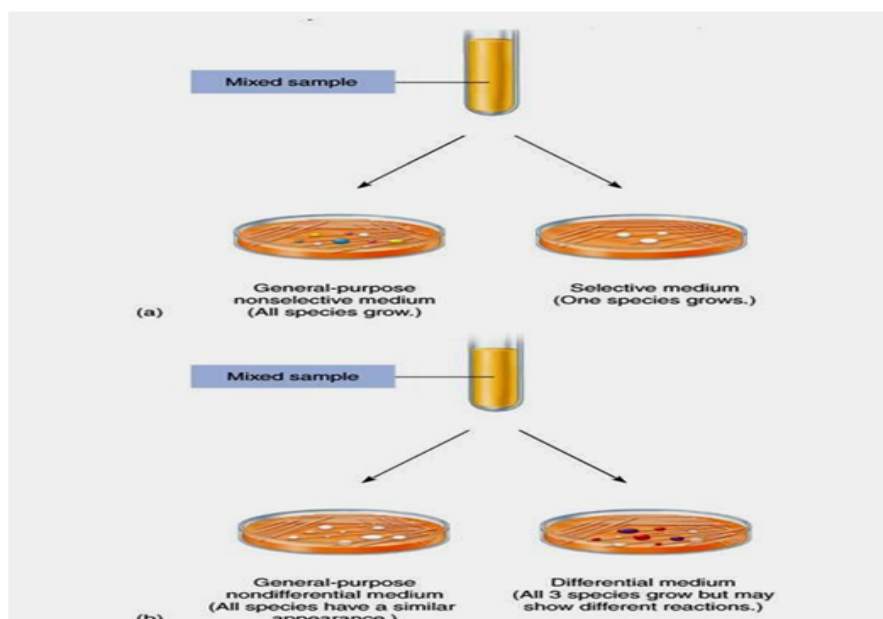

Fig. 17.4 Different types of media used for isolation of bacteria from mixed population

17.5 Composition of Media

In the following sections composition of some commonly used media are given (Table 17.3-17.8).

Table 17.3 Nutrient Agar (General purpose medium)

Beef extract	3 g
Peptone	5 g
Agar	15 g
Distilled water	1 L

Table 17.4 A chemical defined medium for E. coli

Constituent	Amount
Glucose	5.0 g
Sodium Chloride	5.0 g
Ammonium phosphate Monobasic	1.0 g
Potassium phosphate Dibasic	1.0 g
Magnesium Sulfate	0.2 g
Water	1 L

Table 17.5 Plate count agar (standard methods)

Tryptone	5 g
Yeast extract	2.5 g
Dextrose	1 g
Agar	15 g
Distilled water	1 L

Table 17.6 Potato dextrose agar (yeasts and moulds)

Potato infusion	200 g
Dextrose	20 g
Agar	20 g
Distilled water	1 L

Table 17.7 Brilliant green lactose bile broth (selective broth for coliforms)

Peptone	10 g
Lactose	10 g
Oxgall	20 g
Brilliant green	0.0133 g
Distilled water	1 L

Table 17.8 MacConkey agar (selective agar for coliforms)

Proteose peptone or polypeptone	3 g
Peptone or gelysate	17 g
Lactose	10 g
Bile salts No. 3 or bile salts mixture	1.5 g
NaCl	5 g
Neutral red	0.03 g
Crystal violet	0.001 g
Agar	13.5 g
Distilled water	1 L

Lesson 18
## ENVIRONMENTAL FACTORS AFFECTING GROWTH OF MICROORGANISMS

18.1 Introduction

The activities of microorganisms are greatly affected by the chemical and physical conditions of their environments. Different organisms react to their environment in different ways. An environment that is harmful to one microorganism may be beneficial to another. Sometimes an organism can tolerate an adverse condition in which it is unable to grow. There are a number of such factors which affect microbial growth as follows:

- Temperature
- Gas
- pH
- Osmotic pressure
- Other factors
- Microbial association

18.1.1 Temperature

Environmental temperature is one of the most important factors affecting the growth rate of microbes. There is a minimum temperature, below which growth does not occur. As we rise above the minimum, rate of growth increases in accordance with the laws governing the effect of temperature on the chemical reactions that make up growth. These reactions are mostly enzyme catalyzed. The minimum and maximum temperatures for microbial growth vary widely among microorganisms and are usually a reflection of the temperature range and average temperature of their habitat (Fig. 18.1).
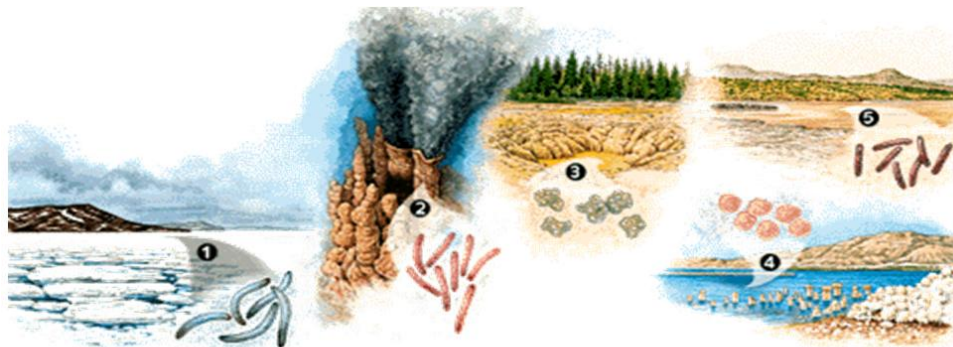
| 1. SEA ICE cold-loving microbes (Psychrophiles) *Polaromonas vacuolata* | 2. DEEP-SEA VENT heat-loving microbes (Thermophiles and Hyperthermophiles) *Methanopyrus kandleri* | 3. SULFURIC SPRING acid-loving microbes (Acidophiles) *Sulfolobus acidocaldarius* | 4. SALT LAKE salt-loving microbes (Halophiles) *Haloferax volcanii* | 5. SODA LAKE alkali-loving microbes (Alkaliphiles) *Natronobacteri um gregoryi* |

Fig. 18.1 Growth of microorganisms in different natural environmental conditions

Temperature affects living organisms in two opposing ways:

- As temperature rises, chemical and enzymatic reactions proceed at a faster rate, and the growth rate increases. Above a certain temperature proteins are irreversibly damaged.
- As temperature is increased within a certain range therefore, growth and metabolic activity increases up to a point where inactivation reactions set in. Above this temperature, cell functions fall sharply to zero.

Each microorganism thus has

- A minimum temperature below which no growth occurs.
- An optimum temperature at which growth is most rapid.
- A maximum temperature, above which growth is not possible.

The optimum temperature is always closer to the maximum rather than the minimum. These three temperatures called the cardinal temperatures are usually characteristic of each type of organism (Fig. 18.2). They are not completely fixed however, because they can be modified by other environmental factors; especially the chemical composition of the medium. The maximum growth temperature usually reflects the inactivation of one or more key proteins in the cell. The factors affecting minimum temperatures are less clear. It may result from the 'freezing' of the cyto-plasmicmembrane, impairing its ability to transport nutrients or form proton gradients. Experiments have shown that adjustment in membrane lipid composition can cause changes in minimum temperature.

18.1.1.1 Variation in cardinal temperature

Cardinal temperatures vary greatly throughout the microbial world. Optimal temperatures vary from 40°C to higher than 100°C. Growth of different bacteria can range from below freezing to above boiling, though no one organism can grow over this whole range. Most bacteria have a temperature range of about 30°C, although some have broader ranges than others.
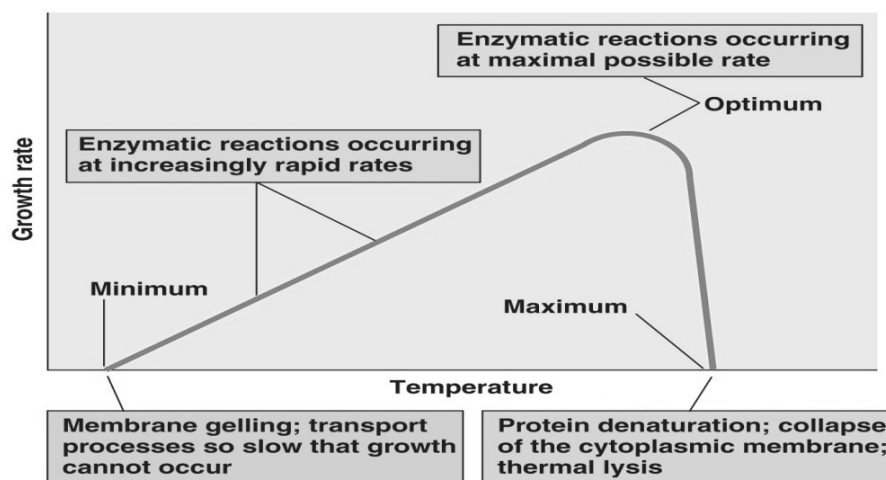

Fig. 18.2 Cardinal temperatures

18.1.1.2 Classification of microorganisms on the basis of growth temperature

Microorganisms can be broadly categorized into following groups on the basis of their growth temperatures as follows (Fig. 19.3):

- Psychrophiles – (cold loving) 0 to 15°C
- Psychrotrophs - (food spoilage) grow between 20 to 30°C
- Mesophiles- (most human pathogens): 20 to 40°C
- Thermophiles- (heat loving): 45 to 80°C
- Hyperthermophiles (Archaea): 89 to 120°C
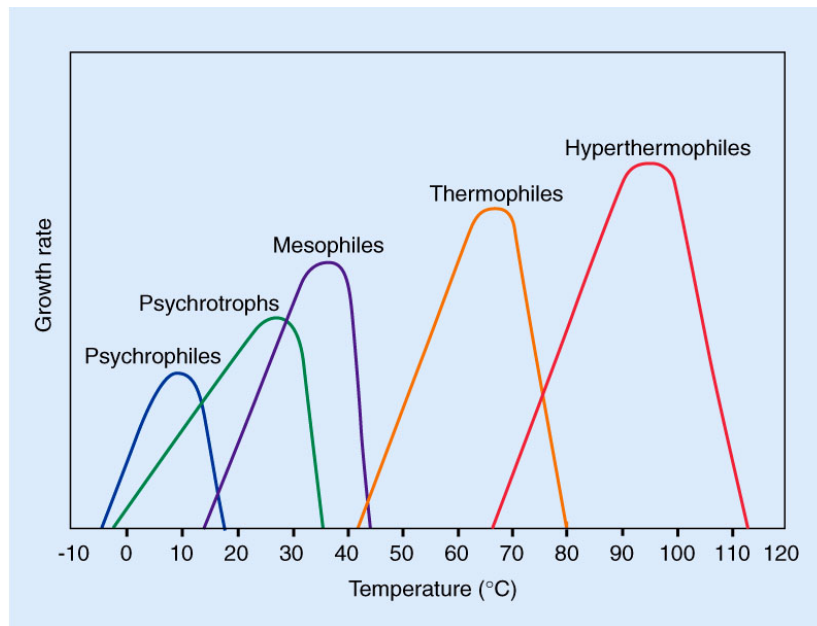
Fig. 18.3 Classes of microorganisms on the basis of growth temperature

How can thermophiles and hyperthermophiles thrive at high temperatures?

1. Enzymes are more heat stable

- Only a few key amino acids are different from mesophiles
- Increase in salt bridges (ionic bonds) between amino acids
- Densely packed hydrophobic interiors
- Example of heat stable enzyme = Taq polymerase used in PCR, isolated from *Thermus aquaticus*

2. Membranes are more heat stable

- Bacteria - saturated fatty acids (decrease fluidity) and stronger hydrophobic environment (greater interaction of fatty acid tails)
- Archaea contain isoprene units - lipid monolayer and ether linkage

18.1.2 Gas requirements

Two gases that influence microbial growth

(1) Oxygen

- Respiration - terminal electron acceptor
- Oxidizing agent - toxic forms

(2) Carbon dioxide

18.1.2.1 Microorganisms classification based on oxygen requirements

Microorganisms vary in their need for or tolerance to oxygen during growth. There are thus four types of organisms with respect to oxygen needs and tolerances (Fig.18.4).

- Aerobes – are capable of growing at full oxygen tension, and many can tolerate elevated levels of oxygen (greater than 21%).
- Microaerophiles – are aerobes that can use oxygen only it is present at reduced levels in air.
- Facultative organisms – are those that under appropriate nutrient and culture conditions can grow in either aerobic or anaerobic conditions.
- Anaerobes –are those organisms that lack respiratory systems and thus cannot use oxygen as the final electron acceptor.

Although oxygen is found as a cellular component, most organisms need molecular oxygen for respiration. In these organisms, oxygen serves as the terminal electron acceptor and such organisms are referred to as 'obligate aerobes', e.g. Nitrobacter. As opposed to this, there are organisms which do not use molecular oxygen as terminal electron acceptor although oxygen is a component of their cellular material. In fact, molecular oxygen is toxic to these organisms and these are, called as 'obligate anaerobes', e.g. clostridia. In these organisms nitrate, sulphate or organic compounds serve as electron acceptors. Some microorganisms can also grow either in the presence or absence of molecular oxygen and these are termed as facultative anaerobes, e.g. E. coli. Some, of these have a fermentative energy yielding metabolism but are not sensitive to the presence of molecular oxygen, while others can shift from a respiratory to a, fermentative metabolism depending upon the presence or absence of oxygen. Obligate anaerobes are unable to detoxify some of the byproducts of oxygen metabolism. Aerobes have enzymes that decompose toxic oxygen products. Anaerobes lack these enzymes.

In addition to these major classes there are organisms which grow best at reduced oxygen pressure but are obligate aerobes and these are called 'microaerophilic', e.g. most lactobacilli.

The Effect of Oxygen on the Growth of Various Types of Bacteria				
	a. Obligate Aerobes	**b. Facultative Anaerobes**	**c. Obligate Anaerobes**	**d. Aerotolerant Anaerobes**	**e. Micro-aerophiles**
Effect of Oxygen on Growth	Only aerobic growth; oxygen required.	Both aerobic and anaerobic growth; greater growth in presence of oxygen.	Only anaerobic growth; ceases in presence of oxygen.	Only anaerobic growth; but continues in presence of oxygen.	Only aerobic growth; oxygen required in low concentration.
Bacterial Growth in Tube of Solid Growth Medium					
Explanation of Growth Patterns	Growth occurs only where high concentrations of oxygen have diffused into the medium.	Growth is best where most oxygen is present, but occurs throughout tube.	Growth occurs only where there is no oxygen.	Growth occurs evenly; oxygen has no effect.	Growth occurs only where a low concent-ration of oxygen has diffused into medium.
Explanation of Oxygen's Effects	Presence of enzymes catalase and superoxide dismutase (SOD) allows toxic forms of oxygen to be neutralized; can use oxygen.	Presence of enzymes catalase and SOD allows toxic forms of oxygen to be neutralized; can use oxygen.	Lacks enzymes to neutralize harmful forms of oxygen; cannot tolerate oxygen.	Presence of one enzyme, SOD, allows harmful forms of oxygen to be partially neutralized; tolerates oxygen.	Produce lethal amounts of toxic forms of oxygen if exposed to normal atmospheric oxygen.

Fig. 18.4 Types of microorganisms on the basis of gaseous requirement

18.1.2.2 Other Gas Requirements

- Microaerophiles - requires less than 10% of atmospheric O_2 e.g. Campylobacter jejuni
- Capnophiles - requires increased CO_2 (5-15%) tension for initial growth e.g. S. pneumonia.

18.1.3 pH

Each organism has a pH range within which growth is possible, and most have well defined pH optima. Most natural environments have pH values between 5 and 9 and most organisms have pH optima in this range. Very few species can grow at pH values below 2 or above 10. Organisms capable of living at low pH are called acidophiles (Fig.18.5). Those capable of living at very high pH are called alkaliphiles. As a group, fungus tend to be more acid tolerant than bacteria. Many grow optimally at pH 5 or below, and a few grow well at pH values as low as 2. Some bacteria are also acidophilic and some cannot grow at neutral pH (obligate acidophiles). These include several species of Thiobacillus, and several genera of the archaea including Sulfolobus.
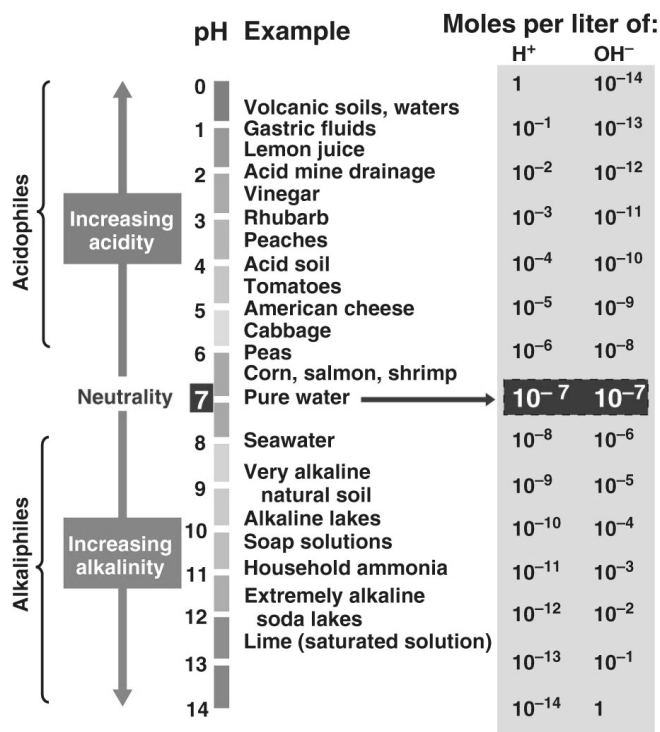
Fig. 18.5 Growth of microorganism at different pH

18.1.3.1 Extracellular versus intracellular pH

Despite the pH requirements of particular organisms for growth, the optimal growth pH represents the pH of the extracellular environment only. The intracellular pH must remain near neutrality to prevent destruction of acid-or alkali-labile macromolecules in the cell. In extreme acidophiles and extreme alkalinophiles, the intracellular pH may vary by several units from neutrality. Most cells grow best between pH 6-8. The internal pH of an extreme acidophile has been measured at 4.6 units while the internal pH of an extreme alkalinophile has been measured at 9.5 units.

18.1.4 Osmotic pressure

Water activity (aw) is a measure of the availability of water in an environment. aw is the ratio of the vapor pressure of the air in equilibrium with a substance or solution to the vapor pressure of pure water. The water activity can vary from 0 to 1. The more dissolved solutes in a mixture the lower the water activity. Osmosis is the process by which water diffuses from a region of low solute concentration (more water) to a region of high solute concentration (less water). Usually cellular cytoplasm has a higher solute concentration than the cells environment. The cell thus usually has what is called a positive water balance and the tendency is for water to diffuse into the cell. If however the cells environment has a low aw there is tendency for water to flow out of the cell.

18.1.4.1 Halophiles

In nature osmotic effects are of interest mainly with high concentrations of salts. Microorganisms found in the sea usually have specific requirement for the sodium ion and also grow optimally at aw of sea water. These are called halophiles (salt loving). Halophiles are divided into mild halophiles, moderate halophiles and extreme halophiles depending on their salt requirements. Halotolerant

microbes are those which can tolerate a reduction in aw but grow best in the absence of the added solute.

Thus on the basis of compatible solute requirement, we can categorize microorganism into following groups (Fig. 18.6):

- Mild halophile – requires 1-6 % salt
- Moderate Halophile – requires 6-15% salt
- Extreme halophiles – requires 15-30% salt
- Osmophiles – are those organisms capable of living in high sugar concentrations
- Xerophiles – are those organisms capable of growing in very dry environments
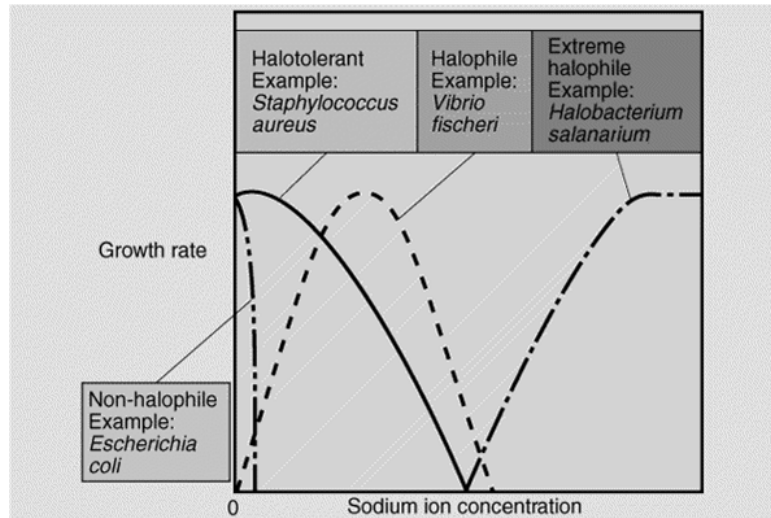
Fig. 18.6 Effect of sodium ion concentration on growth of microorganisms with different salt tolerances

18.1.4.2 Compatible Solutes

When an organism grows in a medium with low water activity, it can obtain water from its environment only by increasing its own internal solute concentration
This is achieved either by

- Pumping inorganic ions into the cell from the environment or
- Synthesizing or concentrating an organic solute

The solute used inside the cell for adjustment of cytoplasmic water activity must be non-inhibitory to the biochemical processes taking place within the cell. These compounds are thus called compatible solutes. Compatible solutes are all highly water soluble sugars or sugar alcohols, other alcohols, amino acids or their derivatives or potassium. Potassium is used only in the case of extreme halophiles, whether bacteria or archaea. Compatible solutes may be synthesized directly by the microorganism or accumulated from the environment e. g. K^+ or glycinebetaine. The concentration of compatible solutes in a cell is a function of the level of external solutes. However, maximal amount of compatible solute made or that can be accumulated is a genetically determined characteristic. Different organisms thus tolerate different water activities. Non-halotolerant,

halotolerant, halophilic, and extremely halophilic microorganisms are essentially defined by their genetic capacity to produce or accumulate compatible solutes.

18.1.5 Other factors

- Radiation (solar, UV, gamma)

  – Can all damage cells; bacteria have pigments to absorb energy and protect themselves.
  – Endospores are radiation resistant.
  – Deinococcus radiodurans: extremely radiation resistant

- Extremely efficient DNA repair, protection against dessication damage to DNA.
- Barophiles/barotolerant: microbes from deep sea; Baro means pressure. Actually require high pressure as found in their environment

Lesson 19
CONTROL OF MICROBIAL GROWTH BY PHYSICAL METHODS

19.1 Introduction

The control of microbial growth is necessary in many practical situations and significant advances in agriculture, medicine, and food science have been made through study of this area of microbiology. 'Control of microbial growth', as used here, means to inhibit or prevent growth of microorganisms. This control is affected in two basic ways: (1) by killing microorganisms or (2) by inhibiting the growth of microorganisms. Control of growth usually involves the use of physical or chemical agents which either kill or prevent the growth of microorganisms. Agents which kill cells are called cidal agents; agents which inhibit the growth of cells (without killing them) are referred to as static agents. Thus, the term bactericidal refers to killing bacteria, and bacteriostatic refers to inhibiting the growth of bacterial cells. A bactericide kills bacteria; a fungicide kills fungi, and so on.

19.2 Mode of Actions of Microbial Control Agents

Two possible antimicrobial effects include:

19.2.1 Alteration of membrane permeability

- The susceptibility of the plasma membrane is due to its lipid and protein components.
- Certain chemical control agents damage the plasma membrane by altering its permeability.

19.2.2 Damage to proteins and nucleic acids

- Some microbial control agents damage cellular proteins by breaking hydrogen bonds and covalent bonds.
- Other agents interfere with DNA and RNA replication and protein synthesis.

Several factors influence the effectiveness of antimicrobial treatment (Fig. 19.1).

- Number of Microbes: The more microbes present, the more time it takes to eliminate population.
- Type of Microbes: Endospores are very difficult to destroy. Vegetative pathogens vary widely in susceptibility to different methods of microbial control.
- Environmental influences: Presence of organic material (blood, feces, saliva) tends to inhibit antimicrobials, pH etc.
- Time of Exposure: Chemical antimicrobials and radiation treatments are more effective at longer times. In heat treatments, longer exposure compensates for lower temperatures.
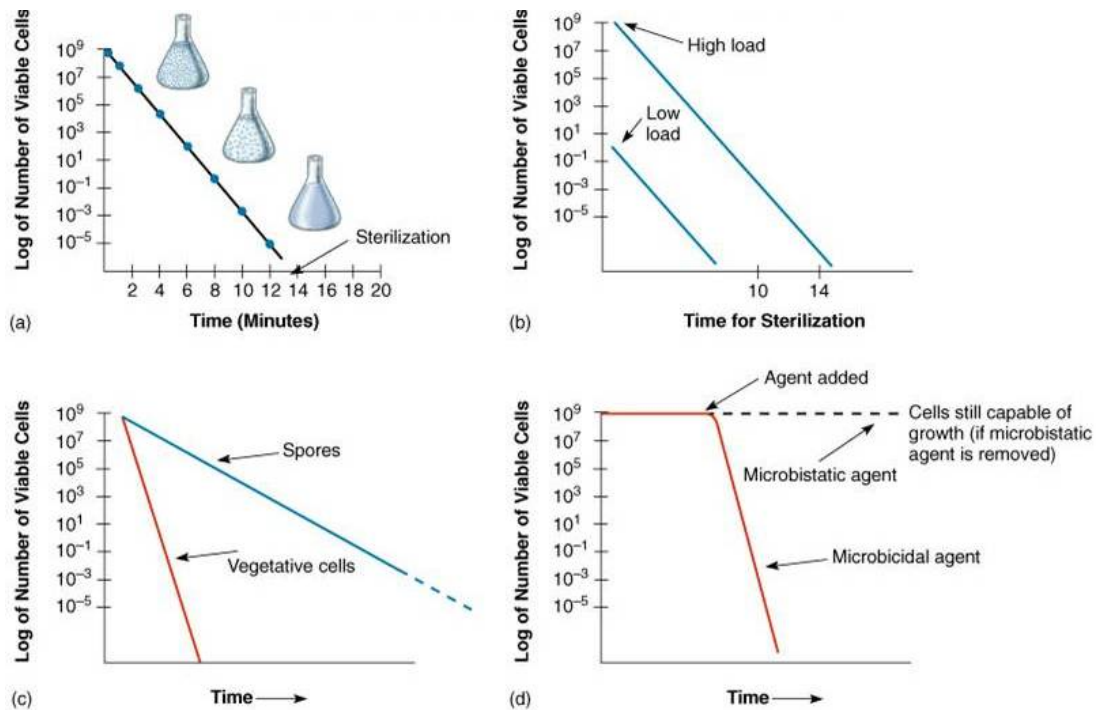
Fig. 19.1 Factors affecting effectiveness of antimicrobial agent

19.3 Physical Methods of Control of Microbial growth

The control methods can be broadly divided into two categories physical and chemical methods(Fig.19.2). In this chapter, former methods are discussed while later being dealt in next chapter.

Physical method can be listed as follows(Table19.1):

- Heat: Moist and Dry Heat
- Filtration
- Low Temperature: Refrigeration, Deep freezing, Lyophilization
- Desiccation
- Osmotic pressure
- Radiation: Ionizing and Non-Ionizing
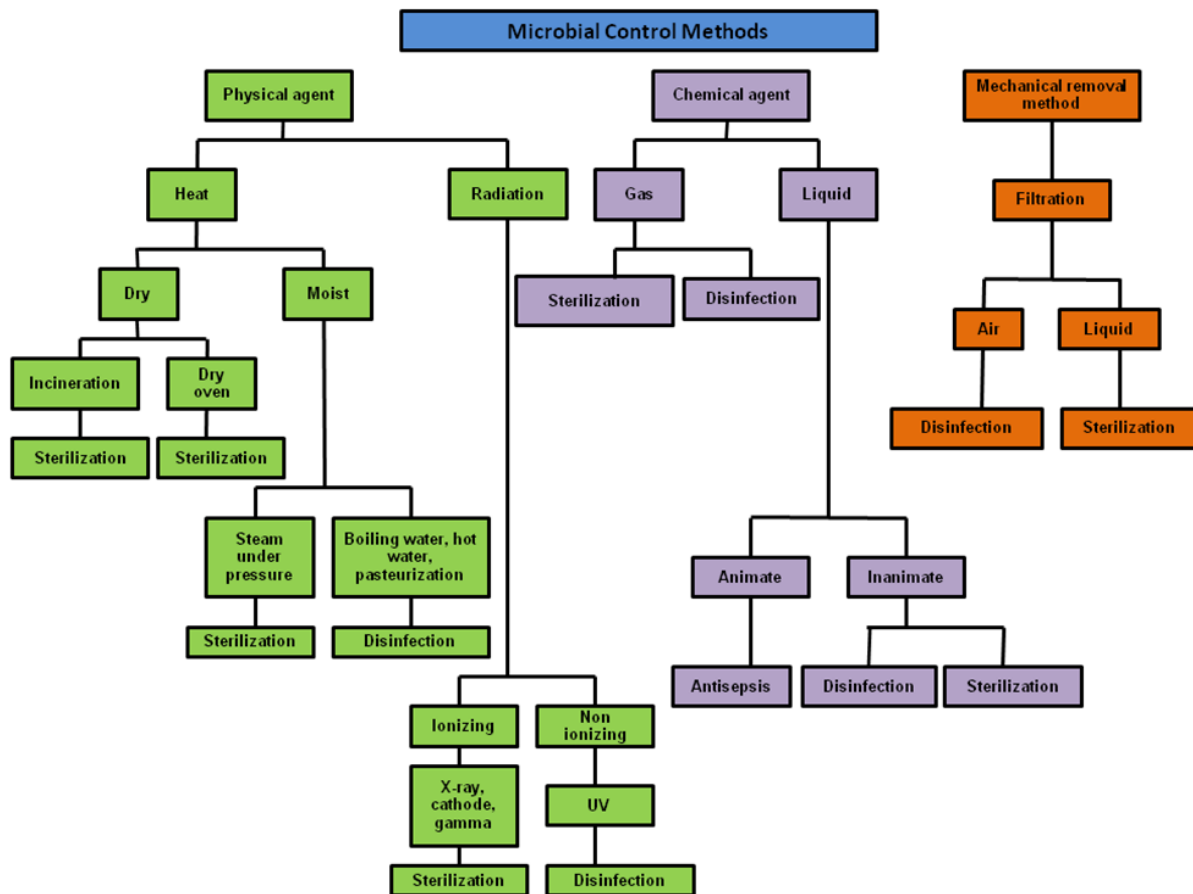
Fig. 19.2 Overview of methods of control of microbial growth

19.4 Physical Methods

19.4.1 Heat

Heat is frequently used to eliminate microorganisms. Moist heat kills microbes by denaturing proteins (enzymes). Dry heat kills organisms by oxidation. For sterilization one must consider the type of heat, and most importantly, the time of application and temperature to ensure destruction of all microorganisms. Endospores of bacteria are considered the most thermoduric of all cells so their destruction guarantees sterility.

Thermal Death Point (TDP) is the lowest temperature at which all the microbes in a liquid culture will be killed in 10 minutes. Thermal Death Time (TDT) is the length of time required to kill all bacterial in a liquid culture at a given temperature. Decimal Reduction Time (DRT) is the length of time required to kill 90% of a bacterial population at a given temperature; D value. Z value is an increase in temperature required to reduce D by 1/10. F value is time in minutes at a specific temperature needed to kill a population of cells or spores.

19.4.1.1 Moist Heat

Moist heat is thought to kill microorganisms by causing denaturation of essential proteins. Death rate is directly proportional to the concentration of microorganisms at any given time. Increasing the temperature decreases TDT, and lowering the temperature increases TDT. Processes conducted at

high temperatures for short periods of time are preferred over lower temperatures for longer times. Approximate effective moist heat conditions are given in (Table 19.2)

Table 19.2 Effective Moist Heat Conditions

Organism	Vegetative cells	Spores
Yeasts	5 min at 70-80°C	5 min at 70-80°C
Molds	30 min at 62°C	30 min at 80°C
Bacteria (Mesophiles)	10 min at 60-70°C	2 min to 12 h at 100°C
Viruses	30 min at 60°C	0.5 -12 min at 121°C

Environmental conditions also influence TDT. Increased heat causes increased toxicity of metabolic products and toxins. TDT decreases with pronounced acidic or basic pH. However, fats and oils slow heat penetration and increase TDT. It must be remembered that thermal death times are not precise values; they measure the effectiveness and rapidity of a sterilization process. Autoclaving 121°C/15 psi for 15 minutes exceeds the thermal death time for most organisms except some extraordinary spore formers.

Common Examples of methods based on moist heat are:

a) Boiling

It involves heating at 100°C for 30 minutes. This method kills everything except some endospores. To kill endospores, and therefore sterilize a solution, very long (> 6 hours) boiling or intermittent boiling is required.

b) Autoclaving

Autoclaving is the most effective and most efficient means of sterilization. All autoclaves operate on a time/temperature relationship. These two variables are extremely important. Higher temperatures ensure more rapid killing. The usual standard temperature/pressure employed is 121ºC/15 psi for 15 minutes (Table 19.3). Longer times are needed for larger loads, large volumes of liquid, and more dense materials. Autoclaving is ideal for sterilizing biohazardous waste, surgical dressings, glassware, many type of microbiologic media, liquids, and many other things. However, certain items, such as plastics and certain medical instruments (e.g. fiber-optic endoscopes), cannot withstand autoclaving and should be sterilized with chemical or gas sterilants. When proper conditions and time are employed, no living organisms will survive a trip through an autoclave. The autoclave is a large pressure cooker; it operates by using steam under pressure as the sterilizing agent (Fig. 19.3). High pressures enable steam to reach high temperatures, thus increasing its heat content and killing power. Most of the heating power of steam comes from its latent heat of vaporization. This is the amount of heat required to convert boiling water to steam. This amount of heat is large compared to that required to make water hot. For example, it takes 80 calories to make

1 liter of water boil, but 540 calories to convert that boiling water to steam. Therefore, steam at 100°C has almost seven times more heat than boiling water.

Table 19.3 Relationship between temperature and pressure of steam at sea level

Pressure (psi in excess of atmospheric pressure)	Temperature
0	100
5	110
10	116
15	121
20	126
30	135
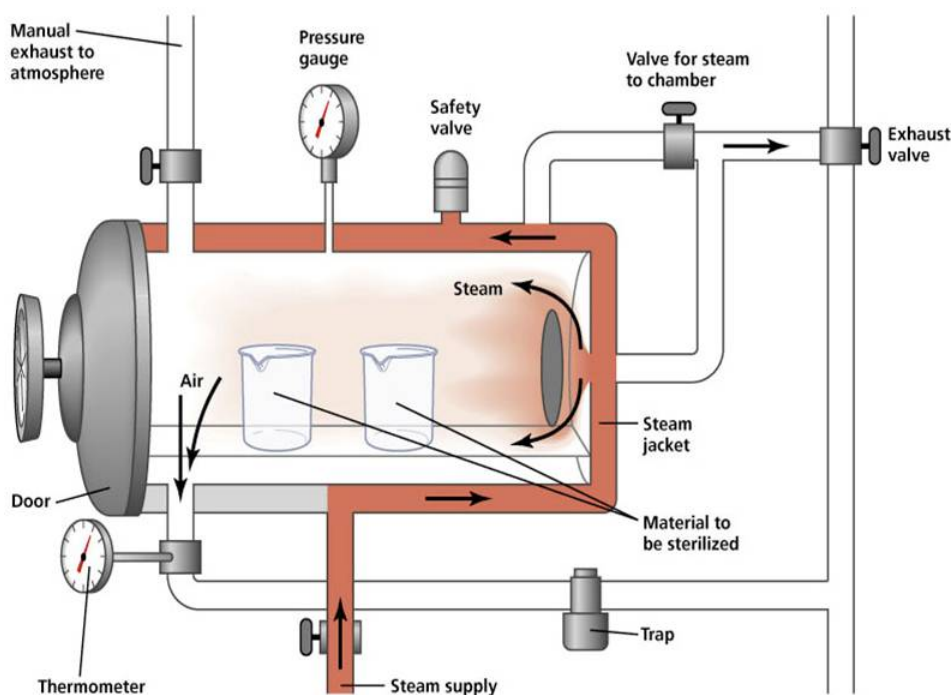
Fig. 19.3 Construction and operation of autoclave

c) Pasteurization

This heat treatment developed by famous microbiologist Louis Pasteur is used to destroy mostly pathogenic bacteria present in liquid medium e.g. milk and wine. Pasteurization is the use of mild heat to reduce the number of microorganisms in a product or food. In the case of pasteurization of milk (Fig. 19.4), the time and temperature depend on killing potential pathogens that are transmitted in milk, i.e. staphylococci, streptococci, Brucella abortus and Mycobacterium tuberculosis. But pasteurization kills many spoilage organisms, as well, and therefore increases the shelf life of milk especially at refrigeration temperatures (2°C).

Milk is usually pasteurized by heating, typically at 63°C for 30 min (batch method) or at 71°C for 15 s (flash method), to kill bacteria and extend the milk's usable life. The process kills pathogens but leaves relatively benign microorganisms that can sour improperly stored milk. Various time-temperature combinations used for pasteurization are given in Table (19.4).

Table 19.4 Time temperature combinations for pasteurization

Process	Treatment
Historical (batch) pasteurization	63°C for 30 min
Flash pasteurization	72°C for 15 s
Ultrahigh temperature pasteurization	134°C for 1 s
Ultrahigh temperature sterilization	140°C for 1-3 s

Fig. 19.4 Pasteurization of milk

19.4.1.2 Dry heat

a) Hot air oven

Basically in the cooking oven (Fig. 19.5a), the rules of relating time and temperature apply, but dry heat is not as effective as moist heat (i.e. higher temperatures are needed for longer periods of time). For example 160°/2h or 170°/1h is necessary for sterilization. The dry heat oven is used for glassware, metal, and objects that won't melt; used on substances that would be damaged by moist heat sterilization e.g. gauzes, dressings or powders.

Fig. 19.5 (a) Hot air oven Fig. 19.5 (b) Bunsen burner

b) Incineration

Burns organisms and physically destroys them (Fig. 19.5b). Used for needles, inoculating wires, glassware, etc. and objects not destroyed in the incineration process.

19.4.2 Filtration

Filtration is the passage of a liquid or gas through a screen like material with pores small enough to retain microorganisms. A vacuum that is created in the receiving flask aids gravity in pulling the liquid through the filter. Some operating theaters occupied by burn patients receive filtered air. High efficiency particulate air (HEPA) filter remove almost all microorganisms larger than 0.3 µm in diameters. Filtration is especially important for sterilization of solutions which would be denatured by heat (e.g. antibiotics, injectable drugs, amino acids, vitamins, etc.). Portable units can be used in the field for water purification and industrial units can be used to 'pasteurize' beverages. Essentially, solutions or gases are passed through a filter of sufficient pore diameter (generally 0.22 µm) to remove the smallest known bacterial cells (Table 19.5, Fig.19.6). Filtration is the primary method of eliminating pathogens from the air supply:

- Operating Rooms
- Burn Units
- Fume Hoods
- Isolation Rooms
- Bio-cabinets
- Pharmaceutical Manufacturing Facilities

Table 19.5 Effective size of membrane filter for exclusion of microorganism

Pore Size(Micrometer)	Microbes Withheld
5	Multicelluar algae and fungi
3	Yeasts and unicellular algae
1.3	Protozoa and small unicellular algae
0.45	Largest bacteria
0.22	Largest viruses and most bacteria
0.025	Largest Viruses and Cell wall less bacteria
0.01	Smallest Viruses
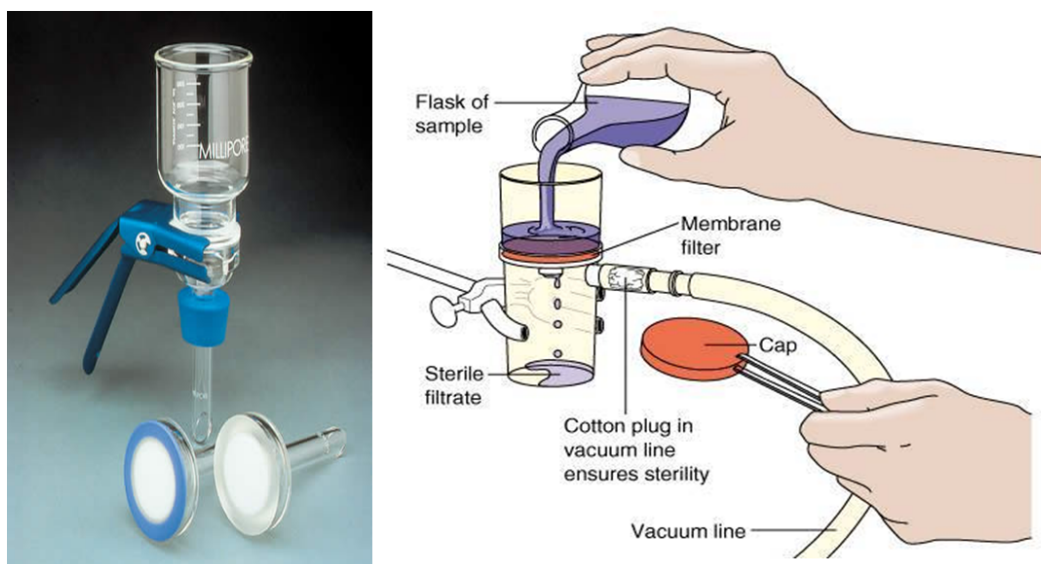
Fig. 19.6 Microfiltration assembly unit

19.4.3 Low temperature

Low temperature (refrigeration and freezing): Most organisms grow very little or not at all at $0^{o}C$. Perishable foods are stored at low temperatures to slow rate of growth and consequent spoilage (e.g. milk). Low temperatures are not bactericidal. Psychrotrophs, rather than true psychrophiles, are the usual cause of food spoilage in refrigerated foods. Although a few microbes will grow in supercooled solutions as low as minus $20°C$, most foods are preserved against microbial growth in the household freezer. The effectiveness of low temperatures depends on the particular microorganism and the intensity of the application. Most microorganisms do not reproduce at ordinary refrigerator temperatures (0-7°C); bacteriostatic. Many microbes survive (but do not grow) at the subzero temperatures used to store foods.

19.4.3.1 Freeze drying

Microbes are placed in a suspending medium and frozen quickly at temperatures between -52 and 95°C. Water is removed by vacuum (sublimation) lyophilization. Powder-like product can be reconstituted to bring culture back to viable conditions.

19.4.4 Desiccation

Drying (removal of H2O): Most microorganisms cannot grow at reduced water activity (aw < 0.90). Drying is often used to preserve foods (e.g. fruits, grains, etc.). Methods involve removal of water from product by heat, evaporation, freeze-drying, and addition of salt or sugar.

19.4.5 Osmotic pressure

The use of high concentrations of salts and sugars in foods is used to increase the osmotic pressure and create a hypertonic environment.

Plasmolysis: As water leaves the cell, plasma membrane shrinks away from cell wall. Cell may not die, but usually stops growing. Yeasts and molds: More resistant to high osmotic pressures. Staphylococci spp. that live on skin are fairly resistant to high osmotic pressure.

19.4.6 Irradiation

Irradiation (UV, X-ray, Gamma radiation): The effects of radiation (Fig. 19.7) depend on its wavelength, intensity, and duration. Ionizing radiation (Gamma rays, X-rays, and high-energy electron beams) has a high degree of penetration and exerts its effect primarily by ionizing water and forming highly reactive hydroxyl radicals. Ultraviolet (UV) radiation, a form of non-ionizing radiation, has a low degree of penetration and causes cell damage by making thymine dimers in DNA that interfere with DNA replication (Fig. 19.8). The most effective germicidal wavelength is 260 nm.
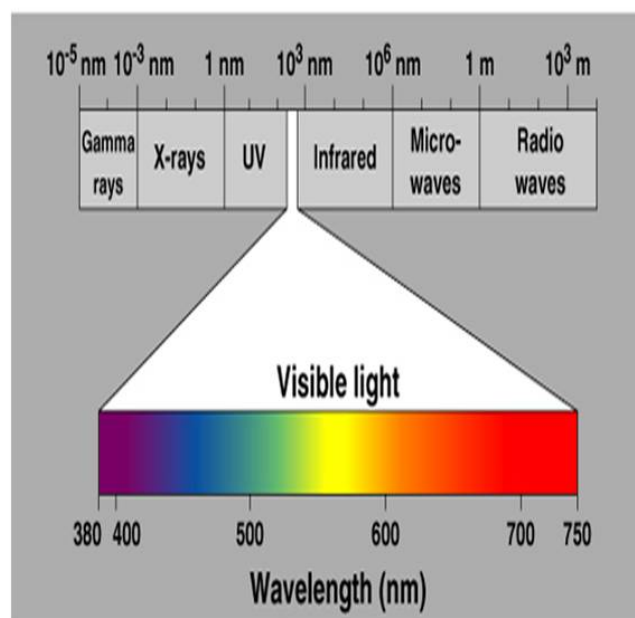
Fig. 19.7 Radiations spectrum
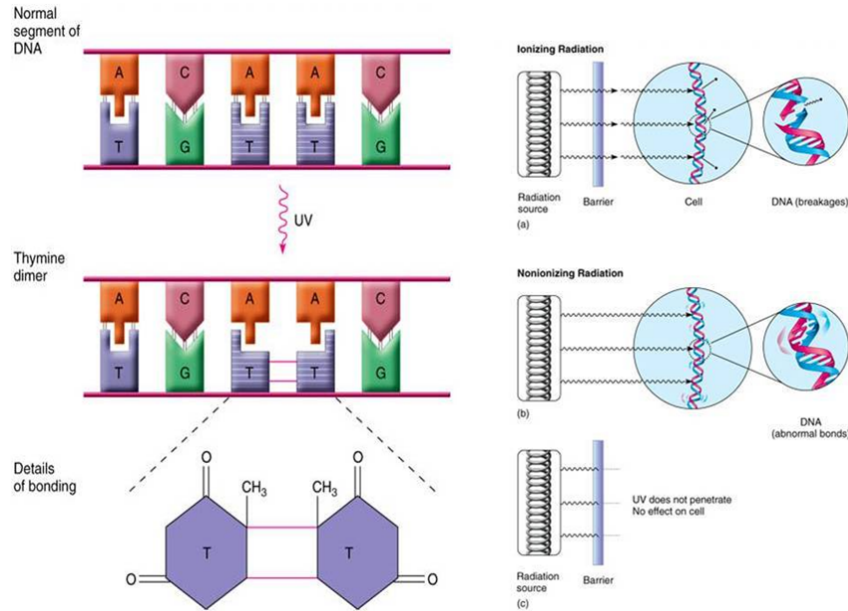
Fig. 19.8 Damage of microbial DNA by UV radiations

In some parts of Europe, fruits and vegetables are irradiated to increase their shelf life up to 500 percent. The practice has not been accepted in the U.S. UV light can be used to pasteurize fruit juices by flowing the juice over a high intensity ultraviolet light source. UV systems for water treatment are available for personal, residential and commercial applications and may be used to control bacteria, viruses and protozoan cysts.

The food and drug administration (FDA) has approved irradiation of poultry and pork to control pathogens, as well as foods such as fruits, vegetables, and grains to control insects, and spices, seasonings, and dry enzymes used in food processing to control microorganisms. Food products are treated by subjecting them to radiation from radioactive sources, which kills significant numbers of insects, pathogenic bacteria and parasites.

According to the FDA, irradiation does not make food radioactive, nor does it noticeably change taste, texture, or appearance. Irradiation of food products to control food-borne disease in humans has been generally endorsed by the United Nation's World Health Organization and the American Medical Association. Two important Disease-causing bacteria that can be controlled by irradiation include Escherichia coli 0157:H7 and Salmonella species.

Microwave cooking ovens were originally researched and developed by German scientists to support mobile operations during the invasion of the Soviet Union. Microwaves can kill microbes indirectly as materials get hot.

19.4.7 Microwave radiation

Wavelength ranges from 1 mm to 1 m. Heat is absorbed by water molecules. It may kill vegetative cells in moist foods. Bacterial endospores, which do not contain water, are not damaged by

microwave radiation. Solid foods are unevenly penetrated by microwaves.

19.4.8 Gamma radiation and electron beam radiation

These radiations are formed of ionizing radiation used primarily in the health care industry. Gamma rays, emitted from cobalt-60, are similar in many ways to microwaves and x-rays. Gamma rays delivered during sterilization break chemical bonds by interacting with the electrons of atomic constituents. Gamma rays are highly effective in killing microorganisms and do not leave residues or have sufficient energy to impart radioactivity.

Electron beam (e-beam) radiation, a form of ionizing energy, is generally characterized by low penetration and high-dose rates. E-beam irradiation is similar to gamma radiation in that it alters various chemical and molecular bonds on contact. Beams produced for e-beam sterilization are concentrated, highly-charged streams of electrons generated by the acceleration and conversion of electricity. E-beam and Gamma radiation are for sterilization of items ranging from syringes to cardiothoracic devices.

Lesson 20
CONTROL OF MICROBIAL GROWTH BY CHEMICAL METHODS

20.1 Introduction

Chemical agents are the disinfectants that kill microorganisms, but not necessarily their spores, but are not safe for application to living tissues; they are used on inanimate objects such as tables, floors, utensils, etc. e.g. hypochlorites, chlorine compounds, lye, copper sulfate, quaternary ammonium compounds, formaldehyde and phenolic compounds (Table 20.1).

Table 20.1 Chemical methods of control of microbial growth

Chemical agents	Mode of action	Remarks	Common use
Phenol	Disruption of plasma membranes and disruption of enzymes	Rarely used; except as a standard of comparison	Seldom used as an antiseptic or disinfectant because of its irritating qualities and disagreeable odor
Phenolics	Disruption of plasma membranes and disruption of enzymes	Environmental surfaces, skin surfaces, instruments and mucous membranes	Derivatives of phenols which are active even in the presence of organic material; O-phenyl phenol is an example.
Bisphenols	Probably Disruption of plasma membranes	Disinfectant hand soaps and skin lotions	Triclosan is an example of Bisphenol; broad spectrum but most effective against gram +ve bacteria
Biguanides (cholrhexidine)	Disruption of plasma membranes	Skin disinfection specially for surgical scubs	Bactericidal to Gram positive bacteria and Gram negative bacteria, nontoxic, persistent
Halogens	Iodine inhibits protein function and it is a strong oxidizing agent; chlorine forms the strong oxidizing agent hypochlorous acid, which alters	Iodine as an effective antiseptic available as a tincture and an iodophor; chlorine gas is used to disinfect water; chlorine compounds are used to disinfect	Iodine and chlorine alone may act as a components of organic and inorganic compounds

Cationic reagents	Enzyme inhibition, protein denaturation and disruption of plasma membranes	Antiseptic for skin, instruments, utensil and glass rubbers	Bactericidal, bacteriostatic, fugicidal, virucidal against enveloped viruses; e.g. Zephiran and Cepacol
Organic acids	Metabolic inhibition; mostly inhibiting molds; action not related to their acidity	Sorbic acid and benzoic acid effective at low pH, parabens much used in shampoos, cosmetics, calcium propionate used in bread; all are mainly antifungal	Widely used to control bacteria and molds in foods and cosmetics
Aldehydes	Protein denaturation	Glutaraldehyde is less irritating than formaldehyde and is used for disinfection of medical equipments	Very effective antimicrobials
Gaseous steriliant	Protein denaturation	Excellent sterilizing agent for the objects that would be damaged by heat	Ethylene oxide is most commonly used
Peroxygens (Oxidizing Agents)	Oxidation	Contaminated surfaces; some deep wounds in which they are most effective against sensitive anaerobes	Ozone is widely used as a supplement for chlorination; Hydrogen peroxide is a good disinfectant but poor antiseptic.

20.1.1 Phenol and phenolics

Phenolics exert their action by injuring the lipid-containing plasma membrane which results in leakage of cellular contents. Mycobacteria are susceptible to phenolics due to their rich lipid content e.g. Cresols (O-phenylphenol, main ingredient in Lysol), bisphenols (Hexachlorophene, used in pHisoHex, effective against Gram positive cocci), triclosan (soap, toothpaste, plastics kitchenware; Gram positive and fungi).

20.1.2 Biguanides

Chlorohexedine damages plasma membranes of vegetative cells and is broad spectrum. These are commonly used for surgical hand scrubs. These are effective against most vegetative bacteria and fungi. Mycobateria, endospores, and protozoan cysts are not affected.

20.1.3 Halogens

Some halogens (iodine and chlorine) are used alone or as components of inorganic or organic solutions. Iodine may combine with certain amino acids to inactivate enzymes and other cellular proteins. Iodine is available as a tincture (in solution with alcohol) or as an iodophor (combined with an organic molecule) like in Betadine. The germicidal action of chlorine is based on the formation of hypochlorous acid when chlorine is added to water. It is an excellent oxidizing agent. Chlorine is used as a disinfectant in gaseous form (Cl_2) or in the form of a compound, such as calcium hypochlorite, sodium hypochlorite, sodium dichloroisocyanurate, and chloramines.

20.1.4 Alcohol

Alcohols exert their action by denaturing proteins and dissolving lipids. In tinctures, they enhance the effectiveness of other antimicrobial agents. Aqueous ethanol (60-90%) and isopropanol are used as disinfectants. Not effective against spores or non-enveloped viruses.

20.1.5 Heavy metals and their compounds

Silver, mercury, copper, and zinc are used as germicidals. They exert their antimicrobial action through oligodynamic action. When heavy metal ions combine with sulfhydryl (-SH) groups, proteins are denatured. Examples are 1% Silver nitrate solution, mercuric chloride, copper sulfate (algicide).

20.1.6 Surface-active agents-soaps and acid anionic detergents

The agents decrease the surface tension among molecules of a liquid; soaps and detergents are examples. Soaps have limited germicidal action but assist in the removal of microorganisms through scrubbing. Acid-anionic detergents are used to clean dairy equipment.

Quaternary Ammonium Compounds: Quats are cationic detergents attached to $NH4^+$. By disrupting the plasma membranes, they allow cytoplasmic constituents to leak out of the cell (Fig. 20.1). Quats are most effective against Gram-positive bacteria. They do not kill endospores or mycobacteria. Examples include Zephiran (benzalkonium chloride) and Cepacol (cetylpyridinium chloride). Pseudomonads are highly resistant, can even live in quats.
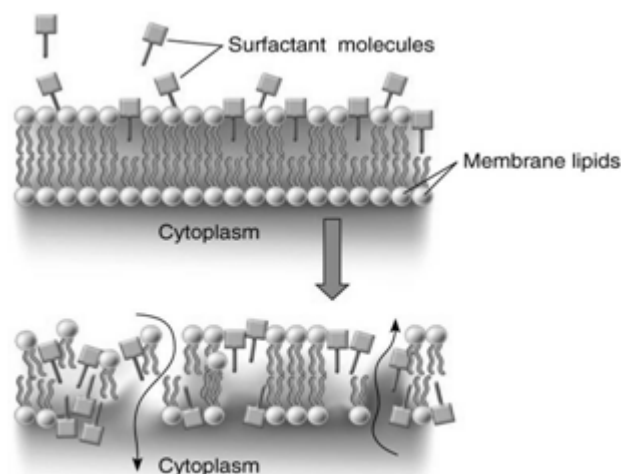
Fig. 20.1 Mode of action of surfactant

20.1.7 Organic acids and derivatives

This class of organic compounds is commonly used as food preservatives. These are effective mostly against mold as they interfere with mold metabolism or the integrity of the plasma membrane.

20.1.7.1 Nitrates

It can be found in some cheeses, adds flavor, maintains pink color in cured meats and prevents botulism in canned foods. Can cause adverse reactions in children, and potentially carcinogenic.

20.1.7.2 Sulfur Dioxide and Sulfites

These are used as preservatives and to prevent browning in alcoholic beverages, fruit juices, soft drinks, dried fruits and vegetables. Sulfites prevent yeast growth and also retard bacterial growth in wine. Sulfites may cause asthma and hyperactivity. They also destroy vitamins.

20.1.7.3 Benzoic acid and sodium benzoate

These are used to preserve oyster sauce, fish sauce, ketchup, non-alcoholic beverages, fruit juices, margarine, salads, confections, baked goods, cheeses, jams and pickled products. They have also been found to cause hyperactivity.

20.1.7.4 Propionic acid and propionates

These are used in bread, chocolate products, and cheese for lasting freshness.

20.1.7.5 Sorbic acid and sorbates

It prevents mold formation in cheese and flour confectioneries

Some common food preservatives along with their applications are listed in Table 20.2.

Table 20.2 Common food preservatives and their application

Preservative	Effective Concentration	Uses
Propionic acid and propionates	0.32%	Antifungal agent in breads, cake, Swiss cheeses
Sorbic acid and sorbates	0.2%	Antifungal agent in cheeses, jellies, syrups, cakes
Benzoic acid and benzoates	0.1%	Antifungal agent in margarine, cider, relishes, soft drinks
Sodium diacetate	0.32%	Antifungal agent in breads
Lactic acid	unknown	Antimicrobial agent in cheeses, buttermilk, yogurt and pickled foods
Sulfur dioxide, sulfites	200-300 ppm	Antimicrobial agent in dried fruits, grapes, molasses
Sodium nitrite	200 ppm	Antibacterial agent in cured meats, fish
Sodium chloride	unknown	Prevents microbial spoilage of meats, fish, etc.
Sugar	unknown	Prevents microbial spoilage of preserves, jams, syrups, jellies
Wood smoke	unknown	Prevents microbial spoilage of meats, fish, etc.

20.1.8 Aldehydes

Aldehydes such as formaldehyde and glutaraldehyde (Gidex) exert their antimicrobial effect by inactivating proteins. They are among the most effective chemical disinfectants.

20.1.9 Gaseous chemosterilants

This class of chemosterilants includes chemicals that sterilize in a closed chamber. Chemicals used for sterilization include the gases ethylene oxide and formaldehyde, and liquids such as glutaraldehyde. Ozone, hydrogen peroxide and peracetic acid are also examples of chemical sterilization techniques are based on oxidative capabilities of the chemical.

20.1.9.1 Ethylene oxide (ETO)

It is the most commonly used form of chemical sterilization. Due to its low boiling point of 10.4°C at atmospheric pressure, ETO behaves as a gas at room temperature. ETO chemically reacts with amino acids, proteins, and DNA to prevent microbial reproduction. The sterilization process is carried out in a specialized gas chamber. After sterilization, products are transferred to an aeration cell, where they remain until the gas disperses and the product is safe to handle. ETO is used for

cellulose and plastics irradiation, usually in hermetically sealed packages. Ethylene oxide can be used with a wide range of plastics (e.g. petri dishes, pipettes, syringes, medical devices, etc.) and other materials without affecting their integrity (Fig.20.2).

Fig. 20.2 An ethylene oxide sterilization gas chamber

Ozone sterilization has been recently approved for use in the U.S. It uses oxygen that is subjected to an intense electrical field that separates oxygen molecules into atomic oxygen, which then combines with other oxygen molecules to form ozone. Ozone is used as a disinfectant for water and food. It is used in both gas and liquid forms as an antimicrobial agent in the treatment, storage and processing of foods, including meat, poultry and eggs. Many municipalities use ozone technology to purify their water and sewage. Los Angeles has one of the largest municipal ozone water treatment plants in the world. Ozone is used to disinfect swimming pools, and some companies selling bottled water use ozonated water to sterilize containers. An ozone fogger for sterilization of egg surfaces is depicted in (Fig.20.2). The system facilitates reaction of ozone with water vapors to create powerful oxidizing radicals. This system is totally chemical free and is effective against bacteria, viruses and hazardous microorganisms which are deposited on egg shells (Fig. 20.3).
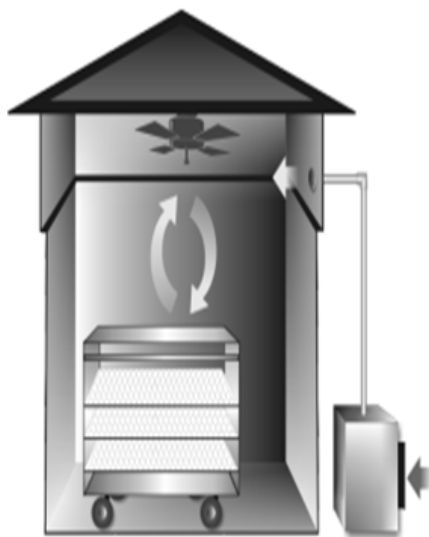

Fig. 20.3 Ozone fogger

20.1.9.2 Low Temperature Gas Plasma (LTGP)

It is used as an alternative to ethylene oxide. It uses a small amount of liquid hydrogen peroxide (H_2O_2), which is energized with radio frequency waves into gas plasma. This leads to the generation of free radicals and other chemical species, which destroy organisms. (Fig. 20.4)

Common antiseptics and disinfectants and their uses are summarized in (Table 20.3)

Table 20.3 Common antiseptics and disinfectants and their uses

Chemical	Action	Uses
Ethanol (50-70%)	Denatures proteins and solubilizes lipids	Antiseptic used on skin
Isopropanol (50-70%)	Denatures proteins and solubilizes lipids	Antiseptic used on skin
Formaldehyde (8%)	Reacts with NH_2, SH and COOH groups	Disinfectant, kills endospores
Tincture of Iodine (2% I_2 in 70% alcohol)	Inactivates proteins	Antiseptic used on skin Disinfection of drinking water
Chlorine (Cl_2) gas	Forms hypochlorous acid (HClO), a strong oxidizing agent	Disinfect drinking water; general disinfectant
Silver nitrate ($AgNO_3$)	Precipitates proteins	General antiseptic and used in the eyes of newborns
Mercuric chloride	Inactivates proteins by reacting with sulfide groups	Disinfectant, although occasionally used as an antiseptic on skin
Detergents (e.g. quaternary ammonium compounds)	Disrupts cell membranes	Skin antiseptics and disinfectants
Phenolic compounds (e.g. carbolic acid, lysol, hexylresorcinol, hexachlorophene)	Denature proteins and disrupt cell membranes	Antiseptics at low concentrations; disinfectants at high concentrations
Ethylene oxide gas	Alkylating agent	Disinfectant used to sterilize heat-sensitive objects such as rubber and plastics
Ozone	Generates lethal oxygen radicals	Purification of water, sewage
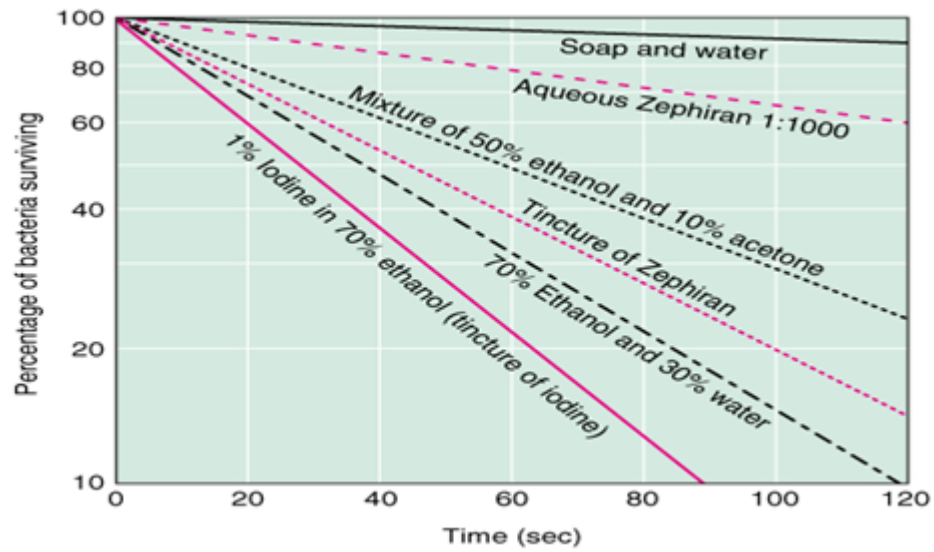
**Fig. 20.4 Relative effectiveness of chemical biocides**

Lesson 21
DNA STRUCTURE

21.1 Introduction

21.1.1 Deoxyribonucleic acid (DNA)

Deoxyribonucleic acid (DNA) is a nucleic acid that contains the genetic instructions used in the development and functioning of all known living organisms with the exception of some viruses. The main role of DNA molecules is the long-term storage of information. DNA is often compared to a set of blueprints, like a recipe or a code, since it contains the instructions needed to construct other components of cells, such as proteins and RNA molecules. The DNA segments that carry this genetic information are called genes, but other DNA sequences have structural purposes, or are involved in regulating the use of this genetic information. Within cells, DNA is organized into long structures called chromosomes. These chromosomes are duplicated before cells divide, in a process called DNA replication. Eukaryotic organisms (animals, plants, fungi, and protists) store most of their DNA inside the cell nucleus and some of their DNA in organelles, such as mitochondria or chloroplasts. In contrast, prokaryotes (bacteria and archaea) store their DNA only in the cytoplasm.

The information in DNA is made up of four bases which combine to form chains. These bases include two purines (Adenine and Guanine) and two pyrimidines (Cytosine and Thymine). These are commonly referred to as A, G, C and T respectively (Fig.21.1)
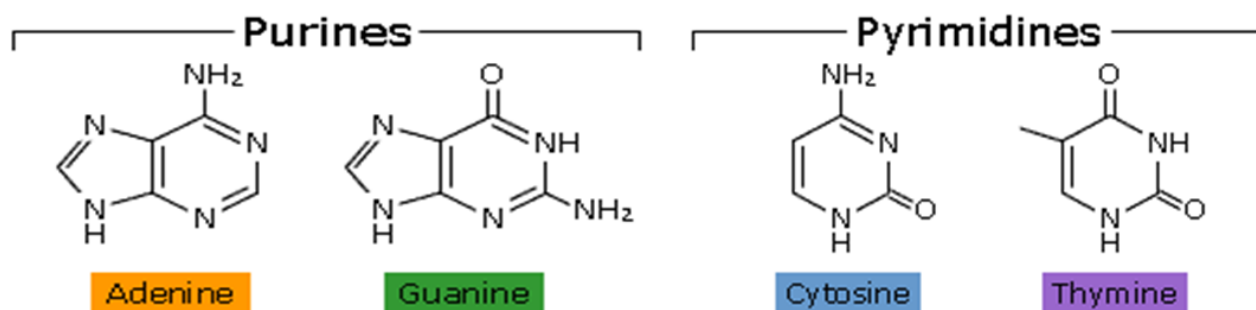
Fig. 21.1 Bases in DNA

Human DNA consists of about 3 billion bases, and more than 99 percent of those bases are the same in all people. The order, or sequence, of these bases determines the information available for building and maintaining an organism, similar to the way in which letters of the alphabet appear in a certain order to form words and sentences.

21.2 Chargaff's Rules

These state that DNA from any cell of all organisms should have a 1:1 ratio of pyrimidine and purine bases and, more specifically, that the amount of guanine is equal tocytosine and the amount of adenine is equal to thymine. This Pattern is found in both strands of the DNA. They were discovered by Austrian chemist Erwin Chargaff.

DNA bases pair up with each other, A with T and C with G, to form units called base pairs. Each base is also attached to a sugar molecule and a phosphate molecule. Together, a base, sugar, and phosphate are called a nucleotide. Nucleotides are arranged in two long strands that form a spiral called a double helix. The structure of the double helix is somewhat like a ladder, with the base pairs forming the ladder's rungs and the sugar and phosphate molecules forming the vertical sidepieces of the ladder.

21.2.1 Structure

In 1953 Watson and Crick postulated a three dimensional model of DNA structure. It consists of two helical DNA chains wound around the same axis to form a right handed double helix. The hydrophilic backbones of alternating deoxyribose and phosphate groups are on the outside of the double helix, facing the surrounding water. The purine and pyrimidine bases of both strands are stacked inside the double helix, with their hydrophobic and nearly planar ring structures very close together and perpendicular to the long axis. The offset pairing of the two strands creates a major groove and minor groove on the surface of the duplex. Each nucleotide base of one strand is paired in the same plane with a base of the other strand. Watson and Crick found that the hydrogen-bonded base pairs, G with C and A with T, are those that fit best within the structure, providing a rationale for Chargaff's rule. It is important to note that three hydrogen bonds can form between G and C, but only two can form between A and T. This is one reason for the finding that separation of paired DNA strands is more difficult the higher the ratio of GC to AT base pairs. Other pairings of bases tend (to varying degrees) to destabilize the double-helical structure. When Watson and Crick constructed their model, they had to decide at the outset whether the strands of DNA should be parallel or antiparallel whether their 5'-3' phosphodiester bonds should run in the same or opposite directions. An antiparallel orientation produced the most convincing model, and later work with DNA polymerases provided experimental evidence that the strands are indeed antiparallel, a finding ultimately confirmed by x-ray analysis. (Fig.21.2)
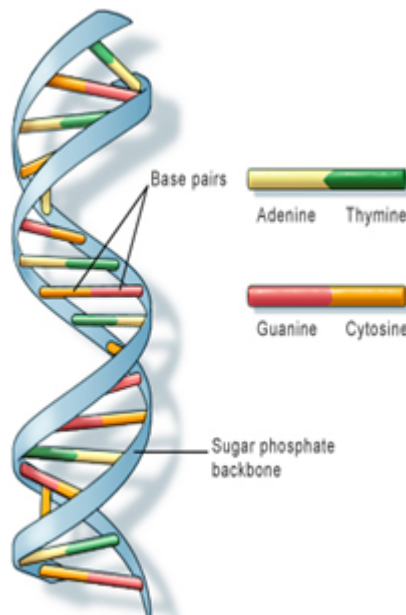
Fig. 21.2 Structure of DNA

21.2.2 The sugars in the backbone

The backbone of DNA is based on a repeated pattern of a sugar group and a phosphate group. The full name of DNA, deoxyribonucleic acid, gives you the name of the sugar present - deoxyribose. Deoxyribose is a modified form of another sugar called ribose. Ribose is the sugar in the backbone of RNA, ribonucleic acid (Fig. 21.3).
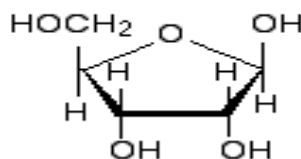
Fig. 21.3 Ribose

This diagram misses out the carbon atoms in the ring for clarity. Each of the four corners where there isn't an atom shown has a carbon atom. Deoxyribose, as the name might suggest, is ribose which has lost an oxygen atom – 'de-oxy' (Fig.21.4).

Deoxyribose has a hydrogen here rather than -OH.
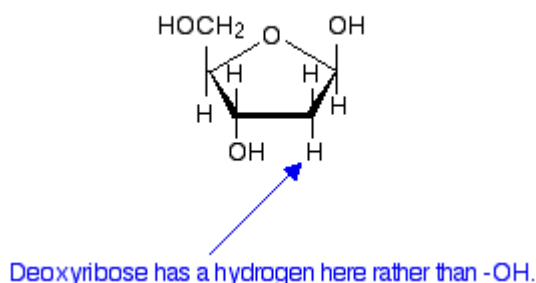
Fig. 21.4 Deoxyribose

The only other thing you need to know about deoxyribose (or ribose, for that matter) is how the carbon atoms in the ring are numbered. The carbon atom to the right of the oxygen as we have drawn the ring is given the number 1, and then you work around to the carbon on the CH2OH side group which is number 5 (Fig. 21.5).

Start numbering on the carbon to the right of the oxygen on the ring . . .

. . . and work around to the side chain carbon.
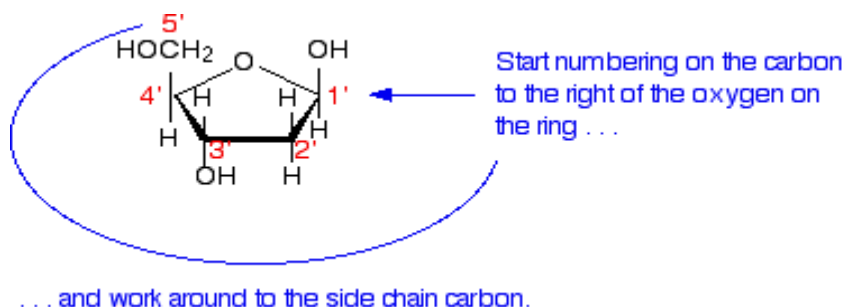
Fig. 21.5 Deoxyribose with carbon numbering

You will notice that each of the numbers has a small dash by it - 3' or 5', for example. If you just had ribose or deoxyribose on its own, that wouldn't be necessary, but in DNA and RNA these sugars are

attached to other ring compounds. The carbons in the sugars are given the little dashes so that they can be distinguished from any numbers given to atoms in the other rings. You read 3' or 5' as '3-prime' or '5-prime'.

21.2.3 Attaching a phosphate group

The other repeating part of the DNA backbone is a phosphate group. A phosphate group is attached to the sugar molecule in place of the -OH group on the 5' carbon (Fig. 21.6).
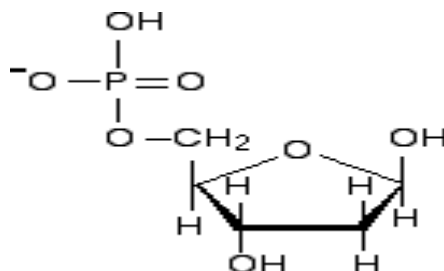
Fig. 21.6 Deoxyribose with Phosphate Group

21.2.4 Attaching a base and making a nucleotide

The final piece that we need to add to this structure before we can build a DNA strand, is one of four complicated organic bases. In DNA, these bases are cytosine (C), thymine (T), adenine (A) and guanine (G).

These bases attach in place of the -OH group on the 1' carbon atom in the sugar ring (Fig. 21.7).
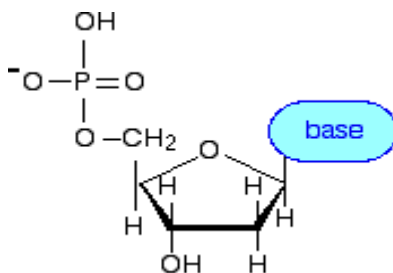
Fig. 21.7 Deoxyribose with Phosphate Group and Base

What we have produced is known as a nucleotide (Fig.21.8).

Here are their structures:
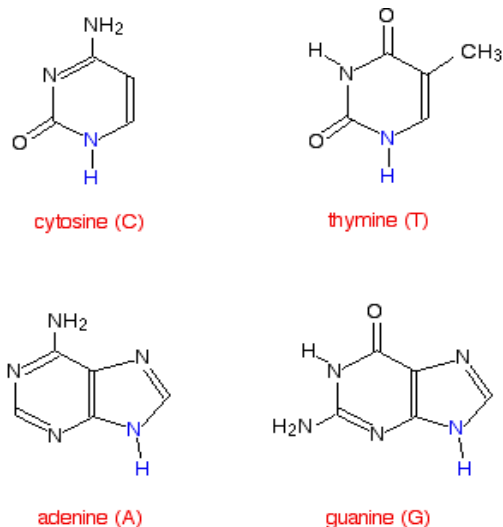
Fig. 21.8 Nucleotides

The nitrogen and hydrogen atoms shown in blue on each molecule show that where these molecules join on to the deoxyribose. In each case, the hydrogen is lost together with the -OH group on the 1' carbon atom of the sugar. This is a condensation reaction - two molecules joining together with the loss of a small one (not necessarily water). For example, here is what the nucleotide containing cytosine would look like: (Fig. 21.9).
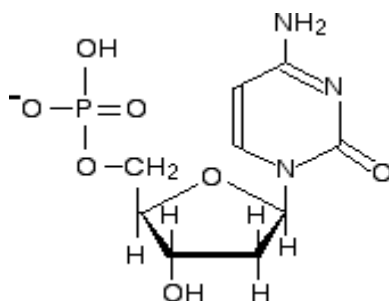
Fig. 21.9 Nucleotide containing cytosine

21.2.5 Joining the nucleotides into a DNA strand

A DNA strand is simply a string of nucleotides joined together. The phosphate group on one nucleotide links to the 3' carbon atom on the sugar of another one. In the process, a molecule of water is lost - another condensation reaction (Fig. 21.10).
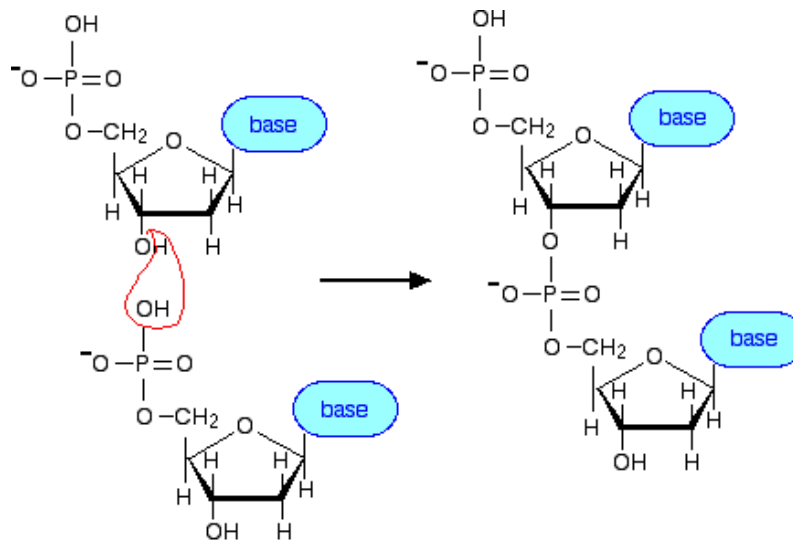
Fig. 21.10 Condensation reaction

21.2.6 Building a DNA chain concentrating on the essentials

What matters in DNA is the sequence the four bases take up in the chain (Fig. 21.11).
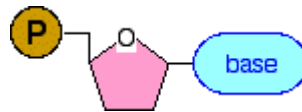
Fig. 21.11 Single chain structure of DNA

There is only one possible point of confusion here - and that relates to how the phosphate group, P, is attached to the sugar ring. Notice that it is joined via two lines with an angle between them.

By convention, if you draw lines like this, there is a carbon atom where these two lines join. That is the carbon atom in the CH_2 group if you refer back to a previous diagram. If you had tried to attach the phosphate to the ring by a single straight line, that CH_2 group would have got lost! Joining up lots of these gives a part of a DNA chain. The diagram below is a bit from the middle of a chain. Notice that the individual bases have been identified by the first letters of the base names. (A = adenine, etc). Notice also that there are two different sizes of base. Adenine and guanine are bigger because they both have two rings. Cytosine and thymine only have one ring each (Fig.21.12).
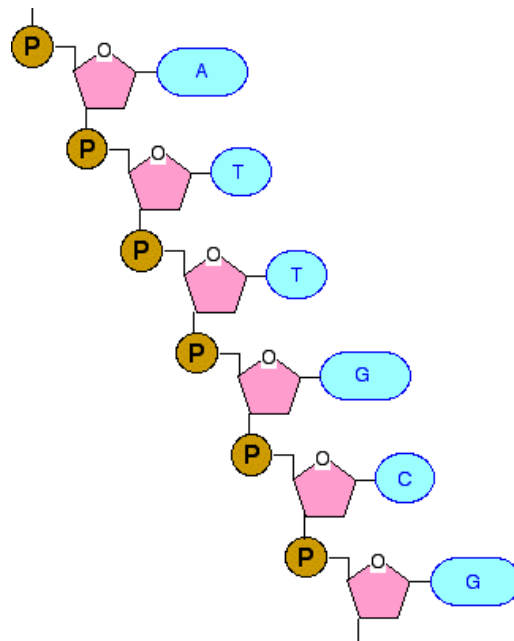
Fig. 21.12 DNA chain

If the top of this segment was the end of the chain, then the phosphate group would have an -OH group attached to the spare bond rather than another sugar ring. Similarly, if the bottom of this segment of chain was the end, then the spare bond at the bottom would also be to an -OH group on the deoxyribose ring.

21.2.7 Joining the two DNA chains together

If you look at the diagram carefully, you will see that an adenine on one chain is always paired with a thymine on the second chain. And a guanine on one chain is always paired with a cytosine on the other one. The first thing to notice is that a smaller base is always paired with a bigger one. The effect of this is to keep the two chains at a fixed distance from each other all the way along (Fig.21.13).

But, more than this, the pairing has to be exactly:

- Adenine (A) pairs with thymine (T);
- Guanine (G) pairs with cytosine (C).

That is because these particular pairs fit exactly to form very effective hydrogen bonds with each other. It is these hydrogen bonds which hold the two chains together (Fig. 21.14).

The base pairs fit together as follows.

The A-T base pair:
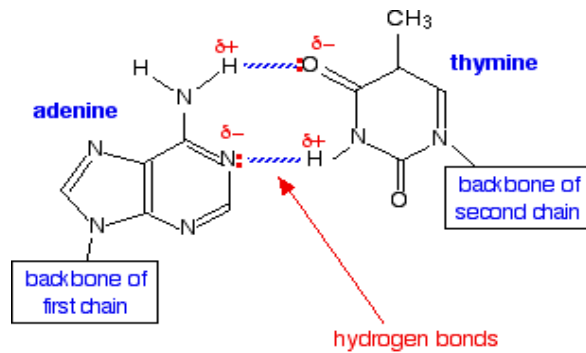
Fig. 21.13 Hydrogen bonding in Nitrogen bases

The G-C base pair:
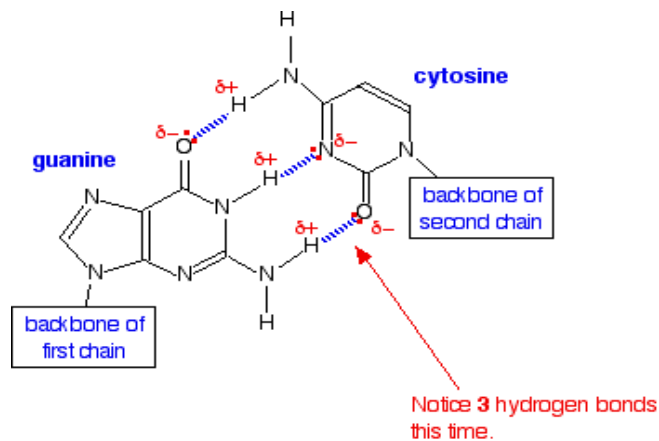
Fig. 21.14 Hydrogen bonding

If you try any other combination of base pairs, they won't fit!
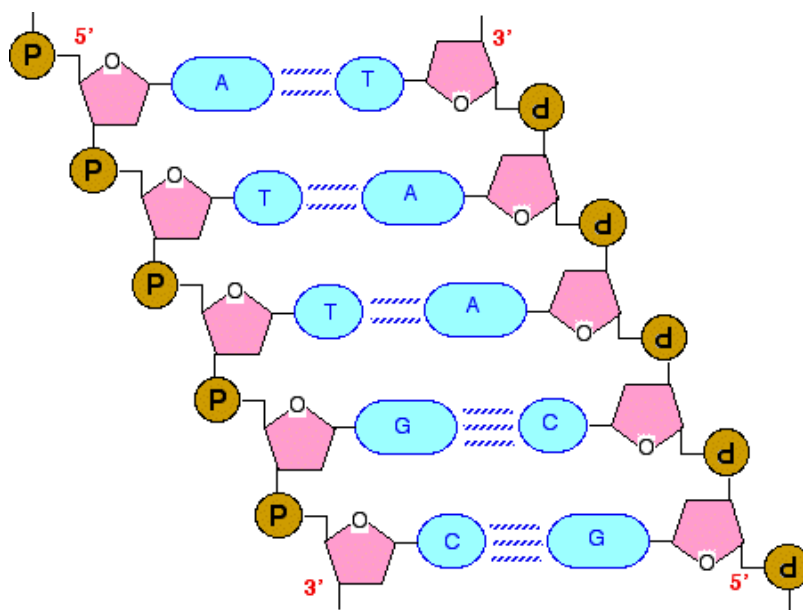
Fig. 21.15 Final structure for DNA showing the important bits

Notice that the two chains run in opposite directions and the right-hand chain is essentially uside-down. The ends of these bits of chain are labeled with 3' and 5' (Fig. 21.15). The genetic code in genes is always written in the 5' to 3' direction along a chain

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Lesson 22
DNA REPLICATION, TRANSCRIPTION, TRANSLATION

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22.1 Introduction

One major question for the human mind is how life continues. One of the most important mechanisms for all life cells to give off springs is undoubtedly the DNA Replication. DNA Replication answers to the question: 'When a cell divides, where the extra DNA comes from?' What 'DNA Replication' is? It is the process that can duplicate the DNA of a cell. The next step is the cell to duplicate. Every cell (of eukaryotes or prokaryotes) has one or more DNA (or RNA) polymer molecules that need to duplicate in order the cell duplication to take place. This is what DNA replication or DNA synthesis succeeds. In the eukaryotes (organisms with cell that have nucleus) the DNA is formed in two strands, each composed of units called nucleotides. The two strands look like two chains that form the DNA double helix. The DNA replication process is capable of opening the double helix and separating the two strands. Then the two strands are copied. As a result two new DNA molecules are created. The next step is the cell division. After that a daughter cell is created. In its nucleus lies a copy of the parental DNA.

22.2 DNA Replication Models

The process of DNA Replication was hiding many secrets. One of the most important was how the two daughter strands are created. In order the hereditary phenomenon to be explained, these strands should be accurately copied and transmitted from the parental cell to the daughter ones. These are three possible models that describe the accurate creation of the daughter chains.

22.2.1 Semiconservative replication

According to this model, DNA Replication would create two molecules. Each of them would be a complex of an old (parental and a daughter strand).

22.2.2 Conservative replication

According to this model, the DNA Replication process would create a brand new DNA double helix made of two daughter strands while the parental chains would stay together.

22.2.3 Dispersive replication

According to this model the replication process would create two DNA double-chains, each of them with parts of both parent and daughter molecules.

22.3 Steps in DNA Replication

1) The first major step in DNA Replication to take place is the breaking of hydrogen bonds between bases of the two anti-parallel strands. The un-wounding of the two strands is the starting point. The splitting happens in places of the chains which are rich in A-T. That is because there are only two bonds between Adenine and Thymine (there are three hydrogen bonds between Cytosine and

<div align="center">

</div>

Guanine). Helicase is the enzyme that splits the two strands. The initiation point where the splitting starts is called 'origin of replication'.The structure that is created is known as 'Replication Fork' (Fig. 22.1).
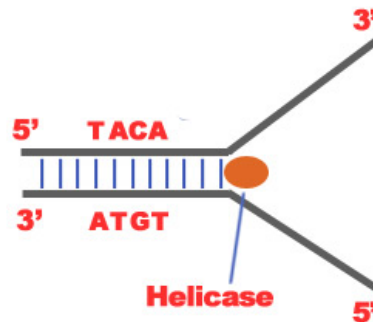
Fig. 22.1 Replication fork

2) One of the most important steps of DNA Replication is the binding of RNA Primase in the the initiation point of the 3'-5' parent chain. RNA Primase can attract RNA nucleotides which bind to the DNA nucleotides of the 3'-5' strand due to the hydrogen bonds between the bases. RNA nucleotides are the primers (starters) for the binding of DNA nucleotides (Fig. 22.2).
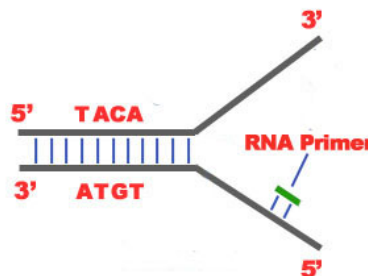
Fig. 22.2 Binding of RNA primase

3) The elongation process is different for the 5'-3' and 3'-5' template.

a) 5'-3' Template: The 3'-5' proceeding daughter strand -that uses a 5'-3' template- is called leading strand because DNA Polymerase A can read the template and continuously adds nucleotides (complementary to the nucleotides of the template, for example Adenine opposite to Thymine etc.) (Fig.22.3).
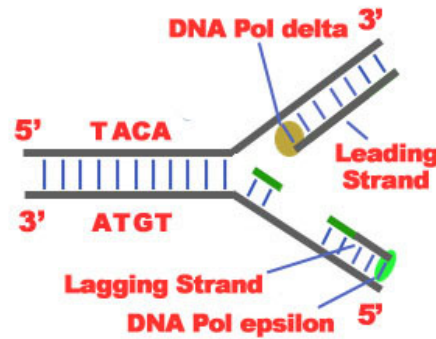
Fig. 22.3 Formation of leading and lagging strands

b) 3'-5'Template: The 3'-5' template cannot be read by DNA Polymerase. The replication of this template is complicated and the new strand is called lagging strand. In the lagging strand the RNA primase adds more RNA primers. DNA polymerase A reads the template and lengthens the bursts. The gap between two RNA primers is called 'Okazaki Fragments'. The RNA primers are necessary for DNA polymerase A to bind nucleotides to the 3' end of them. The daughter strand is elongated with the binding of more DNA nucleotides (Fig. 22.4).
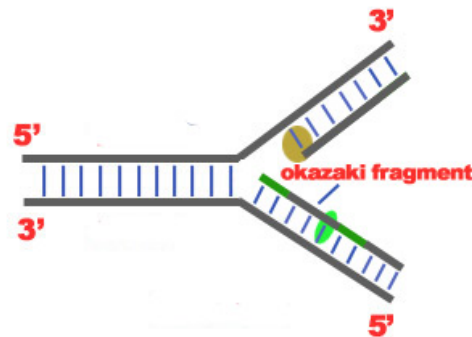
Fig. 22.4 Okazaki fragments

4) In the lagging strand the DNA Pol I - exonuclease reads the fragments and removes the RNA primers. The gaps are closed with the action of DNA Polymerase (adds complementary nucleotides to the gaps) and DNA Ligase (adds phosphate in the remaining gaps of the phosphate - sugar backbone).

Each new double helix is consisted of one old and one new chain. This is what we call semi conservative replication (Fig.22.5)
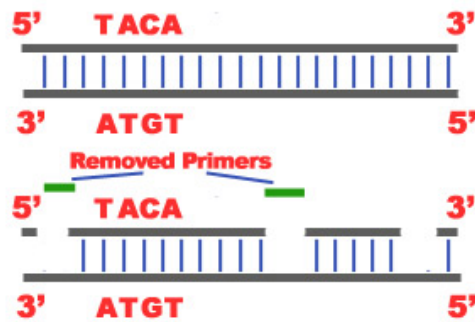
Fig. 22.5 Semi conservative replication-removal of primers

5) The last step of DNA replication is the termination. This process happens when the DNA Polymerase reaches to an end of the strands (Fig. 22.6) We can easily understand that in the last section of the lagging strand, when the RNA primer is removed, it is not possible for the DNA Polymerase to seal the gap (because there is no primer). So, the end of the parental strand where the last primer binds isn't replicated.

6) The DNA Replication is not completed before a mechanism of repair fixes possible errors caused during the replication. Enzymes like nucleases remove the wrong nucleotides and the DNA polymerase fills the gaps.
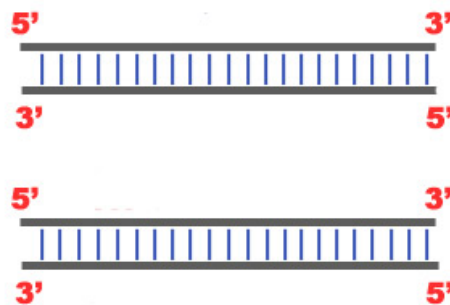
Fig. 22.6 DNA strands

22.4 Transcription

Transcription is the mechanism by which a template strand of DNA is utilized by specific RNA polymerases to generate one of the three different classifications of RNA. These 3 RNA classes are:

- Messenger RNAs (mRNAs): This class of RNAs is the genetic coding templates used by the translational machinery to determine the order of amino acids incorporated into an elongating polypeptide in the process of translation.
- Transfer RNAs (tRNAs): This class of small RNAs form covalent attachments to individual amino acids and recognize the encoded sequences of the mRNAs to allow correct insertion of amino acids into the elongating polypeptide chain.
- Ribosomal RNAs (rRNAs): This class of RNAs is assembled, together with numerous ribosomal proteins, to form the ribosomes. Ribosomes engage the mRNAs and form a

catalytic domain into which the tRNAs enter with their attached amino acids. The proteins of the ribosomes catalyze all of the functions of polypeptide synthesis.

All RNA polymerases are dependent upon a DNA template in order to synthesize RNA. The resultant RNA is, therefore, complimentary to the template strand of the DNA duplex and identical to the non-template strand. The non-template strand is called the coding strand because its' sequences are identical to those of the mRNA. However, in RNA, U is substituted for T.

22.5 Translation

Translation is the RNA directed synthesis of polypeptides. This process requires all three classes of RNA. Although the chemistry of peptide bond formation is relatively simple, the processes leading to the ability to form a peptide bond are exceedingly complex. The template for correct addition of individual amino acids is the mRNA, yet both tRNAs and rRNAs are involved in the process. The tRNAs carry activated amino acids into the ribosome which is composed of rRNA and ribosomal proteins. The ribosome is associated with the mRNA ensuring correct access of activated tRNAs and containing the necessary enzymatic activities to catalyze peptide bond formation.

22.5.1 The genetic code

Shown below are the triplets that are used for each of the 20 amino acids found in eukaryotic proteins. The row on the left side indicates the first nucleotide of each triplet and the row across the top represents the second nucleotide. The wobble position nucleotides are indicated in blue. The three stop codons are highlighted in red (Fig. 22.7).

	U		C		A		G	
U	UUU	Phe	UCU	Ser	UAU	Tyr	UGU	Cys
	UUC	Phe	UCC	Ser	UAC	Tyr	UGC	Cys
	UUA	Leu	UCA	Ser	UAA	End	UGA	End
	UUG	Leu	UCG	Ser	UAG	End	UGG	Trp
C	CUU	Leu	CCU	Pro	CAU	His	CGU	Arg
	CUC	Leu	CCC	Pro	CAC	His	CGC	Arg
	CUA	Leu	CCA	Pro	CAA	Gln	CGA	Arg
	CUG	Leu	CCG	Pro	CAG	Gln	CGG	Arg
A	AUU	Ile	ACU	Thr	AAU	Asn	AGU	Ser
	AUC	Ile	ACC	Thr	AAC	Asn	AGC	Ser
	AUA	Ile	ACA	Thr	AAA	Lys	AGA	Arg
	AUG	Met	ACG	The	AAG	Lys	AGG	Arg
G	GUU	Val	GCU	Ala	GAU	Asp	GGU	Gly
	GUC	Val	GCC	Ala	GAC	Asp	GGC	Gly
	GUA	Val	GCA	Ala	GAA	Glu	GGA	Gly
	GUG	Val	GCG	Ala	GAG	Glu	GGG	Gly

Fig. 22.7 Triplet codons

22.5.2 Order of events in translation

The ability to begin to identify the roles of the various ribosomal proteins in the processes of ribosome assembly and translation was aided by the discovery that the ribosomal subunits will self

assemble in vitro from their constituent parts (Fig. 22.8).

Following assembly of both the small and large subunits onto the mRNA, and given the presence of charged tRNAs, protein synthesis can take place. To reiterate the process of protein synthesis:

- Synthesis proceeds from the N-terminus to the C-terminus of the protein.
- The ribosomes read the mRNA in the 5' to 3' direction.
- Active translation occurs on polyribosomes (also termed polysomes). This means that more than one ribosome can be bound to and translate a given mRNA at any one time.
- Chain elongation occurs by sequential addition of amino acids to the C-terminal end of the ribosome bound polypeptide.

Translation proceeds in an ordered process. First accurate and efficient initiation occurs, then chain elongation, and finally accurate and efficient termination must occur. All three of these processes require specific proteins, some of which are ribosome associated and some of which are separate from the ribosome, but may be temporarily associated with it.

Initiation

- First the 70s ribosome must separate into 50s and 30s subunits.
- The 3 initiation factors IF-1, IF-2, and IF-3 then bind to the 30S ribosomal subunit.
- Then the specific initiator tRNA carrying the N-formylmethionine and the mRNA join the complex (remember base pairing via Shine-Dalgarno sequence). The initiator tRNA is specifically recognized by IF-2.
- At this point in the process we have what is termed a 30s preinitiation complex.
- The 50S subunit binds, triggering the release of the IFs.

Thus, formation of the 70S initiation complex is completed and it is ready for the next phase, elongation.
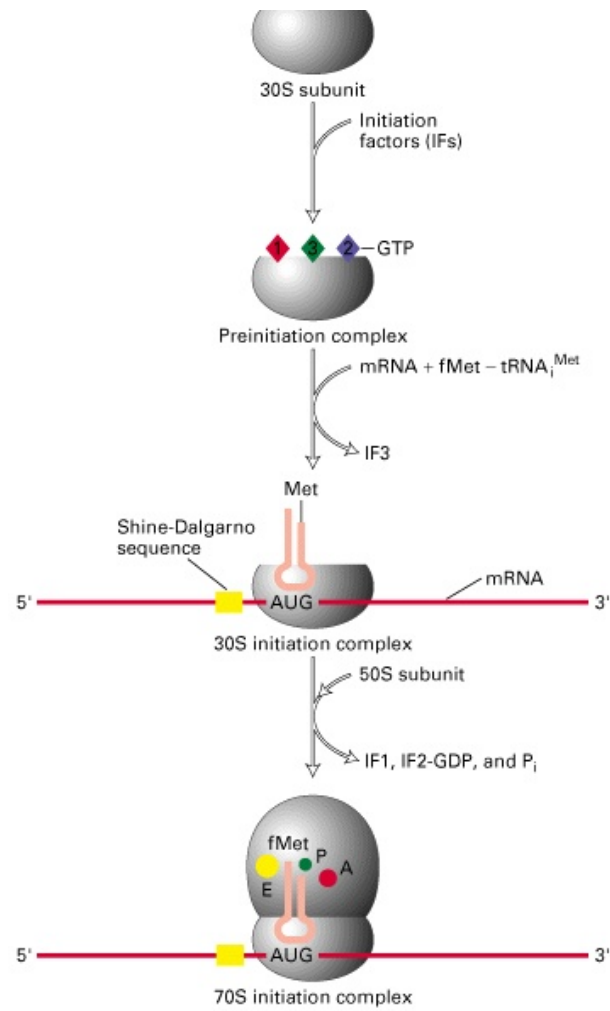
Fig. 22.8 Order of events in translation

Lesson 23
GENETIC MUTATION

23.1 Mutations

A mutation is any change in the sequence of the DNA encoding a gene. Most of these mutations are recognized because the phenotype of the organism has changed Originally genes were thought to be beads-on-string, where each bead was a single entity responsible for a phenotype. This theory lead to the concept that only single mutation was possible for a specific gene. Detailed genetic experiments proved that the gene actually consists of many individual units, and specific changes in these units can lead to several mutant phenotypes. We now know those units are nucleotides. Therefore understanding the nature of mutations is important to our understanding of a gene.

23.2 Germinal and Somatic Mutations

Eukaryotic organisms have two primary cell types: germ and somatic. Mutations can occur in either cell type. If a gene is altered in a germ cell, the mutation is termed a germinal mutation. Because germ cells give rise to gametes, some gamete s will carry the mutation and it will be passed on to the next generation when the individual successfully mates. Typically germinal mutations are not expressed in the individual containing the mutation. The only instance in which it would be expressed is if it negatively (or positively) affected gamete production.

Somatic cells give rise to all non-germline tissues. Mutations in somatic cells are called somatic mutations. Because they do not occur in cells that give rise to gametes, the mutation is not passed along to the next generation by sexual means. To maintain this mutation, the individual containing the mutation must be cloned. Two examples of somatic clones are navel oranges and red delicious apples. Horticulturists first observed the mutants. They then grafted mutant branches onto the stocks of "normal" trees. After the graft was established, cuttings from that original graft were grafted onto tree stocks. In this way the mutation was maintained and proliferated.

Most tissues are derived from a cell or a few progenitor cells. If a mutation occurs in one of the progenitor cells, all of its daughter cells will also express the mutation. For this reason, somatic mutations generally appear as a sector on the mutated individual.

Cancer tumors are a unique class of somatic mutations. The tumor arises when a gene involved in cell division, a protooncogene, is mutated. All of the daughter cells contain this mutation. The phenotype of all cells containing the mutation is uncontrolled cell division. This result in a tumor that is a collection of undifferentiated cells called tumor cells.

23.3 Causes

Two classes of mutations are spontaneous mutations (molecular decay) and induced mutations caused by mutagens.

23.3.1 Spontaneous mutation

In general, the appearance of a new mutation is a rare event. Most mutations that were originally studied occurred spontaneously. This class of mutation is termed spontaneous mutations. The spontaneous mutation rate varies. Large gene provide a large target and tend to mutate more frequently. A study of the five coat color loci in mice showed that the rate of mutation ranged from 2×10^{-6} to 40×10^{-6} mutations per gamete per gene. Data from several studies on eukaryotic organisms shows that in general the spontaneous mutation rate is $2\text{-}12 \times 10^{-6}$ mutations per gamete per gene. Given that the human genome contains 100,000 genes, we can conclude that we would predict that 1-5 human gametes would contain a mutation in some gene. Spontaneous mutations on the molecular level can be caused by:

23.3.1.1 Tautomerism

A base is changed by the repositioning of a hydrogen atom, altering the hydrogen bonding pattern of that base resulting in incorrect base pairing during replication.

23.3.1.2 Depurination

Loss of a purine base (A or G) to form an apurinic site (AP site).

23.3.1.3 Deamination

Hydrolysis changes a normal base to an atypical base containing a keto group in place of the original amine group. Examples include C → U and A → HX (hypoxanthine), which can be corrected by DNA repair mechanisms; and 5MeC (5-methylcytosine) → T, which is less likely to be detected as a mutation because thymine is a normal DNA base.

23.3.1.4 Slipped strand mispairing

It is denaturation of the new strand from the template during replication, followed by renaturation in a different spot (slipping). This can lead to insertions or deletions.

23.3.2 Induced mutation

Spontaneous mutations clearly represent only a small number of all possible mutations. To genetically dissect a biological system further, new mutations were created by scientists by treating an organism with a mutagenizing agent. These mutations are called induced mutations. Induced mutations on the molecular level can be caused by:

- Chemicals
- Radiations
- Transposable elements

23.3.2.1 Chemicals

- Hydroxylamine NH_2OH
- Base analogs (e.g. BrdU)

- Alkylating agents (e.g. N-ethyl-N-nitrosourea). These agents can mutate both replicating and non-replicating DNA. In contrast, a base analog can only mutate the DNA when the analog is incorporated in replicating the DNA. Each of these classes of chemical mutagens has certain effects that then lead to transitions, transversions, or deletions.
- Agents that form DNA adducts (e.g. ochratoxin A metabolites)
- DNA intercalating agents (e.g. ethidium bromide)
- DNA crosslinkers
- Oxidative damage
- Nitrous acid converts amine groups on A and C to diazo groups, altering their hydrogen bonding patterns which leads to incorrect base pairing during replication.

23.3.2.2 Radiation

- Ultraviolet radiation (nonionizing radiation). Two nucleotide bases in DNA – cytosine and thymine – are most vulnerable to radiation that can change their properties. UV light can induce adjacent pyrimidine bases in a DNA strand to become covalently joined as apyrimidine dimer. UV radiation, particularly longer-wave UVA, can also cause oxidative damage to DNA.
- Ionizing radiation
- Radioactive decay, such as 14C in DNA

23.3.2.3 Transposable elements

Scientists are now using the power of transposable elements to create new mutations. Transposable elements are mobile pieces of DNA that can move from one location in a genome to another. Often when they move to a new location, the result is a new mutant. The mutant arises because the presence of a piece of DNA in a wild type gene disrupts the normal function of that gene. As more and more is being learned about genes and genomes, it is becoming apparent that transposable elements are a power source f or creating insertional mutants.

The detailed knowledge of the structure and function of transposable elements is now being applied in the pursuit of new mutations. Stocks are created in which a specific type of element is present. This stock is then crossed to a genetic stock that doe s not contain the element. Once that element enters the virgin stock, it can begin to move around that genome. By carefully observing the offspring, new mutants can be discovered. This approach to developing mutants is termed insertional mutagenesis.

23.4 Classification of Mutations

23.4.1 By effect on structure

The sequence of a gene can be altered in a number of ways. Gene mutations have varying effects on health depending on where they occur and whether they alter the function of essential proteins. Mutations in the structure of genes can be classified as:

23.4.1.1. Point mutations

Often caused by chemicals or malfunction of DNA replication, exchange a single nucleotide for another. These changes are classified as transitions or transversions. Most common is the transition that exchanges a purine for a purine (A ↔ G) or a pyrimidine for a pyrimidine, (C ↔ T). A transition can be caused by nitrous acid, base mis-pairing, or mutagenic base analogs such as 5-bromo-2-deoxyuridine (BrdU). Less common is atransversion, which exchanges a purine for a pyrimidine or a pyrimidine for a purine (C/T ↔ A/G). An example of a transversion is adenine (A) being converted into a cytosine (C). A point mutation can be reversed by another point mutation, in which the nucleotide is changed back to its original state (true reversion) or by second-site reversion (a complementary mutation elsewhere that results in regained gene functionality). Point mutations that occur within the protein coding region of a gene may be classified into three kinds, depending upon what the erroneous codon codes for:

23.4.1.2 Silent mutations

Silent mutations are those that cause no change in the final protein product and can only be detected by sequencing the gene. Most amino acids that make up a protein are encoded by several different codons (see genetic code). So, if for example, the third base in the 'cag' codon is changed to an 'a' to give 'caa', a glutamine (Q) would still be incorporated into the protein product, because the mutated codon still codes for the same amino acid. These types of mutations are 'silent' and have no detrimental effect. (Fig.23.1)

Fig. 23.1Silent mutations

23.4.1.3 Missense mutations

In a missense mutation, the new base alters a codon resulting in a different amino acid being incorporated into the protein chain. This is what happens in sickle cell anaemia. The 17th nucleotide of the gene for the beta chain of haemoglobin is changed from an 'a' to a 't'. This changes the codon from 'gag' to 'ggt' resulting in the 6th amino acid of the chain being changed from glutamic acid to valine. This apparently trivial alteration to the beta globin gene alters the quaternary structure of haemoglobin, which has a profound influence on the physiology and wellbeing of the individual (Fig. 23.2).
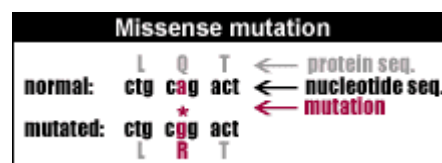
Fig. 23.2 Missense mutations

The picture shows an example of a missence mutation, a substitution of 'a' in the second codon to 'g' leads to an amino acid substitution of glutamine (Q) to arginine (R).

23.4.1.4 Nonsense mutations

In a nonsense mutation, the new base changes a codon that specified an amino acid into one of the stop codons (taa, tag, tga). This will cause translation of the mRNA to stop prematurely and a truncated protein to be produced. This truncated protein will be unlikely to function correctly. Nonsense mutations occur in between 15% to 30% of all inherited diseases including cystic fibrosis, haemophilia, retinitis pigmentosa and duchenne muscular dystrophy (Fig. 23.3).

The picture shows an example of a nonsense mutation, where 'c' in the second codon is changed to 't'. In the normal nucleotide sequence the second codon 'cag' codes for glutamine (Q) whilst in the mutated second codon 'tag' codes for Stop leading to premature termination of the protein.

Fig. 23.3 Nonsense mutations

Insertions add one or more extra nucleotides into the DNA. They are usually caused by transposable elements, or errors during replication of repeating elements (e.g. AT repeats). Insertions in the coding region of a gene may altersplicing of the mRNA (splice site mutation), or cause a shift in the reading frame (frameshift), both of which can significantly alter the gene product. Insertions can be reverted by excision of the transposable element (Fig. 23.4).
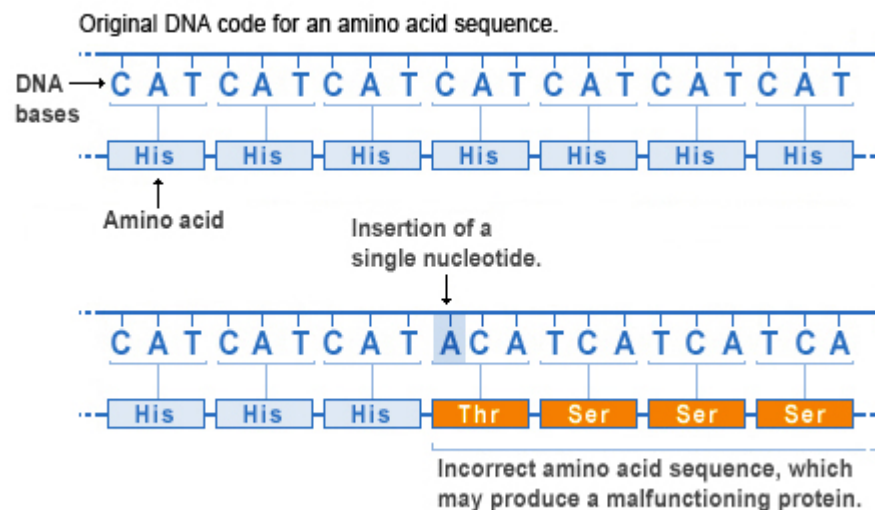
Fig. 23.4 Frameshift mutation

23.4.1.5 Deletions

Deletion removes one or more nucleotides from the DNA. Like insertions, these mutations can alter the reading frame of the gene. They are generally irreversible: though exactly the same sequence might theoretically be restored by an insertion, transposable elements able to revert a very short deletion (say 1–2 bases) in any location are either highly unlikely to exist or do not exist at all. Note that a deletion is not the exact opposite of an insertion: the former is quite random while the latter consists of a specific sequence inserting at locations that are not entirely random or even quite narrowly defined (Fig. 23.5).
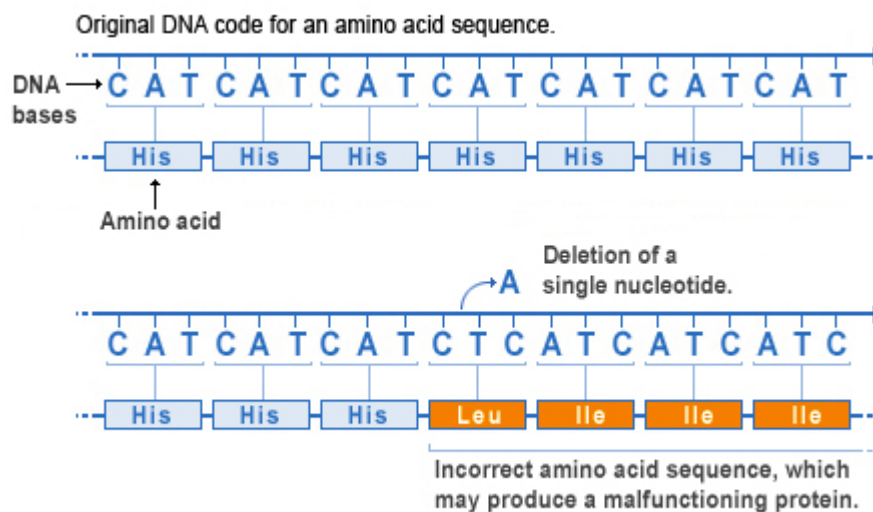
Fig. 23.5 Deletions

23.4.1.6 Large-scale mutations in chromosomal structure, including:

- Amplifications (or gene duplications) leading to multiple copies of all chromosomal regions, increasing the dosage of the genes located within them.
- Deletions of large chromosomal regions, leading to loss of the genes within those regions.
- Mutations whose effect is to juxtapose previously separate pieces of DNA, potentially bringing together separate genes to form functionally distinct fusion genes (e.g. bcr-abl). These include
- Chromosomal translocations
- Interchange of genetic parts from non-homologous chromosomes
- Interstitial deletions
- An intra-chromosomal deletion that removes a segment of DNA from a single chromosome, thereby apposing previously distant genes. For example, cells isolated from a human astrocytoma, a type of brain tumor, were found to have a chromosomal deletion removing sequences between the "fused in glioblastoma" (fig) gene and the receptor tyrosine kinase "ros", producing a fusion protein (FIG-ROS). The abnormal FIG-ROS fusion protein has

constitutively active kinase activity that causes oncogenic transformation (a transformation from normal cells to cancer cells).

- Chromosomal inversions
- Reversing the orientation of a chromosomal segment.
- Loss of heterozygosity
- Loss of one allele, either by a deletion or recombination event, in an organism that previously had two different alleles.

Lesson 24
GENETIC RECOMBINATION SYSTEMS

24.1 Transformation

Transformation is gene transfer resulting from the uptake by a recipient cell of naked DNA from a donor cell. Certain bacteria (e.g. Bacillus, Haemophilus, Neisseria, Pneumococcus) can take up DNA from the environment and the DNA that is taken up can be incorporated into the recipient's chromosome. Bacterial transformation was discovered by Frederick Griffith in 1928. Griffith worked with the pneumococci that cause bacterial pneumonia.

24.1.1 Griffith's experiment (Fig. 24.1)

The transformation process was first demonstrated in 1928 by Frederick Griffith. He experimented on Streptococcus pneumoniae, bacteria that cause pneumonia in mammals. When he examined colonies of the bacteria on petri plates, he could tell that there were two different strains. The colonies of one strain appeared smooth. Later analysis revealed that this strain has a polysaccharide capsule and is virulent, that it, it causes pneumonia. The colonies of the other strain appeared rough. This strain has no capsules and is avirulent. When Griffith injected living encapsulated cells into a mouse, the mouse died of pneumonia and the colonies of encapsulated cells were isolated from the blood of the mouse. When living non-encapsulated cells were injected into a mouse, the mouse remained healthy and the colonies of non-encapsulated cells were isolated from the blood of the mouse. Griffith then heat killed the encapsulated cells and injected them into a mouse. The mouse remained healthy and no colonies were isolated. The encapsulated cells lost the ability to cause the disease. However, a combination of heat-killed encapsulated cells and living non-encapsulated cells did cause pneumonia and colonies of living encapsulated cells were isolated from the mouse. How can a combination of these two strains cause pneumonia when either strand alone does not cause the disease? The living non-encapsulated cells came into contact with DNA fragments of the dead capsulated cells. The genes that code for the capsule entered some of the living cells and a crossing over event occurred. The recombinant cell now has the ability to form a capsule and cause pneumonia. All of the recombinant's offspring have the same ability. That is why the mouse developed pneumonia and died.
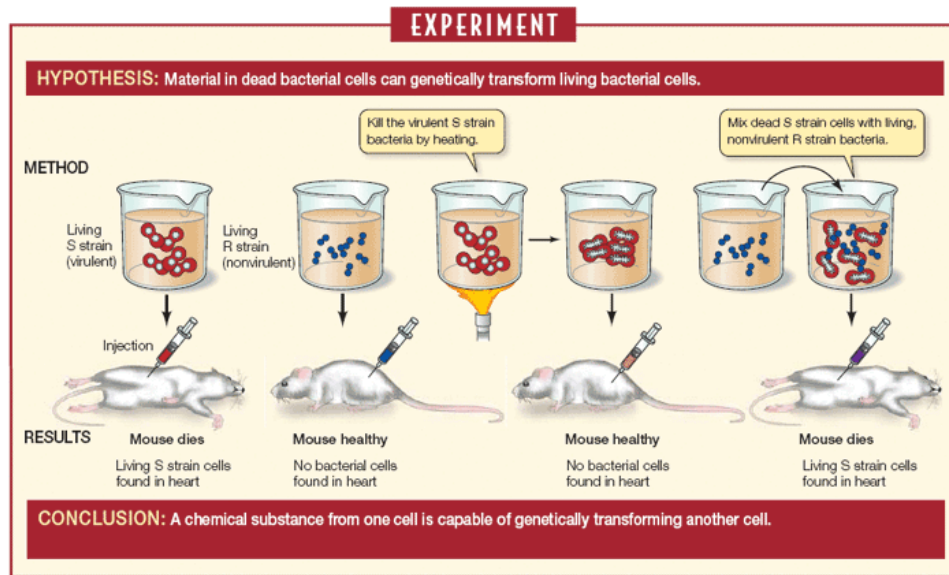
Fig. 24.1 Griffith's Experiment

24.2 Factors Affecting Transformation

24.2.1 DNA size state

Double stranded DNA of at least 5×10^5 daltons works best. Thus, transformation is sensitive to nucleases in the environment.

24.2.2 Competence of the recipient

Some bacteria are able to take up DNA naturally. However, these bacteria only take up DNA a particular time in their growth cycle when they produce a specific protein called a competence factor. At this stage the bacteria are said to be competent. Other bacteria are not able to take up DNA naturally. However, in these bacteria competence can be induced in vitro by treatment with chemicals (e.g. $CaCl_2$).

24.2.2.1 Natural competence

About 1% of bacterial species are capable of naturally taking up DNA under laboratory conditions; many more are able to take it up in their natural environments. Such bacteria carry sets of genes that provide the protein machinery to bring DNA across the cell membrane(s).
DNA material can be transferred between different strains of bacteria, in a process called horizontal gene transfer.

24.2.2.2 Artificial competence

Artificial competence is induced by laboratory procedures and involves making the cell passively permeable to DNA by exposing it to conditions that do not normally occur in nature.

Calcium chloride transformation is a method of promoting competence. Chilling cells in the presence of divalent cations such as Ca2$^+$ (in $CaCl_2$) prepares the cell membrane to become permeable to plasmid DNA. The cells are incubated on ice with the DNA and then briefly heat shocked (e.g., 42°C for 30–120 s) thus allowing the DNA to enter the cells. This method works very well for circular plasmid DNA. An excellent preparation of competent cells will give ~108 colonies per microgram of plasmid. A poor preparation will be about 104/µg or less. Good, non-commercial preparations should give 105 to 106 transformants per microgram of plasmid. The method, however, usually does not work well for linear DNA, such as fragments of chromosomal DNA, probably because the cell's native exonuclease enzymes rapidly degrade linear DNA. Interestingly, cells that are naturally competent are usually transformed more efficiently with linear DNA than with plasmid DNA.

Electroporation is another method of promoting competence. In the method the cells are briefly shocked with an electric field of 10-20 kV/cm that creates holes in the cell membrane through which the plasmid DNA enters. This method is amenable to the uptake of large plasmid DNA. After the electric shock the holes are rapidly closed by the cell's membrane-repair mechanisms.

The efficiency with which a competent culture can take up exogenous DNA and express its genes is known as Transformation efficiency.

24.3 Steps in Transformation

During transformation, DNA fragments (usually about 20 genes long) from a dead degraded bacterium bind to DNA binding proteins on the surface of a competent recipient bacterium. Nuclease enzymes then cut the bound DNA into fragments. One strand is destroyed and the other penetrates the recipient bacterium. This DNA fragment from the donor is then exchanged for a piece of the recipient's DNA by means of Rec A proteins (Fig. 24.2).
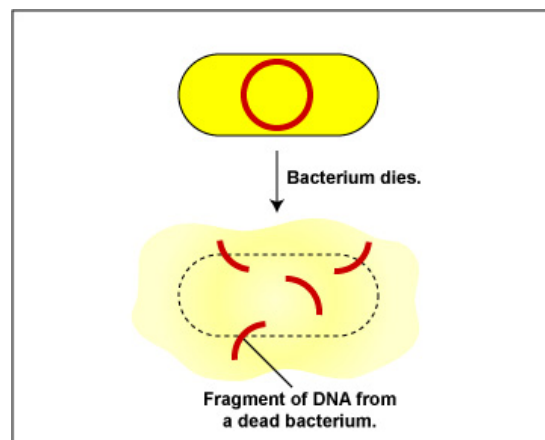
Fig. 24.2(a) A donor bacterium dies and is degraded.
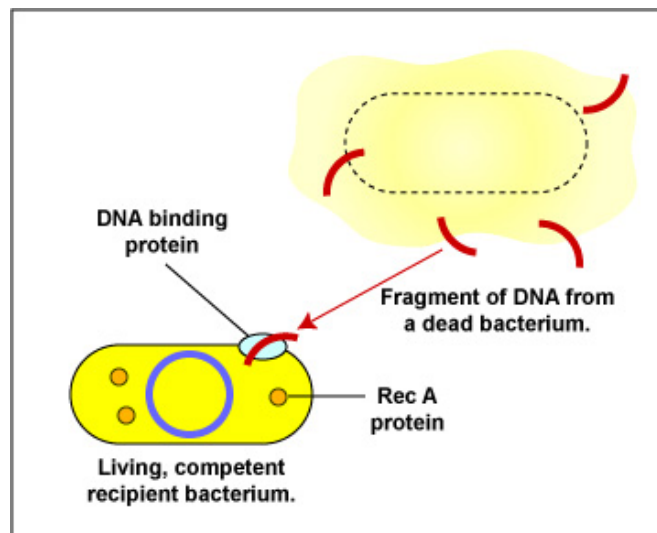
Fig. 24.2(b) A fragment of DNA from the dead donor bacterium binds to DNA binding proteins on the cell wall of a competent, living recipient bacterium.
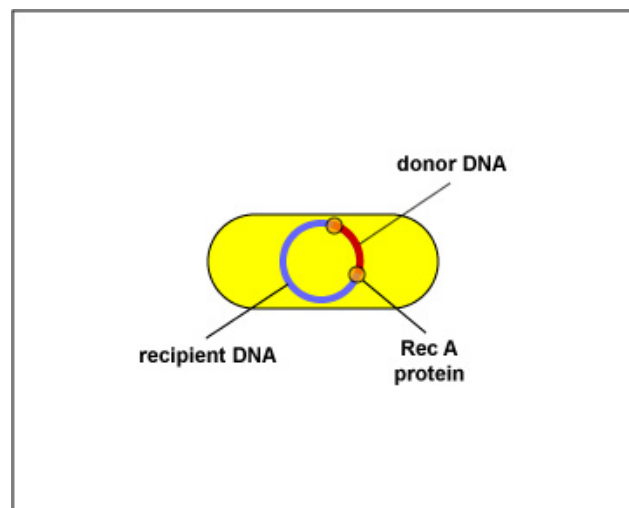
Fig. 24.2 (c) The Rec A protein promotes genetic exchange between a fragment of the donor's DNA and the recipient's DNA
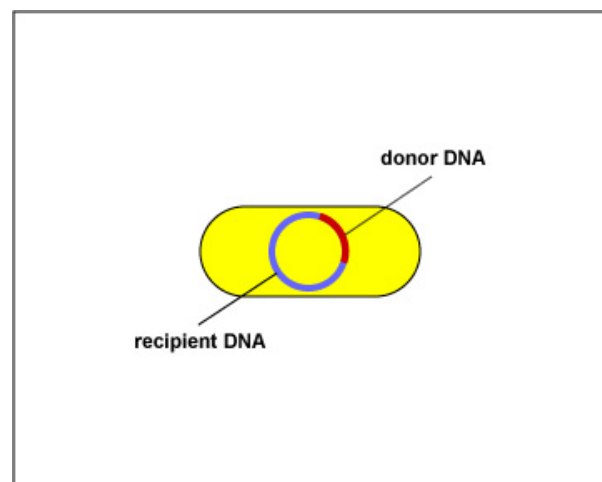
Fig. 24.2 (d) Exchange is complete.

Significance - Transformation occurs in nature and it can lead to increased virulence. In addition transformation is widely used in recombinant DNA technology.

24.4 Conjugation: Bacterial conjugation

Conjugation: Bacterial conjugation is the transfer of genetic material between bacterial cells by direct cell-to-cell contact or by a bridge-like connection between two cells.

Distinguishing characteristics of conjugation:

- DNA transfer requires cell-cell contact.
- DNA transfer occurs via a conjugal pore.
- DNA transfer occurs in one direction - from donor to recipient not vice versa.
- DNA transfer does not require protein synthesis in donor.
- DNA transfer requires energy in donor cell - primarily AT

Do bacteria possess any processes similar to sexual reproduction and recombination? The question was answered in 1946 by the elegantly simple experimental work of Joshua Lederberg and Edward Tatum, who studied two strains of E. coli with different nutritional requirements. Strain A would grow on a minimal medium, only if the medium were supplemented with methionine and biotin; strain B would grow on a minimal medium only if it were supplemented with threonine, leucine, and thiamine. Thus, we can designate strain A as met^- bio^- thr^+ leu^+ thi^+ and strain B as met^+bio^+ thr^- leu^- thi^-. Here, strains A and B are mixed together, and some of the progeny are now wild type, having regained the ability to grow without added nutrients.
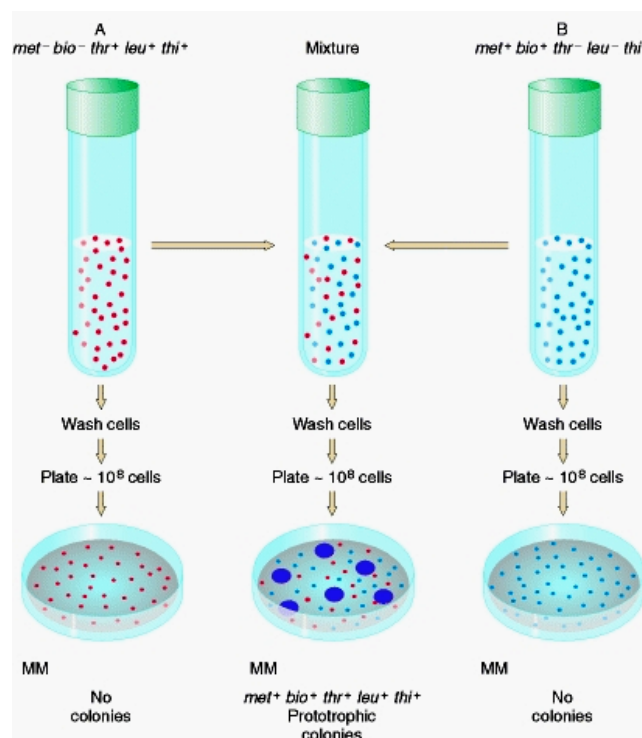
Fig. 24.3 Lederberg & Tatum experiment

Lederberg and Tatum plated bacteria into dishes containing only unsupplemented minimal medium (Fig. 4.3). Some of the dishes were plated only with strain A bacteria, some only with strain B bacteria, and some with a mixture of strain A and strain B bacteria that had been incubated together for several hours in a liquid medium containing all the supplements. No colonies arose on plates containing either strain A or strain B alone, showing that back mutations cannot restore prototrophy, the ability to grow on unsupplemented minimal medium. However, the plates that received the mixture of the two strains produced growing colonies at a frequency of 1 in every 10,000,000 cells plated (in scientific notation, 1×10^{-7}). This observation suggested that some form of recombination of genes had taken place between the genomes of the two strains to produce prototrophs.

But Lederberg and Tatum did not directly prove that physical contact of the cells was necessary for gene transfer. This evidence was provided by Bernard Davis (1950), who constructed a U tube consisting of two pieces of curved glass tubing fuse at the base to form a U shape with a fritted glass filter between the halves (Fig. 24.4). The pores of the filter were too small to allow bacteria to pass through but large enough to allow easy passage of the fluid medium and any dissolved substances. Strain A was put in one arm; strain B in the other. After the strains had been incubated for a while, Davis tested the content of each arm to see if cells had become able to grow on a minimal medium, and none were found. In other words, physical contact between the two strains was needed for wild-type cells to form. It looked as though some kind of gene transfer had taken place, and genetic recombinants were indeed produced.
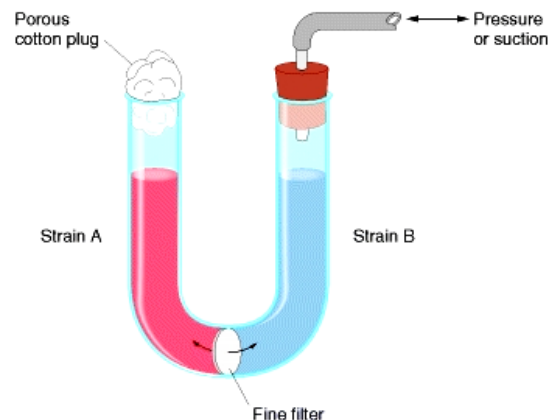
Fig. 24.4 Davis experiment

24.4.1 Discovery of fertility factor

In 1952, William Hayes demonstrated that gene transfer observed by Lederberg and Tatum occurred in one direction. That is, there were definite donor (F+) and recipient (F-) strains, and gene transfer was nonreciprocal. One cell acts as donor, and the other cell acts as the recipient. This kind of unidirectional transfer of genes was originally compared to a sexual difference, with the donor being termed 'male' and the recipient 'female'. However, this type of gene transfer is not true sexual reproduction. In bacterial gene transfer, one organism receives genetic information from a donor; the recipient is changed by that information. In sexual reproduction, two organisms donate equally

(or nearly so) to the formation of a new organism, but only in exceptional cases is either of the donors changed.

By accident, Hayes discovered a variant of his original donor strain that would not produce recombinants on crossing with the recipient strain. Apparently, the donor-type strains had lost the ability to transfer genetic material and had changed into recipient-type strains. In his analysis of this 'sterile' donor variant, Hayes realized that the fertility (ability to donate) of E. coli could be lost and regained rather easily. Hayes suggested that donor ability is itself a hereditary state imposed by a fertility factor (F). Strains that carry F can donate, and are designated F^+. Strains that lack F cannot donate and are recipients. These strains are designated F^-.

24.4.2 F+ conjugation

Genetic recombination in which there is a transfer of an F^+ plasmid (coding only for a sex pilus) but not chromosomal DNA from a male donor bacterium to a female recipient bacterium involves a sex (conjugation) pilus. Other plasmids present in the cytoplasm of the bacterium, such as those coding for antibiotic resistance may also be transferred during this process (Fig. 24.5).

1. The F+ male has an F+ plasmid coding for a sex pilus and can serve as a genetic donor

2. The sex pilus adheres to an F-female (recipient). One strand of the F+ plasmid breaks

3. The sex pilus retracts and a bridge is created between the two bacteria. One strand of the F+ plasmid enters the recipient bacterium

4. Both bacteria make a complementary strand of the F+ plasmid and both are now F+ males capable of producing a sex pilus. There was no transfer of donor chromosomal DNA although other plasmids the donor bacterium carries may also be transferred during F+ conjugation.

Fig. 24.5 Diagrammatic presentation of F^+ Conjugation

24.4.3 Hfr conjugation

Genetic recombination in which fragments of chromosomal DNA from a male donor bacterium are transferred to a female recipient bacterium following insertion of an F^+ plasmid into the nucleoid of the donor bacterium (Fig.24.6).

1. An F+ plasmid inserts into the donor bacterium's nucleoid to form an Hfr male.

2. The sex pilus adheres to an F- female (recipient). One donor DNA strand breaks in the middle of the inserted F+ plasmid.

3. The sex pilus retracts and a bridge forms between the two bacteria. One donor DNA strand begins to enter the recipient bacterium. The two cells break apart easily so the only a portion of the donor's DNA strand is usually transferred to the recipient bacterium.

4. The donor bacterium makes a complementary copy of the remaining DNA strand and remains an Hfr male. The recipient bacterium makes a complementary strand of the transferred donor DNA.

5. The donor DNA fragment undergoes genetic exchange with the recipient bacterium's DNA. Since there was transfer of some donor chromosomal DNA but usually not a complete F+ plasmid, the recipient bacterium usually remains F-

Fig. 24.6 Diagrammatic presentation of Hfr Conjugation

24.4.4 Resistant plasmid conjugation

Genetic recombination in which there is a transfer of an R plasmid (a plasmid coding for multiple antibiotic resistance and often a sex pilus) from a male donor bacterium to a female recipient bacterium (Fig.24.7).

1. The bacterium with an R-plasmid is multiple antibiotic resistant and can produce a sex pilus (serve as a genetic donor).

2. The sex pilus adheres to an F-female (recipient). One strand of the R-plasmid breaks.

3. The sex pilus retracts and a bridge is created between the two bacteria. One strand of the R-plasmid enters the recipient bacterium.

4. Both bacteria make a complementary strand of the R-plasmid and both are now multiple antibiotic resistant and capable of producing a sex pilus.

Fig. 24.7 Diagrammatic representation of resistant plasmid conjugation

24.5 Transduction

Transduction is the process by which DNA is transferred from one bacterium to another by a virus. It also refers to the process whereby foreign DNA is introduced into another cell via a viral vector. This is a common tool used by molecular biologists to stably introduce a foreign gene into a host cell's genome. When bacteriophages (viruses that infect bacteria) infect a bacterial cell, their normal mode of reproduction is to harness the replicational, transcriptional, and translation machinery of the host bacterial cell to make numerous virions, or complete viral particles, including the viral DNA or RNA and the protein coat (Fig.24.8).

■ Bacterial DNA   ■ Viral DNA
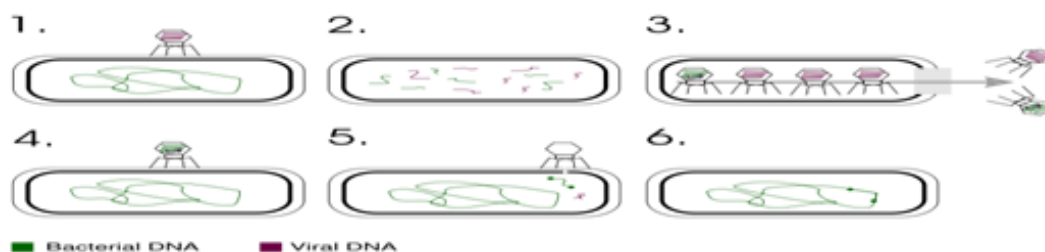
Fig. 24. 8 Mechanism of transduction

24.5.1 Lytic and lysogenic (temperate) cycles

Transduction happens through either the lytic cycle or the lysogenic cycle. After a bacteriophage (or phage, in brief) enters a bacterium, it may encourage the bacterium to make copies of the phage. At the conclusion of the process, the host bacterium undergoes lysis and releases new phages. This cycle is called the lytic cycle. Under other circumstances, the virus may attach to the bacterial chromosome and integrate its DNA into the bacterial DNA. It may remain here for a period of time before detaching and continuing its replicative process. This cycle is known as the lysogenic cycle.

Under these conditions, the virus does not destroy the host bacterium, but remains in a lysogenic condition with it. The virus is called a temperate phage, also known as a prophage. At a later time, the virus can detach, and the lytic cycle will ensue.

24.6 Types of Transduction

- Generalized transduction
- Specialized transduction

24.6.1 Generalized transduction

A DNA fragment is transferred from one bacterium to another by a lytic bacteriophage that is now carrying donor bacterial DNA due to an error in maturation during the lytic life cycle.

During generalized transduction, a phage assumes a lysogenic condition with a bacterium, and the phage DNA remains with the chromosomal DNA. When the phage replicates, however, random fragments of the bacterial DNA are packaged in error by new phages during their production. The result is numerous phages containing genes from the bacterium in addition to their own genes. When these phages enter a new host bacterium and incorporate their DNA to the bacterial chromosome, they also incorporate the DNA from the previous bacterium and the recipient bacterium is transduced (Fig.24. 9). It will express not only its genes, but also the genes acquired from the donor bacterium.
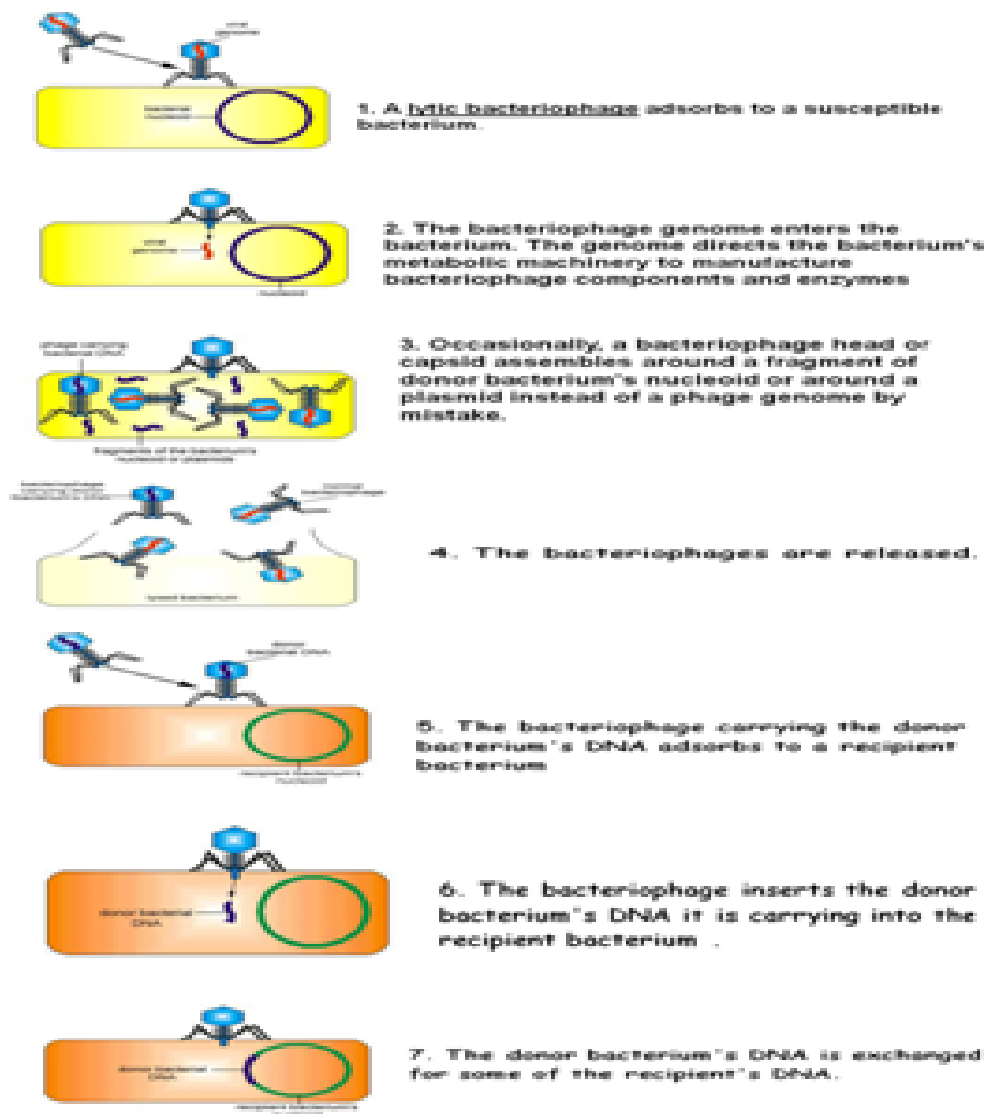
Fig. 24.9 Steps of generalized transduction

24.6.2 Specialized transduction

A DNA fragment is transferred from one bacterium to another by a temperate bacteriophage that is now carrying donor bacterial DNA due to an error in spontaneous induction during the lysogenic life cycle (Fig.24.10).

When the phage DNA breaks away from the bacterial DNA, however, it may take with it a small amount of the bacterial DNA (approx. 5%). When the phage DNA is used as a template for the synthesis of new phage DNA particles, the bacterial genes are also reproduced. When the phages enter new bacterial cells, they carry the bacterial genes along with them. In the recipient bacterium, the phage and donor genes integrate into the bacterial chromosome and transduce the recipient organism. Specialized transduction is an extremely rare event in comparison to generalized transduction because genes do not easily break free from the bacterial chromosome.
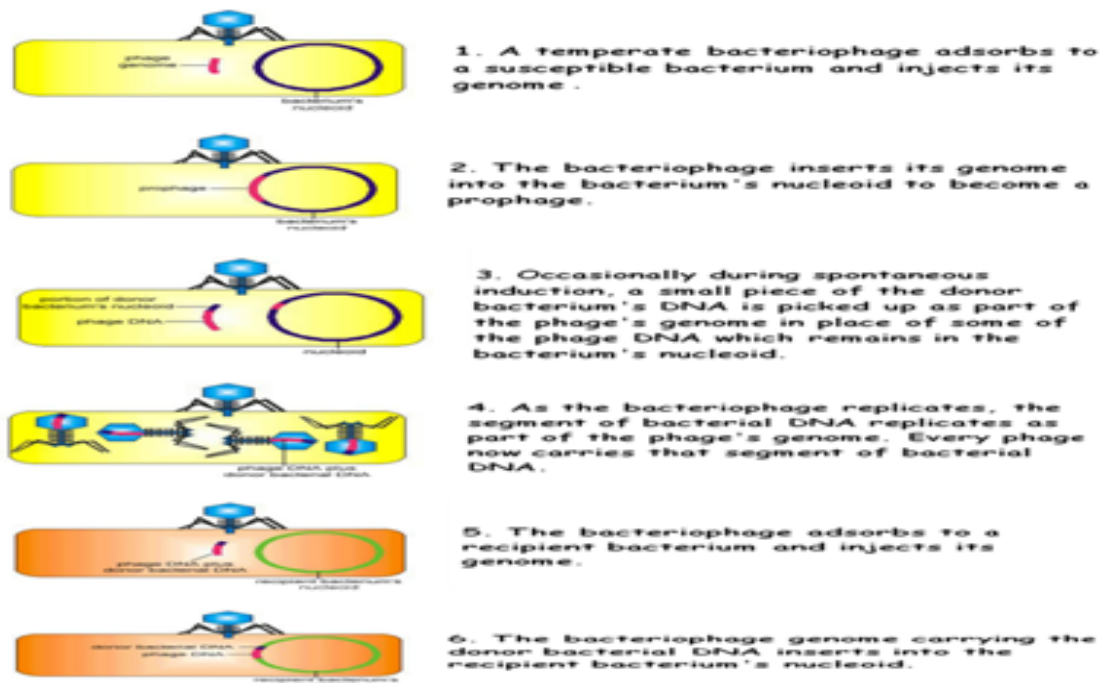
Fig. 24.10 Steps of specialized transduction

Lesson 25
RECOMBINANT DNA TECHNOLOGY

25.1 Introduction

Recombinant DNA is a type of DNA that is artificially created by inserting a strand or more of DNA into a different set of DNA. Recombinant DNA is used in genetic modification to create completely new organisms by adding artificial bits or bits of DNA from other organisms to an existing creature. Recombinant DNA is often referred to as rDNA for short.

The technique for making recombinant DNA was first developed in the early-1970s by Herbert Boyer and Stanley Norman Cohen. Their work was built on the work of Daniel Nathans, Hamilton Smith, and Werner Arber, who discovered restriction endonucleases. In 1978 the three were awarded the Nobel Prize for Medicine for this discovery.

Recombinant DNA technology is a technology which allows DNA to be produced via artificial means. The procedure has been used to change DNA in living organisms and may have even more practical uses in the future. It is an area of medical science that is just beginning to be researched in a concerted effort.

Recombinant DNA technology works by taking DNA from two different sources and combining that DNA into a single molecule. That alone, however, will not do much. Recombinant DNA technology only becomes useful when that artificially-created DNA is reproduced. This is known as DNA cloning (Fig. 25.1).

25.1.1 Here is how recombinant technology works

- Recombinant technology begins with the isolation of a gene of interest. The gene is then inserted into a vector and cloned. A vector is a piece of DNA that is capable of independent growth; commonly used vectors are bacterial plasmids and viral phages. The gene of interest (foreign DNA) is integrated into the plasmid or phage, and this is referred to as recombinant DNA.
- Before introducing the vector containing the foreign DNA into host cells to express the protein, it must be cloned. Cloning is necessary to produce numerous copies of the DNA since the initial supply is inadequate to insert into host cells.
- Once the vector is isolated in large quantities, it can be introduced into the desired host cells such as mammalian, yeast, or special bacterial cells. The host cells will then synthesize the foreign protein from the recombinant DNA. When the cells are grown in vast quantities, the foreign or recombinant protein can be isolated and purified in large amounts.
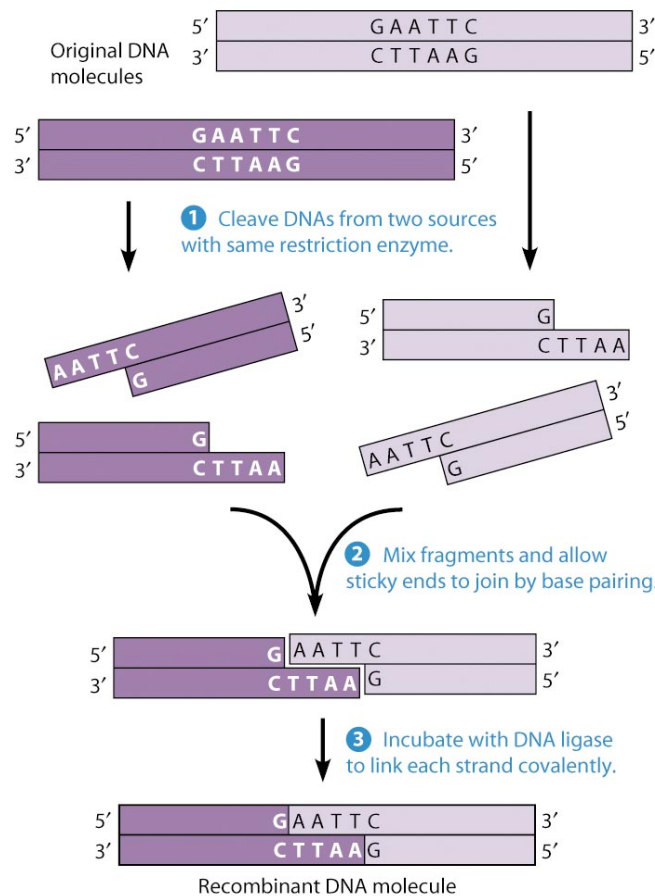
Fig. 25.1 Recombinant DNA molecule

25.1.2 Other uses for recombinant DNA

Recombinant DNA technology is not only an important tool in scientific research, but it has also impacted the diagnosis and treatment of diseases and genetic disorders in many areas of medicine. It has enabled many advances, including:

25.1.2.1 Isolation of large quantities of protein

In addition to the follicle-stimulating hormone (FSH) used in Follistim® AQ Cartridge (follitropin beta injection) and Follistim® AQ Vial (follitropin beta injection) , insulin, growth hormone and other proteins are now available as recombinant products.

25.1.2.2 Identification of mutations

People may be tested for the presence of mutated proteins that may be associated with breast cancer, retino-blastoma, and neurofibromatosis.

25.1.2.3 Diagnosis of affected and carrier states for hereditary diseases

Tests exist to determine if people are carriers of the cystic fibrosis gene, the Huntington's disease gene, the Tay-Sachs disease gene, or the Duchenne muscular dystrophy gene.

25.1.2.4 Transferring of genes from one organism to another

People suffering from cystic fibrosis, rheumatoid arthritis, vascular disease, and certain cancers may now benefit from the progress made in gene therapy.

25.1.2.5 Mapping of human genes on chromosomes

Scientists are able to link mutations and disease states to specific sites on chromosomes.

25.2 Main Concepts and Definitions in Recombinant DNA Technology

Making and replicating a desired piece of DNA

- One little piece of DNA by itself can't be studied. You can't determine its base sequence or its products by looking at it under a microscope.
- To effectively study DNA, one must manufacture a large quantity of a DNA segment of interest, 'magnifying' it for easier study with biochemical methods.
- To do this, recombinant DNA is made by splicing a DNA fragment of interest into a small DNA molecule (such as a bacterial plasmid) called a vector.
- Once this is done, one can make huge numbers of the desired DNA fragment by inserting the vector into a very busy piece of DNA in another live cell (such as a bacterium).
- The bacterium 'works' for you by allowing the vector to replicate. As the bacteria multiply, so does DNA.
- This magnified sample can then be extracted for further study.

A Few Definitions:

- The organism from which the DNA of interest is extracted is called the donor.
- The DNA into which the DNA of interest is inserted (often a bacterial plasmid) is called a vector.
- The organism (or DNA) into which the foreign DNA is inserted is called the recipient.
- An organism containing an artificially inserted, foreign piece of DNA is said to be transgenic (i.e. the recipient becomes transgenic once the new DNA is inserted).

25.2.1 How is it done?

To excise a piece of DNA from a donor organism, restriction enzymes are used. These act somewhat like 'enzymatic scissors', slicing through the DNA at specific, recognized sequences. Once the DNA is excised, DNA ligase is the 'enzymatic glue' used to insert it into replicating DNA of the host cell. Note that DNA ligase isn't picky: it can't tell the difference between foreign and host DNA, and this enables the creation of hybrid DNA-DNA from two separate sources (sometimes different species). A vector molecule with an insert of foreign DNA is a recombinant DNA molecule. DNA made from the combined DNA of two (or more) species is sometimes called chimeric DNA after the beast of Greek mythology. Vectors are often mixed with bacterial strains which take them up and incorporate them into their own genomes, a process known as transformation). Vectors may also be replicated autonomously (without being inserted into the

bacterial DNA) as the bacterium goes about its daily business. By growing the bacterial strain carrying the desired recombinant DNA vector, one can grow a large number of the desired DNA fragment. This is the DNA clone. Once a large DNA clone (remember: a clone is a group of things, not a single individual) has been grown, the researcher can

- characterize the DNA (determine its base sequence)
- make RNA from it
- make protein from it (after you've made the RNA)
- modify the DNA to see what happens when it mutates
- reinsert it into a recipient organism for production of products or further study

25.3 Restriction Enzymes

First discovered in bacteria, restriction enzymes cut DNA at very specific DNA base sequences (called restriction sequences). These enzymes are believed to be a bacterial defense against viruses. Each restriction enzyme recognizes and cleaves a very specific sequence of DNA. Restriction sequences are palindromes: they read the same, forward and backward on the opposite strands. Cutting with restriction enzymes creates highly reactive "sticky ends" that act as attachment points for other fragments of DNA with complementary restriction sequences. By connecting pieces of DNA from two different species (that happen to have the same restriction sites), we create chimeric DNA. Note that restriction sites are a 'happy accident' of nature. They have nothing to do with gene function in the organism in which they are found. In fact, they are a defense mechanism, found primarily in bacteria, which function to fragment and destroy the DNA of invading bacteriophages (i.e. 'bacterium-eating' viruses) before it can incorporate into the bacterial host's genome to do its dirty work. Bacterial DNA is immune to the bacteria's own restriction enzymes: in its normal state a bacterium's own restriction sites are highly methylated (i.e. the bases have many methyl groups (-CH_3 attached), protecting them from the activity of the restriction enzymes.

Restriction enzymes are named for the organism from which they were first isolated. For example

- EcoRI is isolated from E. coli strain RY13
- Eco refers to the genus and species (1st letter of genus; 1st two letters of specific epithet)
- R is the strain of E. coli
- I (Roman numeral) indicate it was the first enzyme of that type isolated from E. coli RY13.
- BamHI is isolated from *Bacillus amyloliquefaciens* strain H
- Sau3A is isolated from *Staphylococcus aureus* strain 3A.

Each enzyme recognizes and cuts specific DNA sequences. For example, BamHI recognizes the double stranded sequence:

5'--GGATCC--3'

3'--CCTAGG--5'

25.3.1 Summary

Most restriction enzymes cut only one specific restriction site. Restriction sites are recognized no matter what the DNA's species. The number of cuts in an organism's DNA made by a particular restriction enzyme depends on the number of restriction sites (specific to that restriction enzyme) in that organism's DNA. A fragment of DNA produced by a pair of adjacent cuts is called a restriction fragment. A particular restriction enzyme will typically cut an organism's DNA in to many pieces, from several thousand to more than a million. There is a great deal of variation in restriction sites, even within a species (Everyone in this room has different numbers and locations of restriction sites. Your restriction site numbers and locations are more similar to those of your close family members than to unrelated humans. Although these DNA variations are not phenotypically expressed, the variants can be considered molecular 'alleles', and they can be detected with sequencing techniques. This is yet another type of genetic variation of interest to the evolutionary biologist.

Lesson 26
MICROBIOTA OF SOIL

26.1 Introduction

Soil microbiology is branch of microbiology which deals with study of soil microorganisms and their activities in the soil. Soil is the outer, loose material of earth's surface which is distinctly different from the underlying bedrock and the region which support plant life. Agriculturally, soil is the region which supports the plant life by providing mechanical support and nutrients required for growth. From the microbiologist view point, soil is one of the most dynamic sites of biological interactions in the nature. It is the region where most of the physical, biological and biochemical reactions related to decomposition of organic weathering of parent rock take place.

26.2 Components of Soil

Soil is an admixture of five major components viz. organic matter, mineral matter, soil-air, soil water and soil microorganisms/living organisms (Fig.26.1). The amount/proposition of these components vary with locality and climate.
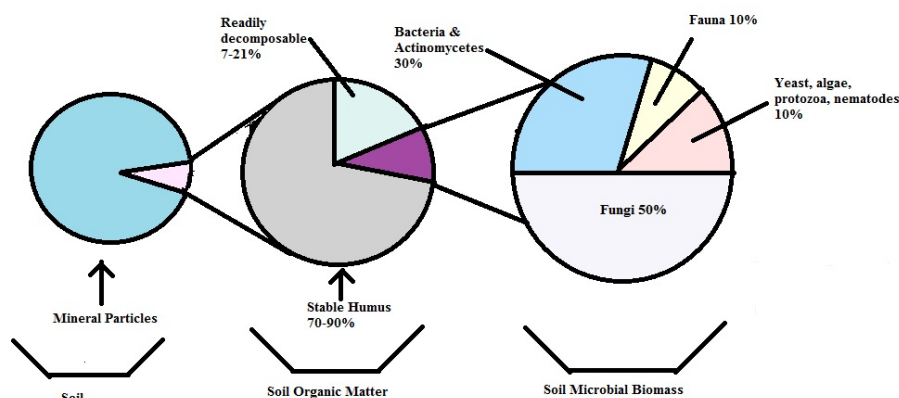
Fig. 26.1 Major components of soil

26.2.1 Mineral/Inorganic Matter

It is derived from parent rocks/bed rocks through decomposition, disintegration and weathering process. Different types of inorganic compounds containing various minerals are present in soil. Amongst them the dominant minerals are Silicon, Aluminum and iron and others like Carbon, Calcium Potassium, Manganese, Sodium, Sulphur, Phosphorus etc. are in trace amount. The proportion of mineral matter in soil is slightly less than half of the total volume of the soil.

26.2.2 Organic matter (components)

These are derived from organic residues of plants and animals added in the soil. Organic matter serves not only as a source of food for microorganisms but also supplies energy for the vital

processes of metabolism which are characteristics of all living organisms. Organic matter in the soil is the potential source of N, P and S for plant growth. Microbial decomposition of organic matter releases the unavailable nutrients in available from. The proportion of organic matter in the soil ranges from 3-6% of the total volume of soil.

26.2.3 Soil Water

The amount of water present in soil varies considerably. Soil water comes from rain, snow, dew or irrigation. Soil water serves as a solvent and carrier of nutrients for the plant growth. The microorganisms inhabiting in the soil also require water for their metabolic activities. Soil water thus, indirectly affects plant growth through its effects on soil and microorganisms. Percentage of soil-water is 25% total volume of soil.

26.2.4 Soil air (Soil gases)

A part of the soil volume which is not occupied by soil particles i.e. pore spaces are filled partly with soil water and partly with soil air (Fig.26.2). Most microbes are in micro colonies on soil particle. They may escape predator activities by refuge in these small pores. These two components (water and air) together, accounts for approximately half the soil's volume. Compared with atmospheric air, soil is lower in oxygen and higher in carbon dioxide, because CO_2 is continuous recycled by the microorganisms during the process of decomposition of organic matter. Soil air comes from external atmosphere and contains nitrogen, oxygen, CO_2 and water vapor (CO_2 > oxygen). CO_2 in soil air (0.3-1.0%) is more than atmospheric air (0.03%). Soil aeration plays important role in plant growth, microbial population, and microbial activities in the soil.

26.2.5 Soil microorganisms

Soil is an excellent culture media for the growth and development of various microorganisms. Soil is not an inert static material but a medium pulsating with life. Soil is now believed to be dynamic or living system. Soil contains several distinct groups of microorganisms and amongst them bacteria, fungi, actinomycetes, algae, protozoa and viruses are the most important. But bacteria are more numerous than any other kinds of microorganisms. Microorganisms form a very small fraction of the soil mass and occupy a volume of less than one percent. In the upper layer of soil (top soil up to 10-30 cm depth i.e. Horizon A), the microbial population is very high which decreases with depth of soil. Each organisms or a group of organisms are responsible for a specific change transformation in the soil. The final effect of various activities of microorganisms in the soil is to make the soil fit for the growth and development of higher plants.
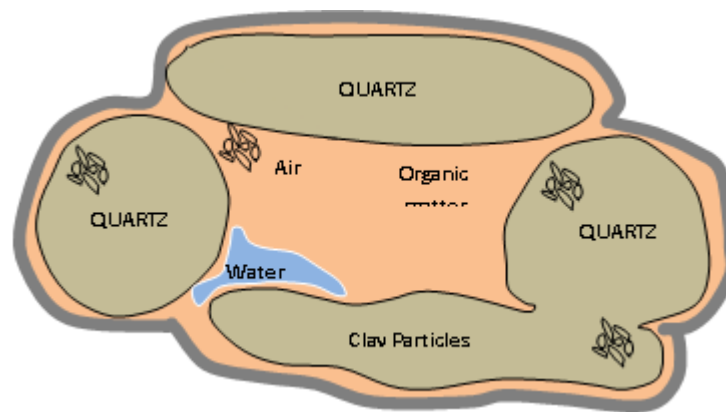
Fig. 26.2 Structure of soil

26.3 Types of Microorganisms in Soil

Living organisms both plants and animals, constitute an important component of soil. The pioneering investigations of a number of early microbiologists showed for the first time that the soil was not an inert static material but a medium pulsating with life. The soil is now believed to be a dynamic or rather a living system, containing a dynamic population of microorganisms. Cultivated soil has relatively more population of microorganisms than the fallow land, and the soils rich in organic matter contain much more population than sandy and eroded soils. Microbes in the soil are important to us in maintaining soil fertility/productivity, cycling of nutrient elements in the biosphere and sources of industrial products such as enzymes, antibiotics, vitamins, hormones, organic acids etc. At the same time certain soil microbes are the causal agents of human and plant diseases.

The soil organisms are broadly classified in to two groups viz. soil flora and soil fauna, the detailed classification of which is as follows.

26.3.1 Soil flora

a) Microflora

- Bacteria
- Fungi, Molds, Yeast, Mushroom
- Actinomycetes, Streptomyces
- Algae e.g. BGA, Yellow Green algae, Golden brown algae.

Bacteria is again classified in

I) Heterotrophic e.g. symbiotic and non - symbiotic N2 fixers, Ammonifier, Cellulose Decomposers, Denitrifiers
II) Autrotrophic e.g. Nitrosomonas, Nitrobacter, Sulphur oxidizers, etc.

b) Macroflora

1. Microfauna: Protozoa, Nematodes

2. Macrofauna: Earthworms, moles, ants and others. As soil inhabit several diverse groups of microorganisms, but the most important amongst them are: bacteria, actinomycetes, fungi, algae and protozoa.

Relative proportion/percentage of various soil microorganisms are: bacteria - aerobic (70%), anaerobic (13 %), actinomycetes (13%), fungi/molds (3%) and others (algae, protozoa, viruses) 0.2-0.8 %.

Major groups of microorganisms are discussed in following sections:

26.4 Bacteria

Amongst the different microorganisms inhabiting in the soil, bacteria are the most abundant and predominant organisms. These are primitive, prokaryotic, microscopic and unicellular microorganisms without chlorophyll. Morphologically, soil bacteria are divided into three groups viz. cocci (round/spherical), bacilli (rod-shaped) and spirilla (cells with long wavy chains). Bacilli are most numerous followed by cocci and spirilla in soil. The most common method used for isolation of soil bacteria is the 'dilution plate count' method which allows the enumeration of only living cells in the soil. The size of soil bacteria varies from 0.5 to 1.0 μ in diameter and 1.0 to 10.0 μ in length. They are motile with locomotory organs flagella. Bacterial population is one-half of the total microbial biomass in the soil ranging from 1,00000 to several hundred millions per gram of soil, depending upon the physical, chemical and biological conditions of the soil.

Winogradsky (1925), on the basis of ecological characteristics classified soil microorganisms in general and bacteria in particular into two broad categories i.e. autochnotus (Indigenous species) and the zymogenous (fermentative). Autochnotus bacterial population is uniform and constant in soil, since their nutrition is derived from native soil organic matter (e.g. Arthrobacter and Nocardia, whereas zymogenous bacterial population in soil is low, as they require an external source of energy, e.g. Pseudomonas and bacillus. The population of zymogenous bacteria increases gradually when a specific substrate is added to the soil. To this category belong the cellulose decomposers, nitrogen utilizing bacteria and ammonifiers. As per the system proposed in the 'Bergey's Manual of Systematic Bacteriology', most of the bacteria which are predominantly encountered in soil are taxonomically included in the three orders, Pseudomonadales, Eubacteriales and Actinomycetales of the class Schizomycetes. The most common soil bacteria belong to the genera Pseudomonas, Arthrobacter, Clostridium Achromobacter, Sarcina, Enterobacter etc. The another group of bacteria common in soils is the Myxobacteria belonging to the genera Micrococcus, Chondrococcus, Archangium, Polyangium, Cyptophaga.

Bacteria are also classified on the basis of physiological activity or mode of nutrition, especially the manner in which they obtain their carbon, nitrogen, energy and other nutrient requirements.

They are broadly divided into two groups i.e. a) Autotrophs and b) Heterotrophs

- Autotrophic bacteria are capable synthesizing their food from simple inorganic nutrients, while heterotrophic bacteria depend on pre-formed food for nutrition. All autotrophic bacteria utilize CO2 (from atmosphere) as carbon source and derive energy either from sunlight (photoautotrophs, e.g. Chromatrum. Chlorobium. Rhadopseudomonas or from the oxidation of

simple inorganic substances present in soil (chemoautotrophs e.g. Nitrobacter, Nitrosomonas, Thiaobacillus).

- Majority of soil bacteria are heterotrophic in nature and derive their carbon and energy from complex organic substances/organic matter, decaying roots and plant residues. They obtain their nitrogen from nitrates and ammonia compounds (proteins) present in soil and other nutrients from soil or from the decomposing organic matter. Certain bacteria also require amino acids, B-vitamins, and other growth promoting substances also.

26.4.1 Functions/role of bacteria

Bacteria bring about a number of changes and biochemical transformations in the soil and thereby directly or indirectly help in the nutrition of higher plants growing in the soil. The important transformations and processes in which soil bacteria play vital role are: decomposition of cellulose and other carbohydrates, ammonification (proteins ammonia), nitrification (ammonia-nitrites-nitrates), denitrification (release of free elemental nitrogen), biological fixation of atmospheric nitrogen (symbiotic and non-symbiotic) oxidation and reduction of sulphur and iron compounds. All these processes play a significant role in plant nutrition.

26.5 Actinomycetes

These are the organisms with characteristics common to both bacteria and fungi but yet possessing distinctive features to delimit them into a distinct category. In the strict taxonomic sense, actinomycetes are clubbed with bacteria the same class of Schizomycetes and confined to the order Actinomycetales. They are unicellular like bacteria, but produce a mycelium which is non-septate (coenocytic) and more slender, tike true bacteria they do not have distinct cell-wall and their cell wall is without chitin and cellulose (commonly found in the cell wall of fungi). On culture media unlike slimy distinct colonies of true bacteria which grow quickly, actinomycetes colonies grow slowly, show powdery consistency and stick firmly to agar surface. They produce hyphae and conidia/sporangia like fungi. Certain actinomycetes whose hyphae undergo segmentation resemble bacteria, both morphologically and physiologically.

Actinomycetes are numerous and widely distributed in soil and are next to bacteria in abundance. They are widely distributed in the soil, compost etc. Plate count estimates give values ranging from 104 to 108 per gram of soil. They are sensitive to acidity/low pH (optimum pH range 6.5 to 8.0) and waterlogged soil conditions. The population of actinomycetes increases with depth of soil even up to horizon 'C' of a soil profiler They are heterotrophic, aerobic and mesophilic (25-30°C) organisms and some species are commonly present in compost and manures are thermophilic growing at 55-65°C temperature (e.g. Thermoatinomycetes, Streptomyces). Actinomycetes belonging to the order of Actinomycetales are grouped under four families viz. Mycobacteriaceae, Actinomycetaceae, Streptomycetaceae and Actinoplanaceae. Actinomycetous genera, which are agriculturally and industrially important, are present in only two families of Actinomycetaceae and Strepotmycetaceae. In the order of abundance in soils, the common genera of actinomycetes are Streptomyces (nearly 70%), Nocardia and Micromonospora although Actinomycetes, Actinoplanes, Micromonospora and Streptosporangium are also generally encountered.

26.5.1 Functions of actinomycetes

- Degrade all sorts of organic substances like cellulose, polysaccharides, protein fats, organic-acids etc.
- Soil, added with organic residues/substances, is first attacked by bacteria and fungi and later by actinomycetes, because they are slow in activity and growth than bacteria and fungi.
- They decompose/degrade the more resistant and indecomposable organic substance/matter and produce a number of dark black to brown pigments which contribute to the dark color of soil humus.
- They are also responsible for subsequent further decomposition of humus (resistant material) in soil.
- They are responsible for earthy/musty odor/smell of freshly ploughed soils.
- Many genera species and strains (e.g. Streptomyces, if actinomycetes synthesize number of antibiotics like Streptomycin, Terramycin, Aureomycin etc.
- One of the species of actinomycetes, Streptomyces scabies causes disease 'potato scab' in potato.

26.6 Fungi

Fungi in soil are present as mycelial bits, rhizomorph or as different spores. Their number varies from a few thousand to a few million per gram of soil. Soil fungi possess filamentous mycelium composed of individual hyphae. The fungal hyphae may be aseptate/coenocytic (Mastigomycotina and Zygomycotina) or septate (Ascomycotina, Basidiomycotina and Deuteromycotina). As observed by C. K. Jackson (1975), most commonly encountered genera of fungi in soil are; Alternaria, Aspergillus, Cladosporium, Cephalosporium Botrytis, Chaetomium, Fusarium, Mucor, Penicillium, Verticillium, Trichoderma, Rhizopus, Gliocladium, Monilia, Pythium, etc. Most of these fungal genera belong to the subdivision deuteromycotina/fungi imperfeacta which lacks sexual mode of reproduction. As these soil fungi are aerobic and heterotrophic, they require abundant supply of oxygen and organic matter in soil. Fungi are dominant in acid soils, because acidic environment is not suitable for the existence of either bacteria or actinomycetes. The optimum pH range for fungi lies-between 4.5 to 6.5. They are also present in neutral and alkaline soils and some can even tolerate pH beyond 9.0

26.6.1 Functions/role of fungi

- Fungi plays significant role in soils and plant nutrition.
- They plays important role in the degradation/decomposition of cellulose, hemi cellulose, starch, pectin, lignin in the organic
- Matter added to the soil.
- Lignin which is resistant to decomposition by bacteria is mainly decomposed by fungi.
- They also serve as food for bacteria.
- Certain fungi belonging to sub-division Zygomycotina and Deuteromycotina are predaceous in nature and attack on protozoa and nematodes in soil and thus, maintain biological equilibrium in soil.
- They also plays important role in soil aggregation and in the formation of humus.
- Some soil fungi are parasitic and causes number of plant diseases such as wilts, root rots, damping-off and seedling blights e.g. Pythium, Phyiophlhora, Fusarium, Verticillium etc.
- Number of soil fungi forms mycorrhizal association with the roots of higher plants (symbiotic association of a fungus with the roots of a higher plant) and helps in mobilization of soil

phosphorus and nitrogen e.g. Glomus, Gigaspora, Aculospora, (Endomycorrhiza) and Amanita, Boletus, Entoloma, Lactarius (Ectomycorrhiza).

26.7 Algae

Algae are present in most of the soils where moisture and sunlight are available. Their number in soil usually ranges from 100 to 10,000 per gram of soil. They are photoautotrophic, aerobic organisms and obtain CO_2 from atmosphere and energy from sunlight and synthesize their own food. They are unicellular, filamentous or colonial. Soil algae are divided in to four main classes or phyla as follows:

- Cyanophyta (Blue-green algae)
- Chlorophyta (Grass-green algae)
- Xanthophyta (Yellow-green algae)
- Bacillariophyta (diatoms or golden-brown algae)

Out of these four classes/phyla, blue-green algae and grass-green algae are more abundant in soil. The green-grass algae and diatoms are dominant in the soils of temperate region while blue-green algae predominate in tropical soils. Green-algae prefer acid soils while blue green algae are commonly found in neutral and alkaline soils. The most common genera of green algae found in soil are: Chlorella, Chlamydomonas, Chlorococcum, Protosiphon etc. and that of diatoms are Navicula, Pinnularia. Synedra, Frangilaria. Blue green algae are unicellular, photoautotrophic prokaryotes containing Phycocyanin pigment in addition to chlorophyll. They do not posses flagella and do not reproduce sexually. They are common in neutral to alkaline soils. The dominant genera of BGA in soil are: Chrococcus, Phormidium, Anabaena, Aphanocapra, Oscillatoria etc. Some BGA posses specialized cells known as 'Heterocyst' which is the sites of nitrogen fixation. BGA fixes nitrogen (non-symbiotically) in puddle paddy/water logged paddy fields (20-30 kg/ha/season). There are certain BGA which possess the character of symbiotic nitrogen fixation in association with other organisms like fungi, mosses, liverworts and aquatic ferns Azolla, e.g. Anabaena-Azolla association fix nitrogen symbiotically in rice fields.

26.7.1 Functions/role of algae or BGA

- Plays important role in the maintenance of soil fertility especially in tropical soils.
- Add organic matter to soil when die and thus increase the amount of organic carbon in soil.
- Most of soil algae (especially BGA) act as cementing agent in binding soil particles and thereby reduce/prevent soil erosion.
- Mucilage secreted by the BGA is hygroscopic in nature and thus helps in increasing water retention capacity of soil for longer time/period.
- Soil algae through the process of photosynthesis liberate large quantity of oxygen in the soil environment and thus facilitate the aeration in submerged soils or oxygenate the soil environment.
- They help in checking the loss of nitrates through leaching and drainage especially in un-cropped soils.
- They help in weathering of rocks and building up of soil structure.

26.8 Protozoa

These are unicellular, eukaryotic, colorless, and animal like organisms (Animal kingdom). They are larger than bacteria and size varying from few microns to a few centimeters. Their population in arable soil ranges from l0,000 to 1,00,000 per gram of soil and are abundant in surface soil. They can withstand adverse soil conditions as they are characterized by 'cyst stage' in their life cycle. Except few genera which reproduce sexually by fusion of cells, rest of them reproduces asexually by fission/binary fission. Most of the soil protozoa are motile by flagella or cilia or pseudopodia as locomotors organs. Depending upon the type of appendages provided for locomotion, protozoa are

- Rhizopoda (Sarcondia)
- Mastigophora
- Ciliophora (Ciliata)
- Sporophora (not common inhabitants of soil)

Rhizopoda consists protozoa without appendages usually have naked protoplasm without cell-wall, pseudopodia as temporary locomotory organs are present some times. Important genera are Amoeba, Biomyxa, Euglypha, etc.

Mastigophora Belongs flagellated protozoa, which are predominant in soil. Important genera are: Allention, Bodo, Cercobodo, Cercomonas, Entosiphon Spiromonas, Spongomions and Testramitus. Many members are saprophytic and some posses chlorophyll and are autotrophic in nature. In this respect, they resemble unicellular algae and hence are known as 'Phytoflagellates'.

Ciliophora are characterized by the presence of cilia (short hair-like appendages) around their body, which helps in locomotion. The important soil inhabitants of this class are Colpidium, Colpoda, Balantiophorus, Gastrostyla, Halteria, Uroleptus, Vortiicella, Pleurotricha etc.

Protozoa are abundant in the upper layer (15 cm) of soil. Organic manures protozoa. Soil moisture, aeration, temperature and pH are the important factors affecting soil protozoa.

26.8.1 Function/role of protozoa

- Most of protozoans derive their nutrition by feeding or ingesting soil bacteria belonging to the genera Enterobacter, Agrobacterium, Bacillus, Escherichia, Micrococcus, and Pseudomonas and thus, they play important role in maintaining microbial/bacterial equilibrium in the soil.
- Some protozoa have been recently used as biological control agents against phytopathogens.
- Species of the bacterial genera viz. Enterobacter and Aerobacter are commonly used as the food base for isolation and enumeration of soil protozoans.
- Several soil protozoa cause diseases in human beings which are carried through water and other vectors, e.g. Amoebic dysentery caused by Entomobea histolytica.

Lesson 27
INTERACTION AMONG SOIL MICROFLORA

27.1 Soil Humus

Humus is the organic residue in the soil resulting from decomposition of plant and animal residues in soil, or it is the highly complex organic residual matter in soil which is not readily degraded by microorganism, or it is the soft brown/dark coloured amorphous substance composed of residual organic matter along with dead microorganisms and comprises B horizon of soil (Fig. 27.1).
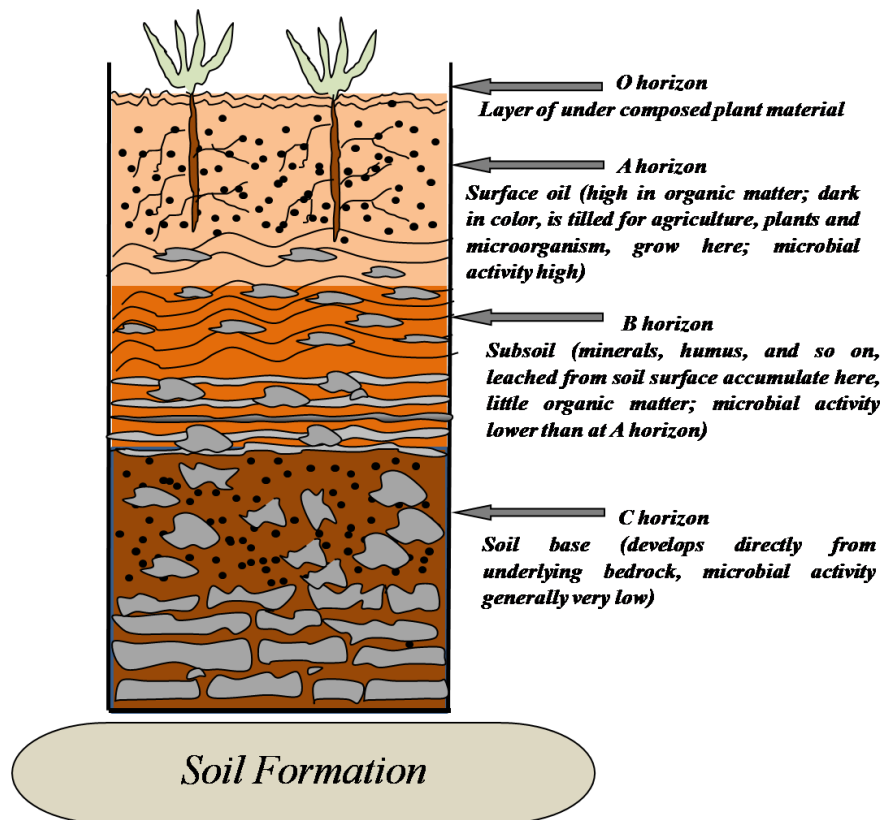
Fig. 27.1 Different horizons of soil

27.1.1 Composition of humus

In most soil, percentage of humus ranges from 2-10%, whereas it is up to 90% in peat bog. On average humus is composed of Carbon (58%), Nitrogen (3-6 %, avg.5%), acids - humic acid, fulvic acid, humin, apocrenic acid, and C:N ratio 10:1 to 12:1. During the course of their activities, the microorganisms synthesize number of compounds which plays important role in humus formation.

27.1.2 Functions of humus

- It improves physical condition of soil
- Improve water holding capacity of soil
- Serve as store house for essential plant nutrients

- Plays important role in determining fertility level of soil
- It tend to make soils more granular with better aggregation of soil particles
- Prevent leaching losses of water soluble plant nutrients
- Improve microbial/biological activity in soil and encourage better development of plant-root system in soil
- Act as buffering agent i.e. prevent sudden change in soil pH/soil reaction
- Serve as source of energy and food for the development of soil organisms
- It supplies both basic and acidic nutrients for the growth and development of higher plants
- Improves aeration and drainage by making the soil more porous

27.2 Interactions among Soil Microorganisms

Living organisms both plant and animal types constitute an important component of soil. Though these organisms form only a fraction (less than one percent) of the total soil mass, but they play important role in supporting plant communities on the earth surface. While studying the scope and importance of soil microbiology, soil-plant-animal ecosystem as such must be taken into account. Therefore, the scope and importance of soil microbiology can be understood in better way by studying following aspects:

27.2.1 Soil as a living system

Soil inhabits diverse group of living organisms, both microflora (fungi, bacteria, algae and actinomycetes) and micro-fauna (protozoa, nematodes, earthworms, moles, ants). The density of living organisms in soil is very high i.e. as much as billions/gm of soil, usually density of organisms is less in cultivated soil than uncultivated/virgin land and population decreases with soil acidity. Top soil, the surface layer contains greater number of microorganisms because it is well supplied with oxygen and nutrients. Lower layer/subsoil is depleted with oxygen and nutrients hence it contains fewer organisms. Soil ecosystem comprises of organisms which are both, autotrophs (algae, BGA) and heterotrophs (fungi, bacteria). Autotrophs use inorganic carbon from CO_2 and are 'primary producers' of organic matter, whereas heterotrophs use organic carbon and are decomposers/consumers.

27.2.2 Soil microbes and plant growth

Microorganisms being minute and microscopic, they are universally present in soil, water and air. Besides supporting the growth of various biological systems, soil and soil microbes serve as a best medium for plant growth. Soil fauna and flora convert complex organic nutrients into simpler inorganic forms which are readily absorbed by the plant for growth. Further, they produce variety of substances like indole acetic acid, gibberellins, antibiotics etc. which directly or indirectly promote the plant growth.

27.2.3 Soil microbes and soil structure

Soil structure is dependent on stable aggregates of soil particles-Soil organisms play important role in soil aggregation. Constituents of soil are viz. organic matter, polysaccharides, lignins and gums, synthesized by soil microbes plays important role in cementing/binding of soil particles. Further,

cells and mycelial strands of fungi and actinomycetes, Vormicasts from earthworm is also found to play important role in soil aggregation. Different soil microorganisms, having soil aggregation/soil binding properties are graded in the order as fungi > actinomycetes > gum producing bacteria > yeasts e.g. fungi like Rhizopus, Mucor, Chaetomium, Fusarium, Cladasporium, Rhizoctonia, Aspergillus, Trichoderma and bacteria like Azotobacter, Rhizobium Bacillus and Xanlhomonas.

27.2.4 Soil microbes and organic matter decomposition

The organic matter serves not only as a source of food for microorganisms but also supplies energy for the vital processes of metabolism that are characteristics of living beings. Microorganisms such as fungi, actinomycetes, bacteria, protozoa etc. and macro organisms such as earthworms, termites, insects etc. plays important role in the process of decomposition of organic matter and release of plant nutrients in soil. Thus, organic matter added to the soil is converted by oxidative decomposition to simpler nutrients/substances for plant growth and the residue is transformed into humus. Organic matter/substances include cellulose, lignins and proteins (in cell wall of plants), glycogen (animal tissues), proteins and fats (plants, animals). Cellulose is degraded by bacteria, especially those of genus *Cytophaga* and other genera (*Bacillus,Pseudomonas*, *Cellulomonas*, *Vibrio*, *Achromobacter*) and fungal genera (*Aspergillus*, *Penicillium*, *Trichoderma*, *Chactomium,Curvularia*). Lignins and proteins are partially digested by fungi, protozoa and nematodes. Proteins are degraded to individual amino acids mainly by fungi, actinomycetes and *Clostridium*. Under anaerobic conditions of waterlogged soils, methane are main carbon containing product which is produced by the bacterial genera (strict anaerobes) *Methanococcus*, *Methanobacterium* and *Methanosarcina*.

27.2.5 Soil microbes and humus formation

Humus is the organic residue in the soil resulting from decomposition of plant and animal residues in soil, or it is the highly complex organic residual matter in soil which is not readily degraded by microorganism, or it is the soft brown/dark coloured amorphous substance composed of residual organic matter along with dead microorganisms.

27.2.6 Soil microbes and cycling of elements

Life on earth is dependent on cycling of elements from their organic/elemental state to inorganic compounds, then to organic compounds and back to their elemental states. The biogeochemical process through which organic compounds are broken down to inorganic compounds or their constituent elements is known as 'Mineralization', or microbial conversion of complex organic compounds into simple inorganic compounds and their constituent elements is known as mineralization. Soil microbes plays important role in the biochemical cycling of elements in the biosphere where the essential elements (C, P, S, N and Iron etc.) undergo chemical transformations (Fig. 27.2 – 27.4). Through the process of mineralization organic carbon, nitrogen, phosphorus, sulphur, iron etc. are made available for reuse by plants.
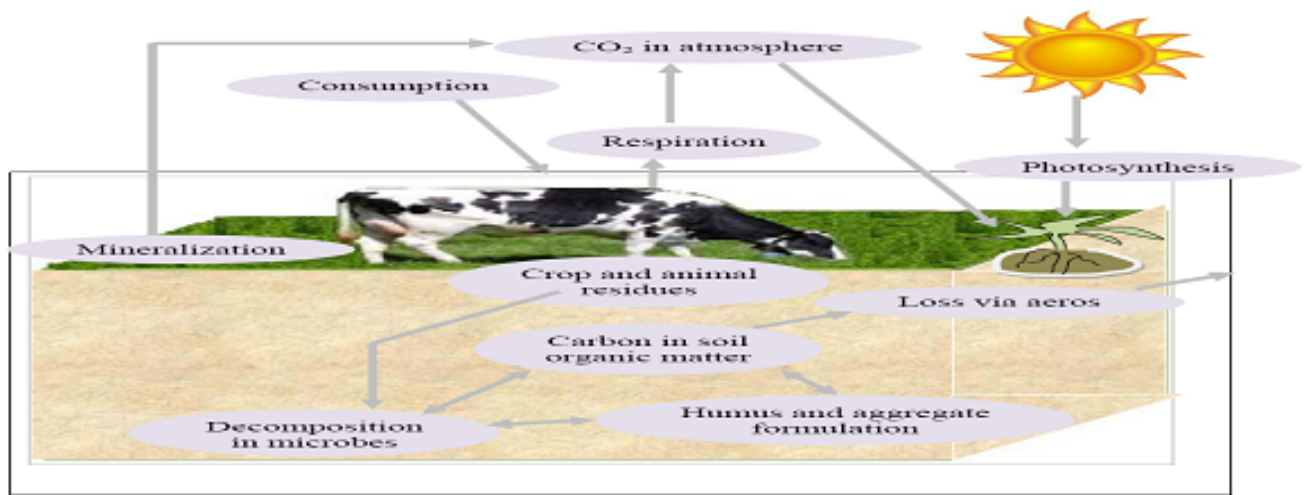
Fig. 27.2 Carbon cycle
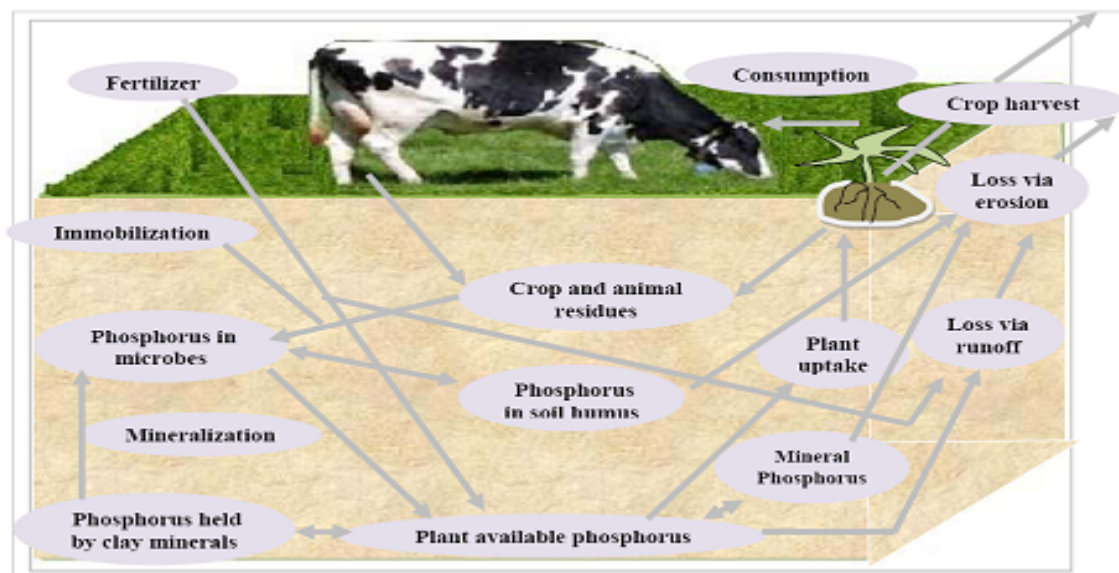
Fig. 27.3 Phosphorous cycle
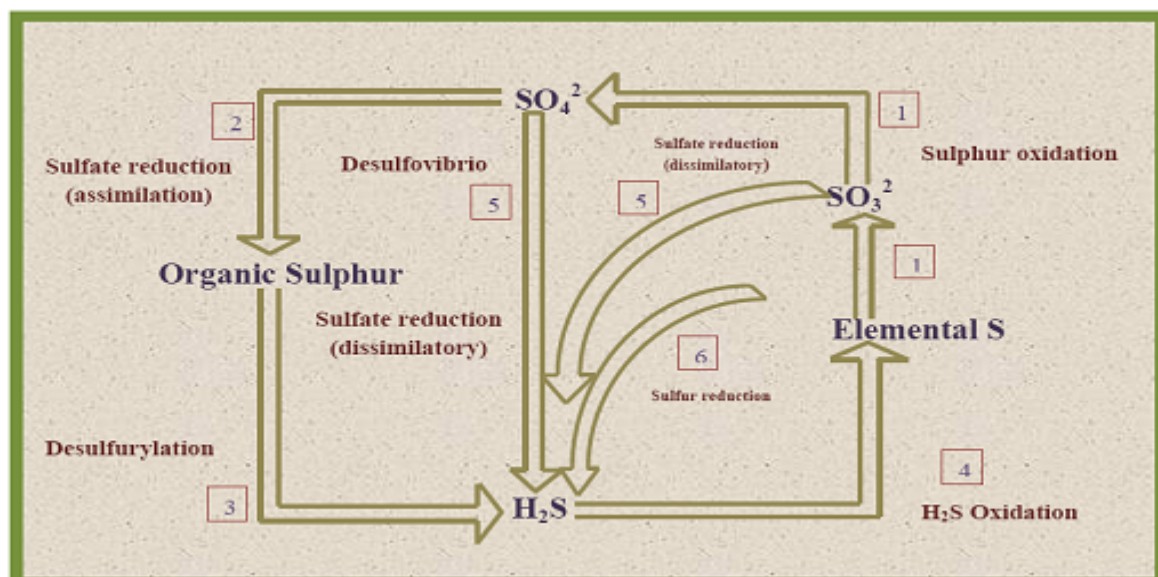
Fig. 27.4 Sulphur cycle

27.2.7 Soil microbes and biological N$_2$ fixation

Conversion of atmospheric nitrogen in to ammonia and nitrate by microorganisms is known as biological nitrogen fixation (Fig. 27.5). Fixation of atmospheric nitrogen is essential because of the reasons:

- Fixed nitrogen is lost through the process of nitrogen cycle through denitrification.
- Demand for fixed nitrogen by the biosphere always exceeds its availability.
- The amount of nitrogen fixed chemically and lightning process is very less (i.e. 0.5%) as compared to biologically fixed nitrogen
- Nitrogenous fertilizers contribute only 25% of the total world requirement while biological nitrogen fixation contributes about 60% of the earth's fixed nitrogen
- Manufacture of nitrogenous fertilizers by 'Haber' process is costly and time consuming. The numbers of soil microorganisms carry out the process of biological nitrogen fixation at normal atmospheric pressure (1 atmosphere) and temp (around 20°C).
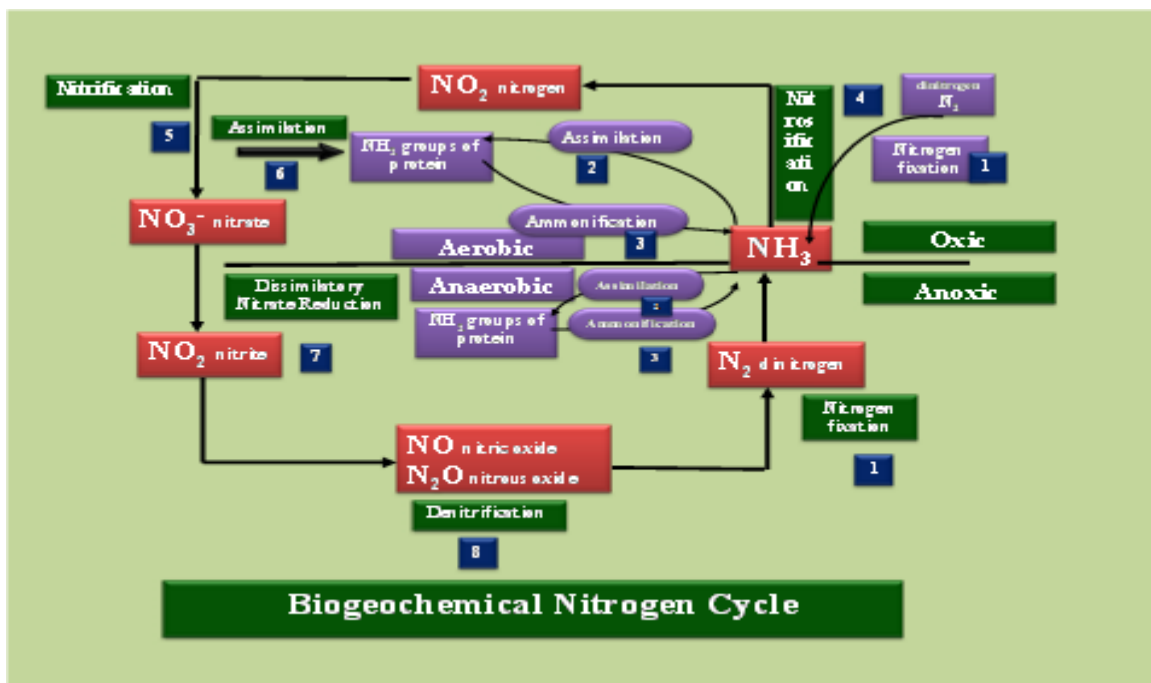
Fig. 27.5 Nitrogen cycle

Two groups of microorganisms are involved in the process of biological nitrogen fixation.

27.2.7.1 Non-symbiotic (free living)

Depending upon the presence or absence of oxygen, non symbiotic N$_2$ fixation prokaryotic organisms may be aerobic heterotrophs (Azotobacter, Pseudomonas, Achromobacter) or aerobic autotrophs (Nostoc, Anabena, Calothrix, BGA) and anaerobic heterotrophs (Clostridium, Kelbsiella, Desulfovibrio) or anaerobic Autotrophs (Chlorobium, Chromnatium, Rhodospirillum,

Meihanobacterium etc.

27.2.7.2 Symbiotic (Associative)

The organisms involved are Rhizobium, Bradyrhizobium in legumes, Azospirillum non legumes, Actinonycetes.

Fig.27.6 Nodulated Roots

Root nodules (Fig. 27.6) occur on the roots of plants (primarily Fabaceae) that associate with symbiotic nitrogen-fixing bacteria. Under nitrogen-limiting conditions, capable plants form a symbiotic relationship with a host-specific strain of rhizobia.

27.2.8 Soil microbes as biocontrol agents

Several ecofriendly bioformulations of microbial origin are used in agriculture for the effective management of plant diseases, insect pests, weeds etc. e.g.: Trichoderma sp and Gleocladium sp are used for biological control of seed and soil borne diseases. Fungal genera Entomophthora, Beauveria, Metarrhizium and protozoa Maltesia grandis. Malameba locustiae etc are used in the management of insect pests. Nuclear polyhydrosis virus is used for the control of Heliothis/American boll worm. Bacteria like Bacillus thuringiensis, Pseudomonas are used in cotton against angular leaf spot and boll worms.

27.2.9 Biodegradation of hydrocarbons

Natural hydrocarbons in soil like waxes, paraffin's, oils etc are degraded by fungi, bacteria and actinomycetes e.g. ethane (C_2H_6) a paraffin hydrocarbon is metabolized and degraded by Mycobacteria, Nocardia, Streptomyces Pseudomonas, Flavobacterium and several fungi.

27.3 Bioremediation

Pesticides are the chemical substances that kill pests and herbicides are the chemicals that kill weeds. In the context of soil, pests are fungi, bacteria insects, worms, and nematodes etc. that cause damage to field crops. Thus, in broad sense pesticides are insecticides, fungicides, bactericides, herbicides and nematicides that are used to control or inhibit plant diseases and insect pests.

Although wide-scale application of pesticides and herbicides is an essential part of augmenting crop yields; excessive use of these chemicals leads to the microbial imbalance, environmental pollution and health hazards. An ideal pesticide should have the ability to destroy target pest quickly and should be able to degrade non-toxic substances as quickly as possible. The ultimate 'sink' of the pesticides applied in agriculture and public health care is soil. Soil being the storehouse of multitudes of microbes, in quantity and quality, receives the chemicals in various forms and acts as a scavenger of harmful substances. The efficiency and the competence to handle the chemicals vary with the soil and its physical, chemical and biological characteristics.

27.4 Effects of Pesticides

Pesticides reaching the soil in significant quantities have direct effect on soil microbiological aspects, which in turn influence plant growth. Some of the most important effects caused by pesticides are: (1) alterations hi ecological balance of the soil microflora, (2) continued application of large quantities of pesticides may cause everlasting changes in the soil microflora, (3) adverse effect on soil fertility and crop productivity, (4) inhibition of N2 fixing soil microorganisms such as Rhizobium, Azotobacter, Azospirillum etc. and cellulolytic and phosphate solubilizing microorganisms, (5) suppression of nitrifying bacteria, Nitrosomonas and Nitrobacter by soil fumigants ethylene bromide, telone, and vapam have also been reported, (6) alterations in nitrogen balance of the soil, (7) interference with ammonification in soil, (8) adverse effect on mycorrhizal symbioses in plants and nodulation in legumes, and (9) alterations in the rhizosphere microflora, both quantitatively and qualitatively.

27.5 Persistence of Pesticides in Soil

How long an insecticide, fungicide, or herbicide persists in soil is of great importance in relation to pest management and environmental pollution. Persistence of pesticides in soil for longer period is undesirable because of the reasons: a) accumulation of the chemicals in soil to highly toxic levels, b) may be assimilated by the plants and get accumulated in edible plant products, c) accumulation in the edible portions of the root crops, d) to be get eroded with soil particles and may enter into the water streams, and finally leading to the soil, water and air pollutions. The effective persistence of pesticides in soil varies from a week to several years depending upon structure and properties of the constituents in the pesticide and availability of moisture in soil. For instance, the highly toxic phosphates do not persist for more than three months while chlorinated hydrocarbon insecticides (e.g. DOT, aldrin, chlordane etc.) are known to persist at least for 4-5 years and some times more than 15 years. From the agricultural point of view, longer persistence of pesticides leading to accumulation of residues in soil may result into the increased absorption of such toxic chemicals by plants to the level at which the consumption of plant products may prove deleterious/hazardous to human beings as well as livestock's. There is a chronic problem of agricultural chemicals, having entered in food chain at highly inadmissible levels in India, Pakistan, Bangladesh and several other developing countries in the world. For example, intensive use of DDT to control insect pests and mercurial fungicides to control diseases in agriculture had been known to persist for longer period and thereby got accumulated in the food chain leading to food contamination and health hazards. Therefore, DDT and mercurial fungicides has been, banned to use in agriculture as well as in public health department.

27.6 Biodegradation of Pesticides in Soil

Pesticides reaching to the soil are acted upon by several physical, chemical, and biological forces. However, physical and chemical forces are acting upon/degrading the pesticides to some extent, microorganism's plays major role in the degradation of pesticides. Many soil microorganisms have the ability to act upon pesticides and convert them into simpler non-toxic compounds. This process of degradation of pesticides and conversion into non-toxic compounds by microorganisms is known as 'biodegradation'. Not all pesticides reaching to the soil are biodegradable and such chemicals that show complete resistance to biodegradation are called 'recalcitrant'. The chemical reactions leading to biodegradation of pesticides fall into several broad categories which are discussed in brief in the following paragraphs.

27.6.1 Detoxification

Conversion of the pesticide molecule to a non-toxic compound. Detoxification is not synonymous with degradation. Since a single chance in the side chain of a complex molecule may render the chemical non-toxic.

27.6.2 Degradation

The breaking down/transformation of a complex substrate into simpler products leading finally to mineralization. Degradation is often considered to be synonymous with mineralization, e.g. Thirum (fungicide) is degraded by a strain of Pseudomonas and the degradation products are dimethlamine, proteins, sulpholipaids, etc.

27.6.3 Conjugation (complex formation or addition reaction)

In which an organism make the substrate more complex or combines the pesticide with cell metabolites. Conjugation or the formation of addition product is accomplished by those organisms catalyzing the reaction of addition of an amino acid, organic acid or methyl crown to the substrate, for e.g. in the microbial metabolism of sodium dimethyl dithiocarbamate, the organism combines the fungicide with an amino acid molecule normally present in the cell and thereby inactivate the pesticides/chemical.

27.6.4 Activation

It is the conversion of non-toxic substrate into a toxic molecule, for e.g. Herbicide, 4-butyric acid (2, 4-D B) and the insecticide Phorate are transformed and activated microbiologically in soil to give metabolites that are toxic to weeds and insects.

27.6.5 Changing the spectrum of toxicity

Some fungicides/pesticides are designed to control one particular group of organisms/pests, but they are metabolized to yield products inhibitory to entirely dissimilar groups of organisms, for e.g. the fungicide PCNB fungicide is converted in soil to chlorinated benzoic acids that kill plants. Biodegradation of pesticides/herbicides is greatly influenced by the soil factors like moisture, temperature, pH and organic matter content, in addition to microbial population and pesticide

solubility. Optimum temperature, moisture and organic matter in soil provide congenial environment for the break down or retention of any pesticide added in the soil. Most of the organic pesticides degrade within a short period (3-6 months) under tropical conditions. Metabolic activities of bacteria, fungi and actinomycetes have the significant role in the degradation of pesticides.

27.7 Criteria for Bioremediation/Biodegradation

For successful biodegradation of pesticide in soil, following aspects must be taken into consideration.

- Organisms must have necessary catabolic activity required for degradation of contaminant at fast rate to bring down the concentration of contaminant,
- The target contaminant must be bioavailability,
- Soil conditions must be congenial for microbial/plant growth and enzymatic activity and
- Cost of bioremediation must be less than other technologies of removal of contaminants.

According to Gales (1952) principal of microbial infallibility, for every naturally occurring organic compound there is a microbe/enzyme system capable its degradation.

27.8 Strategies for Bioremediation

For the successful biodegradation/bioremediation of a given contaminant following strategies are needed.

27.8.1 Passive/intrinsic Bioremediation

It is the natural bioremediation of contaminant by tile indigenous microorganisms and the rate of degradation is very slow.

27.8.2 Biostimulation

Practice of addition of nitrogen and phosphorus to stimulate indigenous microorganisms in soil.

27.8.3 Bioventing

Process/way of Biostimulation by which gases stimulants like oxygen and methane are added or forced into soil to stimulate microbial activity.

27.8.4 Bioaugmentation

It is the inoculation/introduction of microorganisms in the contaminated site/soil to facilitate biodegradation.

27.8.5 Composting

Piles of contaminated soils are constructed and treated with aerobic thermophilic microorganisms to degrade contaminants. Periodic physical mixing and moistening of piles are done to promote

microbial activity.

27.8.6 Phytoremediation

It Can be achieved directly by planting plants which hyper accumulate heavy metals or indirectly by plants stimulating microorganisms in the rhizosphere.

27.8.7 Bioremediation

Process of detoxification of toxic/unwanted chemicals/contaminants in the soil and other environment by using microorganisms.

27.8.8 Mineralization

Complete conversion of an organic contaminant to its inorganic constituent by a species or group of microorganisms.

<div align="center">

Lesson 28
MICROBIOLOGY OF AIR

</div>

28.1 Introduction

The earth's atmosphere is teeming with airborne microorganisms. These organisms are thought to exhibit correlations with air pollution and weather. Most airborne bacteria originate from natural sources such as the soil, lakes, oceans, animals, and humans. Many 'unnatural' origins are also known, such as sewage treatment, animal rendering, fermentation processes, and agricultural activities which disturb the soil. Viable airborne microorganisms are not air pollutants, but should be considered as a factor affecting air quality. Air is an unfavourable environment for microorganisms, in which they cannot grow or divide. It is merely a place which they temporarily occupy and use for movement.

There are 3 elementary limiting factors in the air

- A lack of adequate nutrients
- Frequent deficit of water (desiccation)
- Solar radiation

The atmosphere can be occupied for the longest time by those forms which, due to their chemical composition or structure, are resistant to desiccation and solar radiation. They can be subdivided into the following groups:

- Bacterial resting forms,
- Bacterial vegetative forms which produce carotenoidal dyes or special protective layers (capsules, special structure of cell wall),
- Spores of fungi,
- Viruses with envelopes

28.2 Resting forms of bacteria

Endospores are the best known resting forms. These structures evolve within cells and are covered by a thick multi-layer casing. Consequently, endospores are unusually resistant to most unfavourable environment conditions and are able to survive virtually endlessly in the conditions provided by the atmospheric air. They are only produced by some bacteria, mainly by Bacillus and Clostridium genera. Because each cell produces only one endospore, these spore forms cannot be used for reproduction.

Another type of resting form is produced by very common soil bacteria, the actinomycetes. Their special vertical, filiform cells, of the so-called air mycelium, undergo fragmentation producing numerous ball-shaped formations. Due to the fact that their production is similar to the formation of fungal, they are also called conidia. Contrary to endospores, the conidia are used for reproduction. There are also other bacterial resting forms, among others, the cysts produced by azotobacters - soil bacteria capable of molecular nitrogen assimilation.

28.3 Resistant Vegetative Cells of Bacteria

The production of carotenoidal dyes ensures cells with solar radiation protection. Carotenoids, due to the presence of numerous double bonds within a molecule (-C=C-), serve a purpose as antioxidants, because, as strong reducing agents, they are oxidizedby free radicals. Consequently, important biological macromolecules are being protected against oxidation (DNA, proteins etc.). Bacteria devoid of these dyes quickly perish due to the photodynamic effect of photooxidation. That explains why the colonies of bacteria, which settle upon open agar plates, are often colored. The ability to produce carotenoids is possessed especially by cocci and rod-shaped actinomycetes. Rod-shaped actinomycetes, e.g. *Mycobacteriumtuberculosis*, besides being resistant to light, also demonstrate significant resistance to drying due to a high content of lipids within their cell wall. High survival rates in air are also a characteristic for the bacteria which possess a capsule, e.g. *Klebsiella* genus, that cause respiratory system illnesses.

28.4 Fungal Spores

Spores are special reproductive cells used for asexual reproduction. Fungi produce spores in astronomical quantities, for example the giant puffball (*Calvatia gigantea*) produces 20 billion spores, which get into the air and are dispersed over vast areas. A very common type of spores found in air is that of conidia.

Conidia are a type of spore formed by asexual reproduction. They form in the end-sections of vertical hyphae called conidiophores and are dispersed by wind. The spores of common mould fungi such as*Penicillium* and *Aspergillus* are examples of the above. Spore plants such as ferns, horsetails and lycopods also produce spores. Plant pollen is also a kind of spores.

28.5 Resistant Viruses

Besides cells, the air is also occupied by viruses. Among those that demonstrate the highest resistance are those with enveloped nucleocapsids, such as influenza viruses.
Among viruses without enveloped nucleocapsids, enteroviruses demonstrate a relatively high resistance.

Of course, besides the previously mentioned resistant forms, the air is also occupied by more sensitive cells and viruses, but their survival is much shorter. It is believed, that among vegetative forms, gram-positive bacteria demonstrate greater resistance than Gram negative bacteria (especially for desiccation), mainly due to the thickness of their cell wall. Viruses are usually more resistant than bacteria.

28.6 Factors Affecting Growth of Microorganism in Air

There are several factors which influence the ability of a bioaerosol to survive in air:

- Particular resistance for a given microorganism (morphological characteristics)
- Meteorological conditions (inter alia, air humidity, solar radiation),
- Air pollution,
- The length of time in air.

28.6.1 Resistance of microorganisms

It is a species dependent feature, which relies on the microorganism's morphology and physiology.

28.6.2 Relative humidity

The content of water in air is one of the major factors determining the ability to survive. At a very low humidity and high temperature cells face dehydration, whereas high humidity may give cells protection against the solar radiation. Microorganisms react differently to humidity variations in air, but nevertheless most of them prefer high humidity. The morphology and biochemistry of cell-surrounding structures, which may change its conformation depending on the amount of water in air, are crucial. Actually, an exact mechanism of this is not known. Forms of resting spores with thick envelopes (e.g. bacterial endospores) are not particularly susceptible to humidity variations. Gram-negative bacteria and enveloped viruses (e.g. influenza virus, myxo) deal better with low air humidity which is contrary to gram-positive bacteria and non-enveloped viruses (e.g. enteroviruses) that have higher survival rates in high air humidity.

28.6.3 Temperature

Temperature can indirectly affect cells by changing the relative-air humidity (the higher the temperature, the lower the relative humidity) or a direct affect, causing, in some extreme situations, cell dehydration and protein denaturation (high temperatures) or crystallization of water contained within cells (temperatures below 0°C). Therefore, it can be concluded that low temperatures (but above 0°C) are optimal for the bioaerosol. According to some researchers the optimal temperatures are above 15°C.

28.6.4 Solar radiation

Solar radiation has a negative affect on the survival rate of the bioaerosol, both visible as well as ultraviolet (UV) and infrared radiation due to the following factors:

- Causes mutation,
- Leads to the formation of free radicals, which damage important macromolecules.
- Creates a danger of dehydration.

Visible light rays of about 400-700 nm wavelength, create the so-called photodynamic effect, which produces free radicals within cells, especially compounds such as peroxy and hydroxyl radicals. These radicals demonstrate strong oxidizing activities and may cause damage to crucial macromolecules, e.g. DNA or proteins.

UV radiation has a much larger affect on cells than visible light does, especially the rays of 230-275 nm wavelengths. The mechanism of this effect is based on changes to DNA, both directly (e.g. by creating thymine dimer and consequently causing mutation), as well as indirectly, by creating free radicals as in the case of the visible light.

In addition, infrared (IR) radiation may have a negative effect upon cells contained in air - heating up and consequently dehydration.

28.6.5 Biological aerosols

Microorganisms in air occur in a form of colloidal system or the so-called bioaerosol. Every colloid is a system where, inside its dispersion medium, particles of dispersed phase occur whose size is halfway between molecules and particles visible with the naked eye. In the case of biological aerosols, it's the air (or other gases) that has the function of the dispersion medium, whereas microorganisms are its dispersed phase. However, it is quite rare to have microbes independently occurring in air. Usually, they are bound with dust particles or liquid droplets (water, saliva etc.), thus the particles of the bioaerosol often exceed microorganisms in size and may occur in two phases:

- Dust phase (e.g. bacterial dust) or
- Droplet phase (e.g. formed as the result of water-vapour condensation or uring sneezing).

The dust particles are usually larger than the droplets and they settle faster. The difference in their ability to penetrate the respiratory tract is dependent on the size of the particles; particles of the droplet phase can reach the alveoli, but dust particles are usually retained in the upper respiratory tract. The number of microorganisms associated with one dust particle is greater than in the droplet phase.

The average size of bioaerosols ranges from about 0.02 μm to 100 μm. The sizes of certain particles may change under the influence of outside factors (mainly humidity and temperature) or as a result of larger aggregates forming. By using size criterion, the biological aerosol can be subdivided into the following:

- Fine particles (less than 1μm) and
- Coarse particles (more than 1μm)

Fine particles are mainly viruses, endospores and cell fragments. They possess hygroscopic properties and make-up the so-called nucleus of condensation of water vapour. At high humidity water collects around these particles creating a droplet phase.

Then, the diameter of the particles increases. Coarse particles consist mainly of bacteria and fungi, usually associated with dust particles or with water droplets.

Biological aerosols as a human hazard source .

- What types of dangers are connected to the presence of microorganisms in air?
- Infectious diseases (viral, bacterial, fungal and protozoan),
- Allergic diseases,
- Poisoning (exotoxins, endotoxins, mycotoxins).

Bioaerosols may carry microorganisms other than those which evoke respiratory system diseases. The intestinal microorganisms contained in aerosols may, after settling down, get into the digestive system (e.g. by hands) causing various intestinal illnesses.

28.7 Infectious Airborne Diseases

The mucous membrane of the respiratory system is a specific type of a 'gateway' for most airborne pathogenic microorganisms. Susceptibility to infections is increased by dust and gaseous air-pollution, e.g. SO_2 reacts with water that is present in the respiratory system, creating H_2SO_4, which irritates the layer of mucous. Consequently, in areas of heavy air pollution, especially during smog, there is an increased rate of respiratory diseases.

Bioaerosols may, among other things, carry microbes that penetrate organs via the respiratory system. After settling, microbes from the air may find their way onto the skin or, carried by hands, get into the digestive system (from there, carried by blood, to other systems, e.g. the nervous system). Fungi that cause skin infections, intestinal bacteria that cause digestive system diseases or nervous system attacking enteroviruses are all examples of the above.

28.7.1 Viral diseases

After penetrating the respiratory system with inhaled air, particles of viruses reproduce inside the cuticle cells of both the upper and lower respiratory system. After reproduction some of the viruses stay inside the respiratory system causing various ailments (runny nose, colds, bronchitis, pneumonia), whereas others leave the respiratory system to attack other organs (e.g. chickenpox viruses attack the skin). The most noteworthy viruses are:

Influenza (orthomyxoviruses) Influenza, measles, bronchitis, mumps and pneumonia among newborns (paramyxoviruses)

- German measles (similar to paramyxoviruses)
- Colds (rhinoviruses and koronaviruses)
- Cowpox and true pox (pox type viruses)
- Chickenpox (cold sore group of viruses)
- Foot-and-mouth disease (picorna type viruses)
- Meningitis, pleurodynia (enteroviruses)
- Sore throat, pneumonia (adenoviruses)

28.7.2 Bacterial diseases

Similarly to viruses, some bacteria that find their way to the respiratory system may also cause ailments of other systems. Especially staphylococcus infections assume various clinical forms (bone marrow inflammation, skin necrosis, intestinal inflammation, pneumonia). Often, a susceptible base for development of various bacterial diseases is first prepared by viral diseases, e.g. staphylococcus pneumonia is usually preceded by a flu or mumps. Bacterial airborne diseases include:

- Tuberculosis (*Mycobacterium tuberculosis*),
- Pneumonia (*Staphylococcus, Pneumococci, Streptococcus pneumoniae*, less frequently chromatobars of *Klebsiella pneumoniae*),
- Angina, scarlet fever, laryngitis (*Streptococcus*),
- Inflammation of upper and lower respiratory system and meningitis (*Haemophilus influenzae*),
- Whooping cough (chromatobars of *Bordetella pertussis*),
- Diphtheria (*Corynebacterium diphtheriae*),

- Legionnaires disease (chromatobars of *Legionella* genus, among others *L. pneumophila*),
- Nocardiosis (oxygen actinomycetes of *Nocardia* genus).

28.7.3 Fungal diseases

Many potentially pathogenic airborne fungi or the so-called saprophytes live in soil. They usually have an ability to break down keratin (keratinolysis) - difficult to decompose proteins found in horny skin formations, e.g. human or animal hair, feathers, claws. Some of the keratinolytic fungi, the so-called dermatophytes, cause mycosis of the outer skin (dermatosis), as the break down of keratin enables them to penetrate the epidermis. Other fungi, after penetrating the respiratory system, cause deep mycosis (organ), e.g. attacking lungs. The following are examples of airborne fungi diseases:

- Mycosis (*Microsporum racemosum*),
- Deep mycosis: aspergillosis (*Aspergillus fumigatus*), cryptococcus (*Cryptococcus neoformans*).

28.7.4 Protozoan diseases

Some protozoa, which are able to produce cysts that are resistant to dehydration and solar radiation, may also infect humans by inhalation. The most common example of the above is *Pneumocystis carinii* which causes pneumonia. Dangers connected with pathogenic bioaerosols do not concern only human diseases. Other significant diseases are those that attack cultivated plants or farm animals. The following are examples of the above:

- Blight - grain disease caused by *Puccinia graminis*, and
- Aphthous fever - very infectious disease that attacks artiodactylous animals.

28.8 Basic Sources of Bioaerosol Emission

There are two basic sources of bioaerosol:

Natural sources: These are mainly soil and water, from which microorganisms are being lifted up by the movement of air, and from organisms such as fungi, that produce gigantic amounts of spores that are dispersed by the wind. Therefore, there are always a given number of microorganisms in the air, as a natural background. It is estimated, that the air is considered to be clean, if the concentration of bacteria and fungi cells does not exceed $1000/m^3$ and $3000/m^3$respectively. This latter statement is only true when the concentration of microorganisms consists of saprophytic organisms, not pathogenic organisms. If the concentration of microorganisms in the air exceeds the above values, or contains microorganisms dangerous to humans, then such air is considered to be microbiologically polluted.

Human activities: From the hygienic point of view, living sources of bioaerosols related to human activity, are more important than the natural sources. The emissions from these sources are dangerous due to the following two reasons

- They may distribute pathogenic microorganisms,

- They often cause a high increase of microorganisms in the air, significantly exceeding the natural background.

The emission sources of biological aerosols can have a localized character (e.g. aeration tank) or a surface character (e.g. sewage-irrigated field).

The most important sources of bioaerosol emission are:

- Agriculture and farming-food industry,
- Sewage treatment plants,
- Waste management.

28.9 Microbiology of Inside Air

Bacteria are microscopic organisms found in indoor environments typically come from human sources (skin and respiration) or from the outdoors. Like mold, most of the bacteria found in the air in buildings are saprobes meaning they grow on dead organic matter. As far as building envelopes are concerned the primary concern is about bacteria colonies that may grow in damp areas. Most of the bacteria are shed from human skin surfaces. It is not surprising to find hundreds of thousands of bacteria per gram of dust in carpets. As long as the bacterial types are a mixture of those listed below, there is generally no cause for concern. Bacteria may also enter with outdoor air or floodwater and grow in indoor environmental reservoirs. Common indoor reservoirs are water systems, air handling unit and wet organic material. Inadequately maintained air handling system is an important source for bacterial exposure that may lead to allergic type disease. Air handling system must be check for the contaminated water where chest tightness, cough, and fever are associated with a particular indoor environment.

28.9.1 The most abundant bacteria present include

28.9.1.1 Micrococcus sp

Micrococcus species are human shed bacteria and are caused by the normal shed of skin. It is found in areas of higher occupant density and/or inadequate ventilation. Micrococcus species are generally regarded as being harmless bacteria. Normally, these bacteria are removed through ventilation systems or cleaning procedures such as mopping or vacuuming.

28.9.1.2 Bacillus sp

Bacillus sp mainly associated with soil and dust. Appropriate temperature and moisture with deposited dust on hard surfaces allow for ideal growing conditions. Most are not serious pathogens.

28.9.1.3 Staphylococcus sp

Staphylococcus sp is an inhabitant and shed from of the skin surfaces. Among the *Staphylococcus* species that are commonly found indoors is *Staphylococcus aureus*, which is an important pathogen in hospital environments. It shouldn't be a matter of concern unless it is the predominating colony found on air or surface samples in indoor environment.

28.9.1.4 Gram positive rod

Gram positive rod bacteria mainly associated with soil and dust. Appropriate temperature and moisture allow for ideal growing conditions on carpet, wall, furniture's etc. Most are not serious pathogens. These bacteria can be removed by good house keeping practice and adequate ventilation systems.

28.9.1.5 Gram negative rod

These organisms are rare in indoor environments, if they found in higher concentration may be related to the bio aerosol of contaminated water or other contamination of wet/moist surfaces or materials, or possibly air handling units systems in which they are proliferating. Some Gram negative bacteria (or endotoxin extracted from their walls) have been shown to provoke symptoms of fever. Occasionally, growth in air handling units has been great enough for aerosols to be generated which contained sufficient allergenic cells to have caused the acute pneumonia like symptoms. If there has been a sewage spill or flood, then Gram negative bacteria are to be expected and such environments should be thoroughly cleaned with disinfectant.

Identification of bacteria by cultural analysis is based on morphology (e.g., spherical, rod-shaped, etc.), by staining reactions (e.g. Gram positive or negative) and by the pattern of results from a series of biochemical tests.

Lesson 29
ENUMERATION OF MICROORGANISMS IN AIR, CONTROL OF AIRBORNE MICROORGANISM

29.1 Enumeration of Microorganisms in Air

Various methods commonly applied for enumeration and detection of microorganisms can be subdivided into:

- Microscopic methods
- Culture methods
- Combination of both

29.2 Microscopic Methods

These consist of

- Letting air through a membrane filter or placing a glass coated with a sticky substance (e.g. vaseline), in the path of air
- Staining of the trapped microorganisms and
- Microscopic testing consisting of cell counting

Staining with acridine orange and examination under a fluorescence microscope is often applied. The final result is given as a total number of microbes in 1 m^3 of air. The advantage of this method is that it allows the detection of live and dead microbes in air, as well as those, which do not abundantly flourish in culture media. Due to this, the number of microbes determined is usually higher by one order of magnitude than in culture methods. In addition, it is possible to detect and identify other biological agents e.g. plant pollen, allergenic mites, abiotic organic dust (fragments of skin, feathers, plants, etc.).

However the methods have a serious drawback: inability to determine the species of microbes (bacteria, fungi, viruses).

29.3 Culture Methods

These methods consist of transferring microbes from air onto the surface of the appropriate culture medium. After a period of incubation at optimal temperature, the formed colonies are counted and the result is given as cfu/m^3 of air (colony forming units). Because a colony can form not only from a single cell, but also from a cluster of cells, the air may contain more microbes than suggested by the CFU result. Besides, the method allows the detection of only the cells that are viable and those which are able to grow upon the medium used. Microbes transferred to the culture medium require resuscitation as they were subjected to the influence of unfavourable conditions. Therefore it is recommended to supplement the culture mediums are required to be supplemented with components such as betaine and catalase. Betaine, the methylic derivative of the glycine amino acid, is utilized by bacteria to maintain osmotic balance, and as a donor of methylic groups it is essential during the processes of biosynthesis. Catalase however breaks down harmful peroxides created in air as a result of UV radiation.

However, testing of viruses differs significantly from the methods utilized for other organisms because:

- They may develop only in living cells, therefore they require tissue cultures (e.g. the epithelium of human trachea or monkey's kidney) or, in the case of bacteriophages, bacterial cultures,
- Species identification of detected viruses is meticulous and, among other things, consists of performing electrophoresis or utilizing antiserum that contains antibodies of common viruses,
- Drawing large quantities of air is essential (over 1000 dm^3, at least one order of magnitude higher than in the case of bacteria), as the amount of viruses in air is rather small (this especially concerns the enteroviruses).

After transferring the viruses onto the surface of a single-layer culture, the viruses penetrate the cells, reproduce in them, and after their destruction attack the neighboring cells. Consequently, the areas around the initial places of the cell infections get cleared of cells – this clearing is called plaques. Therefore, the number of viruses detected is given as the number of units that form the plaques, in short pfu/m^3 (plaque forming units). It has to be pointed out though, that such a method only allows the detection of viruses capable of infecting the utilized cells.

29.4 Sampling of Air

There are four basic ways of sampling the air for use in culture methods:

- Koch's sedimentation method
- Filtration method (also used in microscopic methods)
- Centrifugation
- Impact methods

29.5 Sedimentation Method

This 'Settling Plate Technique' based on this approach is the simplest and is often used by air microbiologists. The principle behind this method is that the bacteria carrying particles are allowed to settle onto the medium for a given period of time and incubated at the required temperature. A count of colonies formed shows the number of settled bacteria containing particles. In this method petridishes containing an agar medium of known surface area are selected so that the agar surface is dry without any moisture. Choice of the medium depends upon the kind of microorganisms to be enumerated. For an overall count of pathogenic, commensal and saprophytic bacteria in air blood agar can be used. For detecting a particular pathogen which may be present in only small numbers, an appropriate selective medium may be used. Malt extract agar can be used for molds. The plates are labeled appropriately about the place and time of sampling, duration of exposure etc. Then the plates are uncovered in the selected position for the required period of time. A Petri dish containing agar medium is kept covered and, at the time of sampling, the cover is removed from the Petri dish so that the agar surfaces is exposed to air for a few minutes. The Petri dish is now incubated. One can see a certain number of colonies developing on agar medium (Fig. 29.1). Each colony represents a particle carrying microorganisms which has fallen on the agar surface. The optimal duration of exposure should give a significant and readily countable number of well isolated

colonies, for example about 30-100 colonies. Usually it depends on the dustiness of air being sampled. In occupied rooms and hospital wards the time would generally be between 10 to 60 m. During sampling it is better to keep the plates about I metre above the ground. Immediately after exposure for the given period of time, the plates are closed with the lids. Then the plates are incubated for 24 hrs at 37°C for aerobic bacteria and for 3 days at 22°C for saprophytic bacteria. For molds incubation temperature varies from 10-50°C for 1-2 weeks. After incubation the colonies on each plate are counted and recorded as the number of bacteria carrying particles settling on a given area in a given period of time.

The use of settle plates is not recommended when sampling air for fungal spores, because single spores can remain suspended in air indefinitely. Settle plates have been used mainly to sample for particulates and bacteria either in research studies or during epidemiologic investigations. Results of sedimentation sampling are typically expressed as numbers of viable particles or viable bacteria per unit area per the duration of sampling time (i.e. CFU/area/time); this method can not quantify the volume of air sampled. Because the survival of microorganisms during air sampling is inversely proportional to the velocity at which the air is taken into the sampler, one advantage of using a settle plate is its reliance on gravity to bring organisms and particles into contact with its surface, thus enhancing the potential for optimal survival of collected organisms. This process, however, takes several hours to complete and may be impractical for some situations.

29.5.1 Limitation

Though the method has the advantage of simplicity, it has certain limits.

- In this method only the rate of deposition of large particles from the air, not the total number of bacteria carrying particles per volume, is measured.
- Growth of bacteria in the settled particles may be affected by the medium used since not all microorganisms are growing well on all media.
- Moreover since air currents and any temporary disturbances in the sampling area can affect the count, many plates have to be used.
- Since only particles of certain dimensions tend to settle on to the agar surface and, also, the volume of air entering inside the Petri dish is not known, this technique gives only a rough estimate and can be used only to isolate air-borne microorganisms.
- However, one can gather information about the kind of air-borne microbes occurring in a particular area by repeated use of settling plate technique for a fixed period of time.

Fig. 29.1 Agar plates showing colonies of microbes present in Air

29.6 Filtration Methods

The methods consist of using an aspirator to suck in a given volume of air, passing it through a sterile absorbing substance (liquid or solid) and transferring the filtered microbes onto the appropriate culture medium. After a pre-determined time of incubation the resulting colonies are counted. Most often, a membrane filter or a physiological solution (0.85% NaCl) is utilized for the filtration of air. Filtration using liquids (sometimes classified as the impact method) is one of the most often used and highly valued techniques of sampling bioaerosol (Fig. 29.2). It results in high output of microbe isolation as well as significant survival of the filtered microbes. The method may be utilized in virus testing as long as the remaining microbes are neutralized (e.g. with chloroform) and the liquid is concentrated before its introduction into the cell culture.

The filtration process through membrane filters allows the utilization of both culture methods (filters containing microbes are placed directly upon the culture media or are rinsed and then inoculated) as well as the microscopic methods (filters are stained and observed under a microscope).
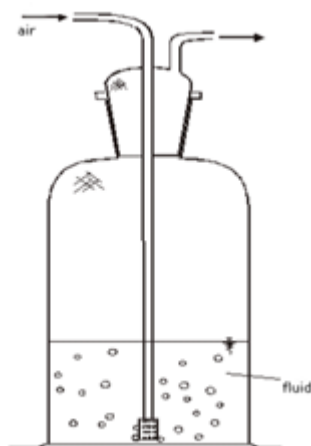
Fig. 29.2 Wash bottle for bioaerosol absorption in filtration methods

These are simple methods for collecting particles from air. The filter can be made of any fibrous or granular material like sand, glass fibre and alginate wool (in phosphate buffer). However, recovery of organisms for culture is not so easy. The membrane filter devices are adaptable to direct collection of microorganisms by filtration of air. These methods are also rather inexpensive and not complicated; they possess two significant advantages over the sedimentation methods:

- The volume of the air tested is known,
- It is possible to detect the very small aerosol that creates the respiratory fraction (nevertheless it is still impossible to determine its size

29.6.1 Tube sampler

This is one of the oldest devices for collecting and enumerating microorganisms in the air. It consists of a tube with an inlet at the top and an outlet at the bottom which is narrower than the top end. Near the bottom there is a filter of wet sand which is supported by a cotton plug below. The entire device can be sterilized. After sterilization the air to be sampled is allowed to pass through the sand and cotton. Microorganisms as well as dust particles containing microorganisms in the air are deposited in the sand filter as the air passes through it. Later the sand is washed with broth and a plate count is made from the broth by taking aliquotes of the broth.

29.6.2 Millipore filter

This type of filters is made of pure and biologically inert cellulose ethers. They are prepared as thin porous, circular membranes of about 150 μm thickness. The filters have different porosity. The assemblage contains a funnel shaped inlet and a tube like outlet. In between these two the filter is fitted. The outlet may be connected to a vacuum pump to suck known amount of air. After collecting required volume of air through the filter, it can directly be placed onto the surface of a solid medium. After incubation colonies formed can be counted.

However, the disadvantage of this method is that it has a significantly low output as the process of passing the air through pores of the filter creates resistance. That's why the method is not recommended for microbe testing, but is routinely put to use in detection of endotoxins in air.

29.7 Centrifugation Methods

29.7.1 Air centrifuge

The first primitive type of air centrifuge was developed by Wells in 1993. The principle of air centrifuge is that the particles from air are centrifuged onto the culture medium. In his air centrifuge sampled air was passed along a tube which was rotated rapidly on its long axis. The inner surface of the tube was lined with culture medium and any bacteria containing particle deposited on it grew into a colony on incubation. A modern version of this centrifuge is the Reuter centrifugal air sampler, which is portable and battery powered. It resembles a large cylindrical torch with an open ended drum at one end. The drum encloses impeller blades which can be rotated by battery power when switched on. A plastic strip coated with culture medium can be inserted along the inner side of the drum. Air is drawn into the drum and subjected to centrifugal acceleration. This causes the

suspended particles to impact on the culture medium. After sampling the strip is removed from the instrument and incubated at 37°C for 48 h. Later the colonies can be counted. Advantage of this sampler is that it is very convenient for transportation and use. However, the disadvantage is that it is less efficient than the slit sampler in detecting particle below 5 mm in diameter. More over the size of the air being sampled cannot be accurately controlled.

29.7.2 Impact methods

These methods consist of using an aspirator to suck in a pre-determined amount (volume) of air, which collides with the nutrient agar at high speed. It causes the microbes in the air to stick to the surface, which after a specific time of incubation, form colonies. The impact methods are the most highly valued and most often used methods of detecting microbes in air. Their biggest advantage is the possibility of detecting and determining the respiratory fraction of the bioaerosol, in other words, determining the size distribution of its particles. The methods can be utilized to test viruses (trapped microbes are swept from the surface of the culture medium and, after the elimination of other microbes with chloroform, introduced into the cell culture).

A disadvantage for the impact method is a decline in the microbes viability caused by the shock of a sudden collision with nutrient agar and also a possibility of the nutrient culture getting overgrown in cases of high air pollution. The above stated methods are usually not cheap. The most widely known device that is based on the impact technique is the Andersen's apparatus, in which the air is drawn in passes through six vertically positioned sieves. A petri dish with nutrient agar is placed underneath each sieve. The speed of the passing air increases as it passes through the consecutive sieves, consequently causing greater impact force as it collides with the sieves. As a result, the heaviest (largest) particles settle upon the first sieve, whereas the lighter (smaller) ones are drawn in by the current of the passing air. As they pass through the consecutive sieves, the increasingly smaller and faster particles collide with the nutrient agar. Consequently the particles of the biological aerosol are sorted according to their size and the colonies are then derived from particles of particular size. This way, by counting the colonies upon the consecutive plates, it is possible to determine the ratio of particles which settle in the upper (higher positioned plates) and lower respiratory system (lower plates).

29.7.2.1 Sampling of measured volume of air

An improvised method wherein a measured volume of air is sampled has also been developed (Fig. 29.3). These are sieve and slit type devices. A sieve device has a large number of small holes in a metal cover, under which is located a petridish containing an agar medium. A measured volume of air is drawn, through these small holes. Airborne particles impinge upon the agar surface. The plates are incubated and the colonies counted. In a slit device the air is drawn through a very narrow slit onto a petridish containing agar medium. The slit is approximately the length of the petridish. The petridish is rotated at a particular speed under the slit. One complete turn is made during the sampling operation
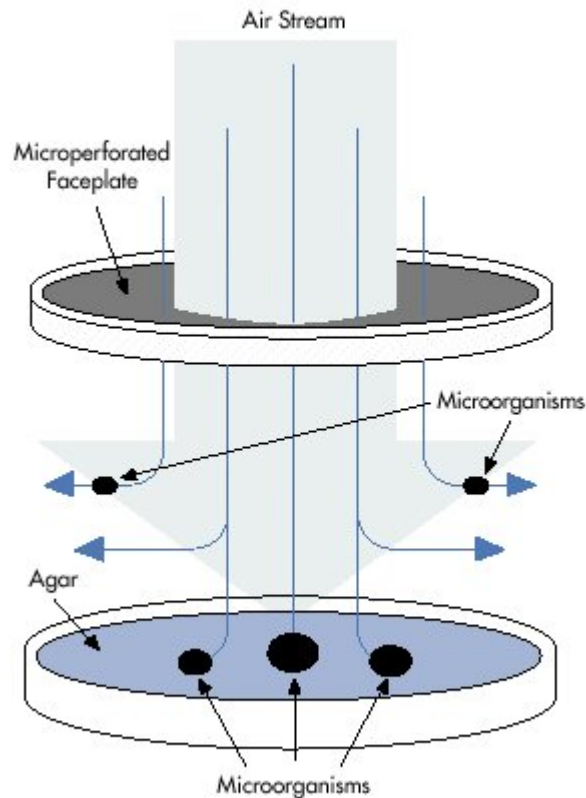
Fig. 29.3 The sieve-impaction sampling method

29.7.2.2 Selection of air sampler

The following factors must be considered when choosing an air sampling instrument:

- Viability and type of the organism to be sampled
- Compatibility with the selected method of analysis
- Sensitivity of particles to sampling
- Assumed concentrations and particle size
- Whether airborne clumps must be broken (i.e. total viable organism count vs. particle count)
- Volume of air to be sampled and length of time sampler is to be continuously operated
- Background contamination
- Ambient conditions
- Sampler collection efficiency
- Effort and skill required to operate sampler
- Availability and cost of sampler, plus back-up samplers in case of equipment malfunction
- Availability of auxiliary equipment and utilities (e.g. vacuum pumps, electricity, and water)

Lesson 30
DISTRIBUTION OF MICROORGANISMS IN AQUATIC ENVIRONMENT

30.1 Introduction

Water occurring in nature contains dissolved salts and gases, especially sea and mineral waters. Water covers 70% of the earth's surface, and thus, it is the most essential habitat of life. The overall volume of inland waters is estimated at 7.5×10^5 km^3, of seas and oceans at 1.4×10^9 km^3, and of glaciers and continental glaciers at 1.8×10^7 km. Water makes up the most crucial component of living organisms (70-90% of cell mass) and fulfils a purpose in taking part in various biological reactions and processes.

30.2 Types of Waters Inhabited by Microorganisms

The biotopes of water microorganisms may be underground and/or surface waters as well as bottom sediments.

- The underground waters (mineral and thermal springs, ground waters) - due to their oligotrophic character (nutrient - deficient) are usually inhabited by a sparse microflora that is represented by a low number of species with almost a complete lack of higher plants or animals.
- The surface waters such as streams, rivers, lakes and sea waters are inhabited by a diverse flora and fauna. Microorganisms in those waters are a largely varied group. Next to the typical water species, other microorganisms from soil habitats and sewage derived from living and industrial pollution occur.
- Bottom sediments are a transient type of habitat i.e. the soil-water habitat that is almost always typically oxygen-free in which the processes of anaerobic decomposition by microorganisms cause the release of hydrogen sulphide and methane into water. In the bottom sediment, anaerobic putrefying microflora, cellulolytic bacteria and the anaerobic chemoautotrophs develop.

30.3 Groups of Water Organisms

Microorganisms occupy surface waters in all of the zones; they may be suspended in water (plankton), cover stationary underwater objects, plants etc. (periphyton), or live in bottom sediments (benthos).

30.3.1 Plankton

The group of organisms that passively float in water not being able to resist the movement and the flow of water mass is called plankton or bioseston. These are of following types:

30.3.1.1 Phytoplankton

Phytoplankton are mainly microscopic algae and blue-green algae. It is a varied community in terms of the systematics and mainly composed of forms smaller than 50 μm. Sea phytoplankton are

dominated by diatoms and dinophyta, whereas fresh water phytoplankton are dominated one by cryptophytes, diatoms, green algae, and blue-green algae.

30.3.1.2 Zooplankton

Zooplankton are small water animals that occur in plankton. There are three systematic groups that occur in fresh waters: rotifers, branchiopods and copepods. The sea water plankton is composed of copepods, ctenophores, urochordata, arrow-worms as well as some species of snails. Most of them are filtrators (condense suspended particles) or predators.

30.3.1.3 Protozoa plankton

Protozoa plankton consists of protozoa which occupy the open water zones like flagellates and ciliates. They are the main consumers of bacteria. Moreover, most ciliates feed upon flagellates, algae and smaller ciliates. The protozoa itself feeds the zooplankton.

30.3.1.4 The heterotrophic bacteria plankton

The heterotrophic bacteria plankton occupy waters which are abundant in organic compounds. The amount of bacteria in open waters varies between 10^5-10^7 cells/ml.

30.3.1.5 Virus plankton

Virus plankton is composed of viruses which are the smallest element of plankton. Their numbers may be very high (from 108 in 1ml) in various fresh and sea water habitats. Viruses are, next to the protozoa, a crucial factor in bacteria mortality.

30.3.2 Periphyton

Periphyton occupy the shore line zones. They are a group of organisms that create outgrowths upon various objects and underwater plants. Most of the time, they usually consist of small algae - diatoms, green algae and bacteria. Moreover, various settled or semi-settled protozoa, eelwarms, oligochaetes, insect larva, and even crustaceans make up the periphyton biocenosis. Periphyton has a characteristic complex biocenosis and many ecological relationships can be observed between its components.

30.3.3 Benthos

The bottom habitat is occupied by a group of organisms called the benthos. The muddy bottom contains an abundance of organic compounds that are created as a result of dead matter decomposition (fallen parts of plants and animals). At great depths the bottom is free from any plants which, due to a lack of light cannot grow. However, the absence of oxygen supports the development of, among others, an oxygen-free putrid microflora. Among the benthos microflora the most numerous are bacteria and fungi (decomposers) as well as some animals (detritophages). Both of the above groups are responsible for decomposition of the organic matter. Benthos of shallow reservoirs may also contain some algae.

30.4 Factors Affecting Growth of Microorganisms in Water

The development of microorganisms in water is influenced by a large number of chemical and physical factors which, in various ways, interact or oppose each other. They have an influence on the size, species and composition of the microbial biocenosis as well as on their appearance and life processes. Within water ecosystems two groups of factors that have a crucial influence on the quantitative and qualitative relationships between microorganisms may be distinguished:

- Abiotic factors - light and thermal energy, water reaction, water flow, climate and the compounds dissolved and suspended in water (dead organic matter, non-organic compounds and gasses such as oxygen, carbon dioxide, methane and others).
- Biotic factors - all water living organisms such as plants, animals, microorganisms and the relationship between them.

30.4.1 Abiotic factors

30.4.1.1 Light energy

Light plays a major role in the process of photosynthesis. The amount of light penetrating different layers of water strictly depends on the position of the sun, transparency, colour and depth of water. The lesser the incidence angle the smaller the loss of sun rays due to reflection. Depending on the level of insolation and water turbidity, the biologically active sun rays usually penetrate water somewhere between 10-150 m. Undoubtedly, sea waters are clearer and less polluted than inland waters, thus light can penetrate much further down through these waters. Sun rays penetrate sea waters down to about 150 m creating the so called photic zone where photosynthesis takes place. Due to different light conditions the development of photoautotrophs isn't identical throughout the entire water mass. The indicator of the illumination quantity is often the lower boundary (limit) of algae occurrence – their greatest development takes place at a depth of 0.5-2 m. Most algae possess an ability to change and adapt their colouring to the light conditions. Light is harmful to those microorganisms which are deprived of any pigments. Both the ultraviolet and the longer wavelength may have a negative effect. For instance, blue light (wave length 366-436 nm) inhibits the process of nitrite oxidation by Nitrobacter vinogradskyi. Light also has an influence upon water fungi development. Blue and green rays have a greater impact than red rays.

30.4.1.2 Temperature

The amount of thermal energy depends, just as in the case of light energy, on the incidence angle (the position of the sun in relation to the water surface). Therefore, it varies with time of day, seasons and latitude. Lotic waters such as rivers have a steady temperature throughout their mass due to constant mixing by the water flow. However, such a water habitat is characterised by daily temperature fluctuations especially in shallow rivers. In lentic (stagnant) waters such as lakes, where the water current is very weak or nonexistent, the temperature fluctuates during the annual cycle. Lakes, especially deep ones, are characterized by vertical stratification (the formation of layers that vary according to their composition and temperature). Illuminated warm and near-surface waters have a lower density than the dark and cold waters from below. The difference in density prevents mixing of the layers. The warm water layer is called the epilimnion. The cooler layers from

below form a thermocline or metalimnion and become cooler with depth. The temperature falls by 1°C with each meter. In the lowest layer - hypolimnion – the water is at 4°C and has the highest density.

The thermocline works as a barrier between the epi- and hypolimnion. The upper waters do not mix throughout the year due to their different density. Water is only moved within the epilimnion layer by the wind. The biogenes present near the bottom are not available for the organisms living in the upper layers thus, in late summer; the top layer has a deficit of trophic substances. In the autumn the surface waters begin to cool down, slowly falling while pushing the warmer waters upwards, which also cool down. As the waters continue to exchange (autumn circulation) and are mixed by the wind they oxygenate and at the same time lose CO_2 by releasing it to the atmosphere especially from the bottom waters. A slight inversion of temperature occurs in the winter since the water at less than 4°C has a lower density than the 4°C water and it rises towards the surface. Different circulation occurs in the spring as the surface waters warm up. Then, the entire body of water is rich in oxygen and biogenes. The mixing of water also causes organisms to move.

30.4.1.3 Water movement

Mixing of water is of great importance to both the temperature distribution and for the balance of the chemical composition (gasses, nutrients, substances that equalize the osmotic pressure, water pH etc.). The movement of water is caused by the following:

- variations in density caused by different temperatures and contents of soluble
- suspended compounds
- winds
- difference in the levels at the bottom (lotic waters)
- specific hydraulic engineering processes.

30.4.1.4 Pressure

Pressure is an important ecological factor that strongly influences the life of microorganisms among other things by affecting the activity of the cells enzymatic systems. In water the hydrostatic pressure gradually increases with depth at 1 atm per 10 m. Thus, in large oceans and some deep lakes the pressure is quite high - in most seas it's at about 100 atm and in some Pacific trenches it may reach even 1100 atm. The group of abyssal microorganisms, which occur at depths of 10,000 m, are called barophilic. They grow and develop not only under great pressures, but also at very low temperature (3 -5°C) but their growth is very slow. Most fresh water and soil bacteria do not develop when the pressure exceeds 200 atm (barophobic microorganisms).

30.4.1.5 pH of water

An optimal pH for water-bacteria is between 6.5 and 8.5. The pH of most lakes is 7.0, rivers - 7.5, and the surface layer of the seas 8.2. Because of the high content of carbonates and their buffer properties, the pH of water does not usually fluctuate significantly. But when there is a rapid growth of photosynthesising organisms the pH may increase rather considerably. Some mineral springs and inland waters with a high content of humus compounds may be acidic. In such conditions the number of acidophilic fungi increases. Relatively large changes in pH can be observed in eutrophic

lakes where the pH varies between 7-10, which has an obvious influence on the populations of bacteria and fungi.

30.4.1.6 Salinity

Most microorganisms that live in clean rivers and lakes are halophobic and in natural conditions do not live in waters in which the salinity exceeds 10%. There aren't many halophilic organisms which may grow in waters of higher salinity. Due to the salinity, sea is thought of as a separate (distinct) biotope; the predominant number of bacteria and fungi living in seas are halophilic. Their life processes depend on a specific concentration of NaCl thus, most of the organisms living in such habitats cannot survive anywhere else. The major mass of salt (99%) is composed of the following elements: Cl, Na, S, Mg, Ca and K. The concentration of salt in sea water is on average 35%. The optimal salinity range for most halophilic bacteria and fungi varies between 25-40%. In the oceans is on average 32-38%, however in closed seas (salty lakes) the range is much greater. For instance, the Caspian Sea contains a low level of salt (1.1-1.3%), whereas the Dead Sea's salinity ranges up to 28%. An increase in salinity has an influence on the generation cycle of bacteria and fungi, and on their morphological and physiological properties. Lakes with a high concentration of salts are extreme biotopes and their biotic groups are low in species variation (the main microorganisms are the bacteria, blue-green algae, flagellates).

30.4.1.7 Other non-organic substances

The life cycle of water microorganisms is also dependent on non-organic substances other than NaCl, among which phosphorus and nitrogen compounds play a major role.

Besides free nitrogen, many mineral compounds of this element, such as nitrates, nitrites and ammonium salts, occur in surface waters. Algae and heterotrophic bacteria most often use nitrates and ammonium salts. The maximum amounts of nitrogen which are tolerated by various algae species are different. For instance, diatoms (such as Asterionella) may reproduce at high concentrations – even at 100 μg N/l, whereas the maximum level for Pediastrum algae is only 2 μg N/l. It is similar for the bacteria - the maximum amount is different for various species. The most important element which limits the development of algae is phosphorus. Its content in water is rather low (0.01-0.1 mg P_2O_5/l). Mineral phosphorus occurs in waters in diluted forms (orthophosphate) and in the form of insoluble salts - calcium phosphate, magnesium phosphate etc. Algae may store phosphorus in their cells in amounts exceeding their requirement. The influence of an increasing phosphate concentration by the introduction of pollutants is a reason for water blooming. In oligotrophic lakes as well as in seas that are nutrient-deficient, it is difficult to detect any presence of ammonium ions, nitrites, nitrates and phosphates since these elements are utilized by phytoplankton immediately after their production. Within the photic zones of many tropical seas the deficiencies in nitrogen and phosphorus compounds last throughout the year, whereas in temperate zones it undergoes seasonal changes.

On the other hand, in the deep waters of some large lakes and seas the accumulation of nitrates and phosphates occurs as a result of heterotrophic microorganism activities.

Ammonium ions and nitrites are the energy substrates for the nitrification bacteria whereas, oxygen

combined in nitrates may be utilized by a number of denitrifying bacteria to oxidize the organic substances in anaerobic conditions. Other life essential salts are the compounds of S, Mg, Ca, K, Fe and Si. They are utilized by microorganisms to build cell structures and for the activation of enzymes.

30.4.1.8 Gases-In water reservoirs

Gases-In water reservoirs, besides salts and organic substances, small quantities of diluted gas can be found. Water possesses an ability to dilute gases but the solubility decreases as the temperature and salinity increase; it is lower in sea waters than in the fresh water basins. It mainly concerns oxygen, carbon dioxide and nitrogen. The main source of the above gases is the atmosphere from which gases diffuse into the upper layers of water until a state of saturation is obtained. In addition, gases diluted in water and sediments may be created during biochemical processes. In this way oxygen is released by green plants as a result of photosynthesis, CO_2 during respiration, free nitrogen during denitrification, hydrogen sulphide as a result of desulfurication, and hydrocarbons as a result of fermentation processes.

30.4.1.9 Organic substances

Organic substances are either secreted by living cells or the products of their autolysis. However, the greatest amounts of organic compounds are introduced into water by sewage. Organic compounds occur in water in the form of solutions or as suspended matter. First of all they serve as food for heterotrophic bacteria and fungi. Microorganisms that often occur on the surface of the suspensions, especially upon the particles of the detritus which absorb the organic substances from water, enjoy favourable feeding conditions. The development and metabolic changes of microorganisms are influenced, more by the content of readily available organic compounds (such as carbohydrates, organic acids, proteins and lipids) rather than the amount of the organic substances in general. Their depletion from water occurs rather quickly. When there is a lack of organic substances bacteria do not reach their proper size and their cell division is slowed down.

30.4.1.10 Trophicity of surface waters

Trophicity is the water's abundance of biogenic elements and soluble simple organic compounds. Trophicity determines the primary production rate and the size of the biomass. Major indicators of water trophicity are: phosphorus and nitrogen concentration, chlorophyll concentration, water transparency and oxygen conditions near the bottom. With reference to the water trophicity, the following kinds of water reservoirs may be distinguished: oligotrophic (low nutrient concentration), mesotrophic (medium nutrient concentration), eutrophic (rich in nutrients/fertile), and hypertrophic (very rich in nutrients). The abundance of nutrients in water reservoirs changes with time.

This process of water fertility increases from oligothrophic through mesotrophic to eutrophic waters in a process called eutrophication. When the above process is moderate and its effects are beneficial, then it is considered to be a fertilization process.

However when the process is excessive and its effects aren't beneficial, then it is considered to be biogenic substance pollution. Nutrient deficient low-fertile waters are those which have a low

content of phosphorus and nitrogen – it is these two elements that are the most crucial. Therefore the waters contain low numbers of phyto- and zooplankton organisms and they are clean and clear. In waters which contain a low amount of plankton very little dead matter falls to the bottom. Therefore, its decomposition does not deplete the oxygen reserves near the bottom. Up to a certain level, the increase in water fertility in turn causes an increase in the number of most organisms in water and consequently an intensification of life manifestations. When the level of nutrients is too high the organic matter produced disturbs the ecosystem's homeostasis. Some enzymes released by bacteria cause decomposition of the other bacteria and algae. Owing to a release of organic substances the plankton microorganisms grow abundantly. The increased use of oxygen causes a deficit of oxygen in deeper waters and consequently, the development of anaerobic microorganisms and the appearance of methane and hydrogen sulphide. Thus, high vitality means increased production in water basins, biomass development of phytoplankton and at the same time lower oxygen concentration in deeper layers. Water blooming is a consequence of eutrophication and it is caused by the reproduction of algae in the upper layers of water. The above process causes changes in water colour, its turbidity, water quality deteriorates, and toxic compounds are produced. The process of natural eutrophication proceeds very slowly – it takes up to a few thousand years. Accelerated eutrophication, however, is caused by human activities. As a result, excess amounts of nitrogen and phosphorus get into waters from various sources such as industrial and municipal wastes, as well as fields that have been fertilized with phosphorus and nitrogen fertilizers. Such activities greatly increase the concentration of biogenes creating favourable conditions for algae reproduction. In such polluted waters, faecal and pathogenic bacteria may survive for a longer period of time. Precipitation in highly industrial and polluted areas has also had an influence upon eutrophication.

30.4.2 Biotic factors

Mutual interactions exist between individual members of the biocenosis that inhabit surface waters. As a result, the organisms may support each other (synergism) or inhibit each other (antagonism).

30.4.2.1 Competition for food

The organisms which most efficiently find and take in food may have an advantage over others. For a given habitat with a typical supply of nutrients, the number of microorganisms quickly increases. However, in many cases, the abundant production of products of metabolism (inhibitors) decreases the number of competitors, sometimes eliminating them entirely. Such situations occur, for instance, when the pH is significantly altered by acidification or alkalization, and when antibiotic substances are released.

30.4.2.2 Co-operation

In feeding and growth processes, co-operation between the microorganisms is often observed. It allows quicker development of mixed microorganism cultures. Biodegradation is a multistage process when consecutive reactions are conducted by different specialized microorganisms. The process prevents the accumulation of the metabolism by-products. Owing to this co-operation, the biodegradation of persistent organic compounds (ligninocellulose) becomes possible.

30.4.2.3 Predation

Bacteria and fungi are food for lower animals. This is why in some water reservoirs their numbers may vary a lot. Most protozoa feed on bacteria. It has been confirmed that their biomass increases along with the increase in bacterial numbers. Numerous multi-cellular organisms also utilize bacteria as their food. These mainly include filtrating animals such as sponges. In bottom sediments many animals feed upon fungi. Blue-green algae which are a part of the benthos are often eaten by turbellarians, nematodes, crustaceans and insect larva. Blue-green algae are eaten by zooplankton, without the latter water blooming and release of toxic substances would occur.

30.4.2.4 Parasitism

Water microorganisms are attacked and destroyed by viruses, bacteria and fungi. The presence of bacteriophages has been affirmed in inland and sea waters. They are especially numerous in sewage and are probably the reason for a quick depletion of bacteria in rivers, lakes and in inshore waters that were polluted with sewage. Another reason for the limitation of bacterial numbers is the presence of the Vibrio bacteria which belong to Bdellovibrio genera and lead a parasitic existence. They attach themselves to host cells and reproduce utilizing their energy and consequently digest the cells content. After lysing the host's cell wall, they free themselves and infect further bacteria.

Lesson 31
MICROBIOLOGY OF AQUATIC WATER

31.1 Bacteria

Bacteria that occur in water habitats may be divided into the following:

- Autochthonous (native) constantly occupying water habitats
- Allochthonous (foreign) finding their way from the soil or the air as well as microorganisms that get into the water basins along with municipal and industrial sewage.

31.1.1 Autochthonous bacteria

We can distinguish photoautotrophs, chemoautotrophs and chemoorganoautotrophs as follows:

a) photosynthesizing bacteria (photoautotrophs)

Purple and green bacteria are among the photosynthesizing autotrophs. Due to their metabolism these bacteria can be divided into the following groups:

- Filiform green bacteria (*Chloroflexaceae*),
- Sulfuric green bacteria (*Chlorobiaceae*),
- Sulfuric purple bacteria (*Chromatiaceae* and*Ectothiorhodaceae*),
- Non-sulfuric purple bacteria (*Rhodospirillaceae*),
- Heliobacteria (*Heliobacteriaceae*).

The photosynthesis of bacteria is carried out slightly differently to that of plants. Most importantly, it is an oxygen-free process which requires the presence of reduced mineral compounds and it is not accompanied by a release of oxygen but by a production of oxidized non-organic or organic compounds. The assimilating pigments of bacteria are categorized by the ability to absorb infrared light that is not absorbed by green plants. The photosynthesis in surface waters is conducted mainly by algae and plants and the role of the bacterial photosynthesis is less important.

b) Chemosynthesizing bacteria (chemoautotrophs)

Chemoautotrophs get energy from the oxidation processes of non-organic compounds.

Depending on the nature of the oxidized substrate the following can be distinguished: nitrifying, ferruginous, sulfuric and hydrogen bacteria.

- The role of the nitrifying bacteria in surface waters is the oxidation of ammonia and nitrite to nitrate. In greater concentrations the above compounds may be harmful to water organisms as well as to humans (in cases when such water is utilized for water supply systems). Moreover, the production of nitrate is a fundamental process that supplies water plants with a source of nitrogen.
- Ferruginous bacteria grow in waters when the content of bivalent iron ranges between 0.15-8.5 mg/dm^3. Their negative influence includes corrosion and fouling of plumbing, sewage systems

and different metal constructions. The most common ferruginous bacteria are the *Leptothrix ochracea* and*Crenothrix polyspora* and they belong to the filamentous bacteria which are categorized by the fact that the single cells form thread-like forms surrounded by a gelatinous sheath of various thickness. Stored ferruginous substances in cells change the coloration of cells threads into a yellow or dark-brown shade. The ferruginous bacteria are very common in fresh bodies of water. Especially in waters from wells and springs where it is possible to observe their clusters with the naked eye. Moreover, they occur abundantly in muddy streams, marshes and ponds.

- Sulfuric bacteria occur mainly in waters containing hydrogen sulfide which is toxic for most microorganisms, whereas for this group it is one of the crucial compounds for survival. These bacteria can be found in mineral springs that contain hydrogen sulfide of geological origin as well as in highly polluted waters where it is produced as a result of oxygen-free protein decomposition or desulfurication processes. The typical representatives of the sulfuric bacteria are: bacteria that move in sliding motions *Beggiatoa alba* and fixed to the bottom*Thiothrix nivea*. The forms of individual sulfuric bacteria are:

-*Thiobacillus thioparus* - stores sulfur derived from oxidation of thiosulfate

-*Thiobacillus thiooxidans* - grows in acidic habitats of pH 1.0 - 4.0

-Thiobacillus ferroxidans - besides thiosulfates and tetrationans, it possesses the ability to decompose ferruginous salts.

-*Thiobacillus denitrificans* - is a relative anaerobe and it has ability to utilizenitrates as the electron acceptor during the oxidation of hydrogen sulfide. In aerobic conditions the above function is performed by the oxygen.

- Hydrogen bacteria have ability to oxidize hydrogen using oxygen as a final acceptor of electrons. Most often they feed heterotrophically and switch to autotrophic feeding when hydrogen is present in the habitat. The most widespread species belong to the genus *Hydrogenomonas. Micrococcus denitrificans* belongs to a group of the hydrogen bacteria and they conduct the oxidation of hydrogen while simultaneously reducing nitrate down to molecular nitrogen. *Desulfovibrio desulfuricans* also oxidizes hydrogen while reducing sulphate down to hydrogen sulphide.
- Heterotrophic bacteria (chemoorganotrophs) A predominant part of autochthonous bacteria which occur in water basins are the chemoorganotrophic bacteria which belong to a group of saprophytes that feed upon dead plant and animal organic matter. Typical bacteria plankton that occupy an entire water mass are the cilliated Gramnegative rods and they represent the following genera: Pseudomonas, *Achromobacter Alcaligenes*,*Vibrio* and *Aeromonas*, as well as the Gram-positive cocci that belong to the *Micrococcus* genus, Treponema and spiral bacteria of the *Spirillum* genus. The underwater parts of higher plants and the underwater fixed particles are colonized by numerous stem-like bacteria (e.g. *Caulobacter*), sheathed, filiform, and gemmating bacteria (e.g. *Hyphomicrobium*), which are one of the microorganisms forming the periphyton. Organisms which usually grow in bottom sediments are oxygen-free putrefactive bacteria, then oxygen-free cellulolytic bacteria and finally oxygen-free chemoorganotrophs such as*Desulfovibrio* genus that reduce sulfate down to the

hydrogen sulphide. In addition there are some less numerous oxygen-free methane generating bacteria which reduce organic compounds down to methane

31.1.2 Allochthonous bacteria

Waters of high fertility and also highly polluted surface waters are abundant in saprophytes and parasitic bacteria from among which, the following are predominant: Gram negative intestinal rods of*Escherichia coli* as well as the *Proteus* genus, *Klebsiella* and*Enterobacter*, and also rods of Pseudomonas *aeruginosa* and of the*Arthrobacter* genus. Moreover, Gram-positive rods (bacilli) of the*Bacillus*, *Corynebacterium* and *Clostridium* genera, which are washed out from the soil and get into the bodies of water during heavy rainfalls, also belong to the allochthonous bacteria. Municipal wastes are the main source of pathogenic bacteria. Moreover, during the infiltration processes and surface run-offs, soil bacteria find their way into the waters as well. The role of air in water contamination is significant in densely populated areas of cities and industrial regions.

31.2 Water Fungi

In contrast to bacteria which grow best in waters of pH between 6 and 8, fungi occur only in waters below pH 6.0. Usually fungi occur in shallow waters, right on or just below the surface, which is closely connected to the fact that the organisms require significant amounts of oxygen). The predominant fungi in water environments are represented by mold fungi which belong to the *Oomycota* class (*Leptomitus*, *Phytophthora*) and to the class of *Zygomycota* (*Mucor*and *Rhizopus*). Relatively frequently fungi belonging to *Ascomycota*as well as the *Deuteromycota* are found in surface waters. Almost all fungi are heterotrophs that decompose organic matter; waters are occupied by both saprophytes and parasites which colonize water plants and animals. They have more diverse shapes than bacteria and they differentiate into larger cells and more complicated structures. In addition to unicellular ones there are also multi-cellular fungi with large mycelium.

Fungi usually do not occur in clean waters. They grow in abundance on the bottom of waters polluted by sewage (e.g. *Leptomitus lacteus*).

31.3 Blue-green Algae

Blue-green algae are a group of organisms previously considered to be algae. Currently they are classified to the *Procaryota* kingdom and the sub-kingdom of *Eubacteria*. There are unicellular, colonial (loose cells connected with a single mucus envelope) and filamentous forms. The prokaryotic organisms contain a nucleoid instead of an isolated nucleus. In contrast to other bacteria they are capable of conducting oxygen photosynthesis. They contain chlorophyll and sometimes disguise it in other photosynthesizing pigments: ficocyanine and alloficocyanine. Characteristically the blue-green coloring of blue-green algae comes from the combination of chlorophyll and ficocyanine. Blue-green algae reproduce mainly through proliferation by cell fission. Their characteristic trait is that they possess gaseous vacuoles which allow movement in water to places of better illumination. Some (*Anabaena*) are capable of binding atmospheric nitrogen in structures called heterocysts. Due to their resistance to extreme environmental conditions they are ubiquitous.

They can be found in deserts and in hot springs. Blue-green algae can cause blooming in lakes and other water reservoirs. Some of them produce toxic metabolites.

31.4 Algae

Algae are the simplest autotrophic eukaryotes that incorporate over 20 thousand species. Algae occur in fresh and sea waters. They are important producers of organic matter and oxygen. Algae live in the form of single cells or they create multicellular body of various shapes called thallus (threads, spheres, multilayer clusters). The composition of algae community changes significantly with respect to quality and quantity, depending on the content of the mineral salts in any given reservoir as well as on the characteristics of the substances that make up the main pollutant.

The following are the characteristic algae that occur in oligotrophic waters: diatoms of the following genera: *Asterionella*, *Tabellaria*,*Melosira* and some other algae (*Dinobrion*). In eutrophic waters the content of algae is completely different. Most of all, such waters contain only a vestigial number of diatoms, and instead of them the algae from the *Dinophyta* class as well as the *Spirogyra* genus appear.

31.5 Water Protozoa

Protozoa live in all types of waters, from small puddles, to inland waters, to the seas. They feed heterotrophically absorbing the dissolved organic compounds or feeding upon bacteria. They are most numerous in highly polluted waters and are the element of activated sludge. When the pollution level is not too high ciliates become predominant, and that concerns both the free-swimming ones (e.g. *Paramecium*) and the settled ones (e.g. *Vorticella*). Protozoa can be subdivided into four classes:

31.5.1 Flagellata – flagellates

These move utilizing long flagella. They feed heterotrophically and occur in polluted waters or in inefficiently functioning activated sludge. Besides dissolved substances, they may also absorb bacteria or unicellular algae. Flagellates live individually or in colonies. There are parasitic forms among them too. This is exemplified by a human parasite *Giardia lamblia* and the *Trypanosoma gambiense*which is transferred on to humans by the Tsetse fly causing African sleeping-sickness and neurological disturbances.

31.5.2 Ciliata–ciliates

Most of the representatives lead a free-swimming life style (*Paramaecium*, *Euplotes*), others crawl or are attached to the bottom. They feed upon bacteria, algae and organic substances. Ciliates occur in large numbers in polluted waters and in activated sludge. Some, such as *Balantidum* coli which causes dysentery, are parasites of animals and humans.

31.5.3 Rhizopoda-amoebae

The cells move around utilizing the plasmatic pseudopodia which are used for locomotion and for

capture of food. Some amoebae have a changeable shape, others however, have a constant shape as they are equipped with a mini-skeleton or an outer shell. Some amoebae lead a parasitic life (*Entamoeba histolytica*).

31.5.4 Sporozoa

Only parasites belong to this class and representatives are*Cryptosporidium parvum* causing intestinal diseases and Plasmodium malariae causing the *malaria*. The second parasite attacks the red blood cells. This pathogen is carried by the *Anopheles*mosquito.

31.6 Water Transmitted Pathogenic Microorganisms

31.6.1 Bacteria

The groups of obligate pathogenic bacteria, which occur in polluted surface waters, contain rods causing typhoid fever (*Salmonella typhi*), as well as other Gram negative bacteria of the *Salmonella*genus (the cause of various infections of the digestive tract). Bacterial dysentery caused by Gram negative rods of the Shigella genus are not as common as the above. In surface waters of tropical countries, bacteria of the *Vibrio* genus (cholera) frequently occur. Moreover, *Mycobacterium tuberculosis* causing tuberculosis and treponema of the *Leptospira* can be also found in polluted waters. The latter bacteria cause bacterial jaundice. In surface waters which are described as opportunistic microorganisms (facultatively, pathogenic). These belong to the *Pseudomonas*, *Aeromonas,Klebsiella, Flavobacterium, Enterobacter, Citrobacter, Serratia, Acinetobacter, Proteus* and *Providencia* genera. All of the rods are part of the usual flora of the intestine and are not typically pathogenic for as long as they occur in human or animal digestive tracts. In some cases though, these bacteria find their way into other organs becoming a potential cause of different illnesses such as inflammation of urinary and respiratory systems and also sepsis which is a general infection of all internal organs.

31.6.2 Viruses

Besides pathogenic bacteria of surface waters, into which municipal and industrial sewage is disposed, the waters also contain significant amounts of other pathogenic microorganisms such as the polio virus. They are responsible for causing the Heine-Medina disease (polio). Enteroviruses, which cause intestinal infections, occur even in slightly polluted rivers.

31.6.3 Protozoa

Infections of the digestive tract caused by protozoa may come from contaminated water. Most parasitic protozoa produce cysts which are able to survive inside their host in unfavorable conditions. When the conditions improve, cysts transform into so called trophozoits, the vegetative form occurring in humans.

31.6.4 Parasitic fungi

In polluted surface waters parasitic fungi can also occur, for

example*Microsporum* sp., *Trichophyton* sp. and *Epidermophyton* sp. They are dermatophytes causing ringworm and other cutaneous infections.

A list of water borne bacteria and viruses along with associated diseases is provided in (Table 31.1 – 31.2) respectively.

31.7 Indicator Organisms

In routine laboratory work, which conducts sanitary-epidemiological supervision, it is impossible to constantly monitor water for all pathogenic and potentially pathogenic microorganisms, which may be found in water. Therefore, routine monitoring concentrates mainly on detecting bacteria that indicate faecal contamination of water. The sanitary quality of water may be checked by utilizing the saprophytic microflora that occupy the human large intestine. The following indicators of water contamination have been adopted:

- Coliforms
- Faecal coliforms
- Faecal streptococci
- Bacilli of *Clostridium* genus, sulphite-reducing bacteria
- Staphylococci – coagulase positive
- *Pseudomonas aeruginosa*

31.7.1 Coliforms

Bacteria of the coli group are mainly made up of strains of*Escherichia coli* as well as the genera: *Enterobacter*, *Citrobacter* and*Klebsiella*. They are detected on media containing lactose at 37°C.

Faecal coliforms (thermotolerant) are mainly strains of *Escherichia coli* and only some of the strains of *Enterobacter*, *Citrobacter* and*Klebsiella*, which have an ability to ferment lactose at 44°C.

The presence of coliforms or faecal coliforms in a water sample indicates relatively recent contamination of water with faecal matter, sewage, soil or with decaying plants. For most types of waters a quantitative determination of both groups of coliforms is recommended.

31.7.2 Faecal streptococci

While in a water environment, faecal streptococci are characterized by a slightly longer period of survival and resistance to most disinfecting products than the coliforms. Faecal streptococci include microorganisms of *Enterococcus* and *Streptococcus* genera, which belong to the serological group of Lancefield D. Detection of faecal streptococci in a test sample, significantly exceeding the coli group bacteria, may suggest water contamination with animal faecal matter or sewage from animal farms.

31.7.3 Bacilli of Clostridium genus

The detection of sulphite reducing bacteria (mainly strains of *Clostridium perfringens*) may suggest less recent contamination with faecal matter; their endospores are able to survive for many years in unfavourable conditions. Sulphite reducing clostridia are a good indicator of properly conducted water treatment processes - coagulation, sedimentation, and filtration. Endospores of these bacteria as well as the cysts of parasitic protozoa (*Cryptosporidium parvum*, *Giardia lamblia*) ought to be eliminated in those stages of water treatment, because they are especially resistant to the disinfecting agents. Conducting analysis of a water sample, in order to detect bacteria of the *Clostridium* genus, is technically less complicated than searching for parasitic protozoa and it ensures that the treated water is free from protozoa and from the eggs of pathogenic worms (Helminthes).

31.7.4 *Pseudomonas aeruginosa*

Currently, detection of *Pseudomonas aeruginosa* bacteria in drinking water, running water, swimming pools and surface waters is recommended in addition to the above elements of sanitary analysis. They are Gram-negative rods that do not produce spores. Their characteristic trait is the ability to produce a blue-green pigment - pyocyanin as well as a fluorescent pigment – fluorescein. Representatives of this species were isolated from human faeces and in cases of infection from urinary tracts, inner ear, suppurating wounds etc. These bacteria pose a potential pathogenic danger for both humans and animals. In addition, they are widely distributed in surface waters and soil. It is also important that the species may live in chlorinated water because it is, to some extent, resistant to disinfection.

31.7.5 *Staphylococci*

The *Staphylococcus* genus is mainly used to assess sanitary quality of swimming pools. Recreation waters are the cause of infections of respiratory tracts, skin and eyes. For this reason microbiological analysis based on standard indicators (coliforms) is insufficient. Some researchers have recommended *Staphylococcus aureus* to be used as an additional indicator of sanitary quality of recreational waters, because its presence is associated with human activity in these waters.

31.8 Total Number of Bacteria

In routine analysis the total number of bacteria present in 1 ml of water is also determined by an agar plate method. One set of plates is incubated at 37°C for 48 h (mesophilic bacteria). Another set of plates is incubated at 22°C for 72 h (psychrophilic bacteria). After incubation the colonies are counted and the amount of cfu/ml (colony forming units) can be calculated.

31.8.1 Total number of psychrophilic bacteria

Non pathogenic water bacteria grow mainly at lower temperatures. It is important that Gram-negative bacteria in water produce lipopolysaccharides in their cell wall which can be toxic – like endotoxins of pathogenic bacteria. Because of this, their numbers in water should be constantly monitored. A large increase in their numbers is evidence of the presence of easily available organic compounds in the water. Theoretically, the presence of 0.1 mg organic carbon in water can result in an increase of bacteria up to 108 cfu in 1 ml. Phosphorus is also a factor which stimulates the

growth of microorganisms. Adding even small amounts of this element (<50 mg/l) causes 10 times the acceleration of bacterial growth in a water treatment plant.

31.8.2 Total number of mesophilic bacteria

More dangerous are high numbers of bacteria growing at 37°C, because among this high population, pathogenic forms may be found which are dangerous for human health.

High number of bacteria in samples of water can prove that water treatment processes proceed badly or that polluted water is siphoned.

Table 31.1 Bacterial infections

Disease type	Species or genera of bacteria
Typhoid	*Salmonella typhi*
Paratyphoid	*Salmonella paratyphi*
Animal salmonellosis	*Salmonella* sp.
Bacterial dysentery	*Shigella* sp.
Cholera	*Vibrio cholerae, Vibrio cholerae type eltor*
Stomach and intestine catarrhs	Enteropatogenic *Escherichia coli, Klebsiella pneumoniae, Aeromonas hydrophila, Plesiomonas shigelloides, Pseudomonas aeruginosa, Vibrio parahaemolyticus, Campylobacter (Vibrio) fetus* subsp. *jejuni, Clostridium perfringens, Bacillus cereus*
Yersiniosis	*Yersinia enterocolitica*
Tularemia	*Pasteurella (Francisella) tularensis*
Leptospirosis	*Leptospira* sp.
Skin infections	*Pseudomonas aeruginisa, Mycobacterium (M. balnei, M. phlei, M. marinum, M. kansasii, M. fortuitum, M. cholonei, M. gorgonae*
Bacteremia conjunctivitis, ear and upper-respiratory system infection	*Psudomonas aeruginosa, Pseudomonas cepacia*
Fever (pyrogens)	Gram negative water rods (*Pseudomonas, Achromobacter, Xantomonas, Moraxella Acinetobacter)*
Legionnaires disease	*Legionella pneumophila*

Table 31.2 Intestinal viruses which may be transmitted by water and diseases caused by them

| Viruses	Number of types	Diseases
Poliovirus	3	Palsies, meningitis, fever
ECHO	34	Meningitis, respiratory system diseases, rash, diarrhea, fever
Coxsackie A	23	Herpangina, respiratory system diseases, meningitis, fever
Coxsackie B	6	Cardiac muscle inflammation, innate heart defects, rash, fever, meningitis, respiratory system diseases, pleurodinia
Enteroviruses	4	Meningitis, encephalitis, respiratory system diseases, acute hemorrhage conjunctivitis, fever
Hepatitis virus, type A	1	Hepatitis type A
Norwalk virus	1	Epidemic diarrhea, fever
Parvovirus	3	Accompany the respiratory system diseases
Adenoviruses	41	Respiratory system disease, eye infections, diarrhea
Rotaviruses	4	Epidemic diarrheas (mainly among children)
Reoviruses	3	Respiratory system diseases

Lesson 32
WASTE WATER TREATMENT AND DISPOSAL

32.1 Introduction

Waters become polluted as a result of domestic and industrial sewage disposal into surface waters, which contain huge amounts of various compounds that affect the biocenosis of water reservoirs. Besides sewage, pollution is also caused by rain run-offs which wash away different fertilizers and crop protection products. Moreover, the pollutants also transfer into waters from the surrounding air. This usually results from industrial dust which falls directly into the water or is washed away from the ground surface by rain. Important gases are: sulphur dioxide, nitrogen oxides, carbon oxides and dioxides which get into the waters mainly in highly industrial areas. Some of the above compounds undergo microbiological decomposition relatively easily, becoming food for heterotrophic microorganisms, others are resistant to such decomposition and are harmful or toxic to microorganisms. Examples of these are the following: cyclic compounds, engine oil, lubricants, chlorinated hydrocarbons, pesticides, and among the mineral pollutants – heavy metal salts.

32.2 Self-purification of Surface Waters

Self-purification encompasses complex co-operation between physical and biochemical factors such as: sedimentation (settling), oxidation, an exchange of volatile substances between the atmosphere and water, and the release of gaseous products of metabolism into the atmosphere. However, the critical role is played by the biological factors.

A wide range of microorganisms and higher organisms participate in self-purification processes. Bacteria and fungi are the most crucial as they are capable of mineralising various mineral components. Proteins, simple and complex sugars, fats, cellulose, lignin, wax and others undergo degradation during the process of self-purification. As a result of mineralization the following compounds are created: H_2O, CO_2, NO_3^-, SO_4^{2-}, PO_4^{3-}, and other simple compounds. With the progression of self-purification the populations of microorganisms that act in the environment change (Fig. 32.1).
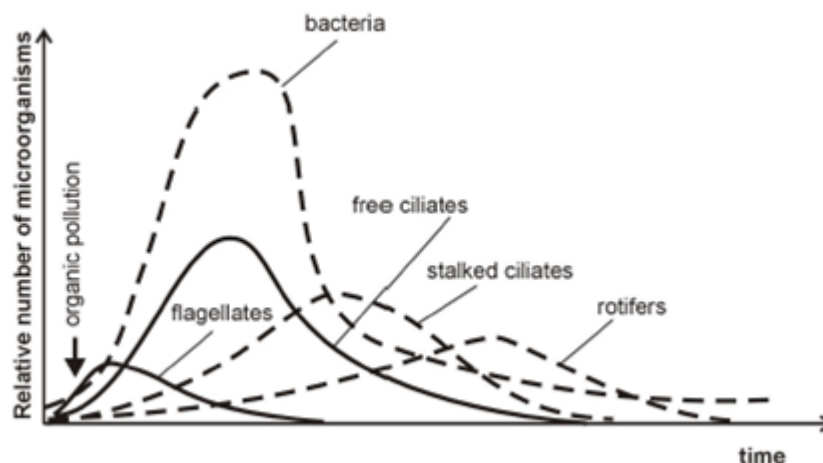
Fig. 32.1 Succession of microorganisms during the self-purification process

The self-purification process utilizes large amounts of oxygen during the biochemical processes. The amount of oxygen that is used up in any specified time by water microorganisms is called the biochemical oxygen demand (BOD). By analyzing the BOD, it is possible to determine the concentration of the organic compounds dissolved in water which are susceptible to biological oxidation. The discharge of impurities into the water reservoir creates a sudden change in chemical, biological and physical conditions. Simultaneously, right below the area of the discharge, the process of self-purification begins. The process leads to the formation of zones containing characteristically gradually decreasing levels of pollution.

32.3 Types of Sewage

- Domestic sewage contains large amounts of faecal matter, plant and animal wastes, surface-active agents, urea. The sewage comes from households, public lavatories and industrial facilities posing a serious hygienic and epidemiological threat,
- Industrial (technological), evolve during all types of industrial processes (manufacturing and processing),
- Precipitation (rain and melt waters) contain various atmospheric impurities (dusts, microorganisms, gaseous substances), surface run-offs, streets and paved surfaces run-offs (oils, liquid fuels, bacteria, small particle suspensions), microbiological impurities (bacteria, viruses, fungi).

32.4 Methods of the Wastewater Treatment

Depending on the type of pollutants, there are different methods of purification used prior to reintroduction into a receiving body of water. The methods are classified as follows:

- Mechanical – in this method only non-soluble pollutants are removed by utilizing the following processes: gravitational and centrifugal sedimentation, flotation, source filtration, separation in hydrocyclones, which allow the removal of organic and mineral suspensions as well as floating bodies;
- Physical-chemical – utilizes the following operations and processes: coagulation, coprecipitation, sorption, ion exchange, electrolysis, reverse osmosis, ultrafiltration;
- Chemical – utilizes neutralization, oxidation, reduction;
- Biological – consists of sewage purification (elimination of organic pollutants as well as biogenic and some refraction compounds) during biochemical processes of mineralization conducted naturally by microorganisms in a water habitat (e.g. sprinkling of wastewater onto agricultural lands), or in special devices (on trickling filters or activated sludge).

32.5 Stages of Sewage Treatment

A typical process of sewage treatment consists of four stages of purification:

- Mechanical (stage I of purification),
- Biological (stage II of purification),
- Elimination of biogenic compounds (stage III of purification),
- Water renovation (stage IV of purification).

Stage I of purification, primary treatment the so-called initial or mechanical purification. The goal of this stage is the removal of solid impurities. This stage is considered to be the preparation of sewage for further purification. By utilizing simple mechanical operations the following impurities are removed during the first stage:

- floating solid impurities,
- settling suspensions,
- oils and fats.

Stage II of purification, secondary treatment includes biological purification, which leads to the biodegradation of soluble organic impurities, colloidal systems and suspensions not removed during the first stage. The intensification of purification processes is obtained by utilizing trickling filters and activated sludge.

Stage III of purification, tertiary treatment includes processes used to thoroughly clean sewage. The largest impurities removed during this process are the biogenic compounds. The nitrogenous compounds are removed during the process of biological nitrification and de-nitrification, whereas the compounds of phosphorus are eliminated by a process of chemical precipitation. The role of thorough cleaning of sewage in this stage is the prevention of water eutrophication.

Stage IV of purification (water renovation) includes the processes of residual sewage removal, which are left over from the previous stages of purification. Water regeneration involves a set of methods which confer the properties of natural water onto the sewage so that it can be utilized in industrial facilities. Water regeneration allows the recycling of sewage, which is a significant element in water resource management, especially in regions low in water. There are several systems of water regeneration, from very simple ones, that use rapid filters or straining through microsieves, to very complex physico-chemical processes: coagulation, membrane processes and disinfection, sedimentation, expelling of ammonium, recarbonization, absorption, ion exchange, and water demineralization.

32.6 Techniques Used for Wastewater Treatment

32.6.1 Use of trickling filters (Fig. 32.2 & 32.3)

The treatment of sewage by trickling filters is conducted in reservoirs filled with loose, grainy and porous material. Sewage is sprayed upon the upper layer of the bed with sprinklers and then left to seep through its content.
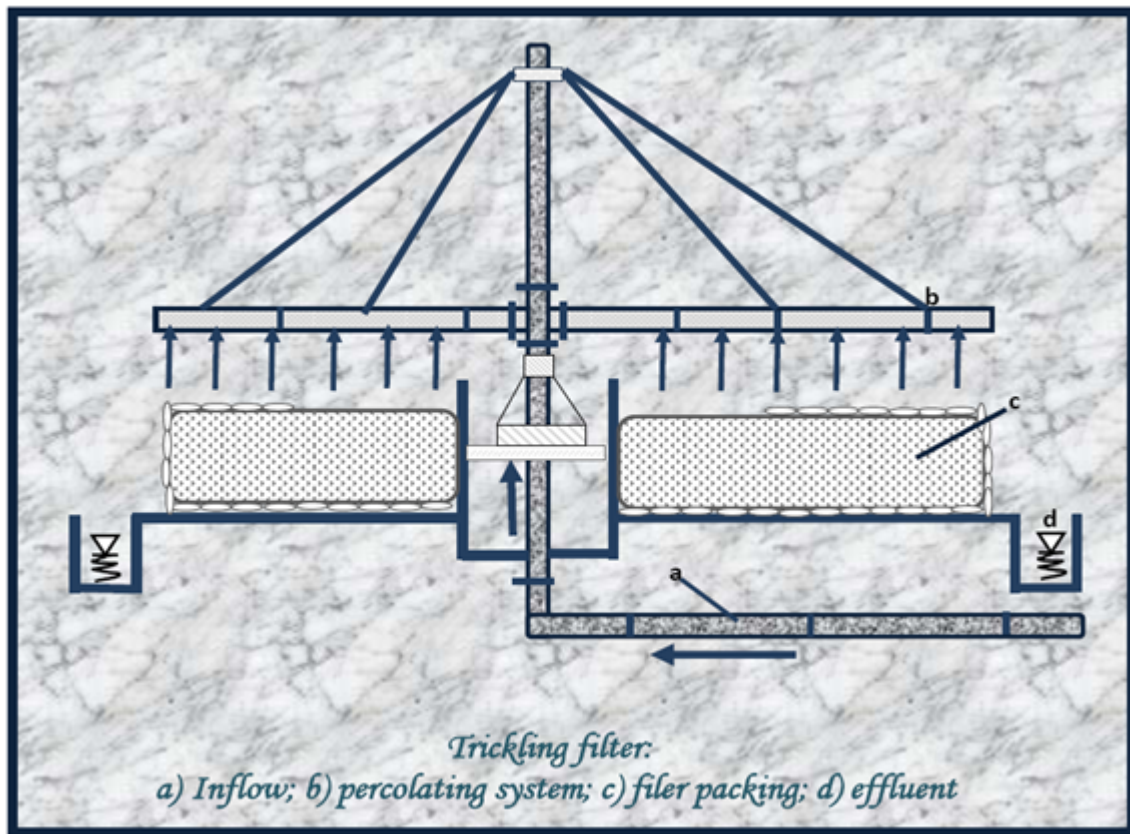
Trickling filter:
a) Inflow; b) percolating system; c) filer packing; d) effluent

Fig. 32.2 Trickling filters

A mucous biological film forms upon the content of the bed. The film is composed of microorganisms such as: bacteria, protozoa and fungi. The role of the filter involves a constant supply of sewage and its flow through the trickling filter while maintaining contact with the biological film. During the flow, the sewage undergoes mineralization as a result of aerobic decomposition by microorganisms. The biological film is initially composed of zoogleal bacteria which produce mucous sheets. With time, the composition of species of the mucous membrane changes due to their succession. Besides bacteria, the following appear: fungi, protozoa, annelida and fly larvae. Depending on the amount of treated sewage, the trickling filters may be subdivided into percolating and flushing filters.

The trickling filter process
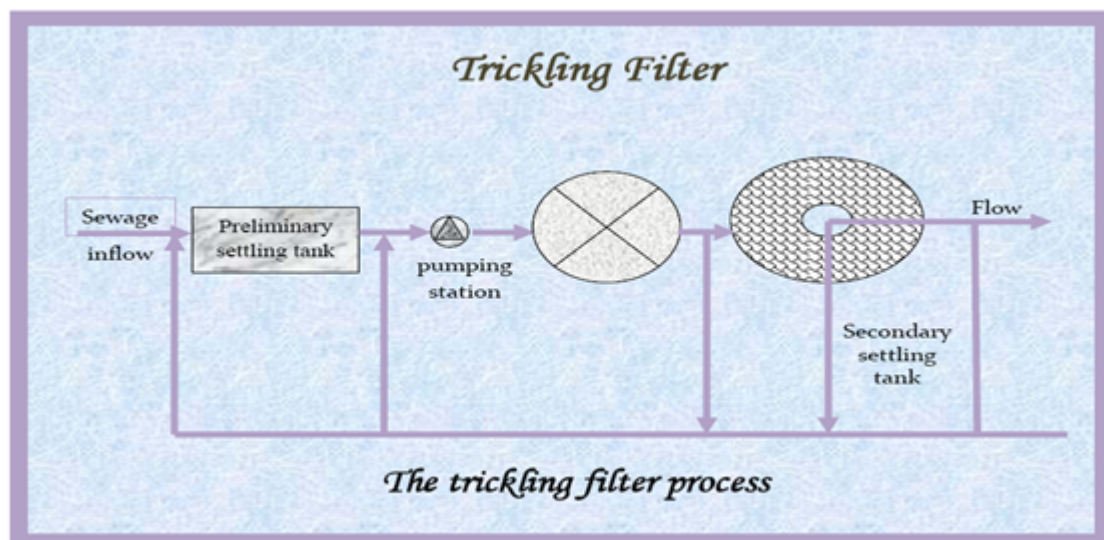
Fig. 32.3 Filter process

Depending on the amount of the organic load the following types of biofilters are distinguished

- Low loaded may be filled with natural or artificial material. The supplied organic material is less than 0.4 kg $BZT5/m^3 \times d$. In percolating filters the film is more developed and the biological process of decomposition is almost complete. In the final phase of purification, intensive processes of nitrification occur, which lead to an increase of nitrates in a run-off to the secondary settling tank.
- Mid-loaded are filled with natural-synthetic material and work with a load between 0.4-0.65 kg $BZT5/m^3 \times d$. In order to ensure an adequate concentration of the supplied sewage the recirculation of part of the purified sewage is utilized with this type of filters. The reduction of organic compounds upon these filters is adequate, and the processes of nitrification partially occur. The introduction of additional processes of purification is not necessary.
- High loaded (flushed) are filled with natural-synthetic material, the filter is loaded with: 0.65-1.6kg $BZT5/m^3 \times d$. In flushing filters, the intensity of sewage flow is greater; however the biofilm is composed almost entirely of bacteria and does not develop as much as in the above stated case. Flowing sewage washes out used and dead biological material from the filter. The washed out material is transported in the form of flocy sediment. Only a partial mineralization of organic compounds occurs on that type of filter and the nitrification process is inhibited. A low content of nitrates in effluent from filters testifies to partial mineralization of organic compounds. In complex systems, after these types of filters, re-purification is utilized, as the quality of the purified sewage does not usually meet the required standards.

32.6.2 Activated sludge

The process of activated sludge relies on sewage purification by freely suspended matter. It consists of producing 50-100 μm flocs with highly developed surface areas. The floc is made up of brown or beige mineral nucleus, while on its surface it contains heterotrophic bacteria within the mucous envelopes. The method of activated sludge requires delivery of oxygen into the substrate for bio-oxidation of organic pollutants, which should be >0.5 mg/dm3 in order to ensure proper oxygen conditions for the bacteria.

32.6.2.1 Activated sludge characteristics

Activated sludge is a type of flocculent suspended matter created during the aeration of sewage. Treating sewage with activated sludge consists of mineralization of organic compounds, conducted mainly by bacteria and following the same biochemical processes as observed in self-purification. However, the speed of the process is much greater. These results from the fact that the conditions of intensive aeration, triggered during sewage flow through aeration tanks are conducive to the development of impurity-decomposing bacteria.

Agglomeraters (flocs) which consist of heterotrophic bacteria coagulated with mucous, form during the process of aeration in aeration tanks (flocculation). The floccules absorb impurities contained in sewage, whereas microorganisms in floc decompose the absorbed substances. Activated sludge has

a spongy, loose structure, made of small openings of various shapes. Undisturbed floccules easily settle and thus allow the separation of the activated sludge from sewage.

Biocenosis of activated sludge is, for the most part, composed of heterotrophic bacteria.In small percentages - and only under particular conditions and in some arrangements - it's made up of chemolithotrophic bacteria, especially nitrifying bacteria. The most common species of activated sludge are: Zooglea ramigera, Pseudomonas fluorescens, Pseudomonas putida as well as bacteria of Achromobacter, Bacillus, Flavobacterium and Alcaligenes genera. The process of selection occurs naturally. The conditions in aeration tank, especially the chemical composition, pH-value and air conditions, are the determining factors for the diversity of the bacterial complex standards.

In unfavorable conditions (overloading of aeration tanks with easily available substrates, high oxygen deficit) excess development of flocs occurs causing the so-called active-sludge swelling. There are two distinguishable types of swelling: fibrous and non-fibrous swelling.

Fibrous swelling is caused by excess filiform bacteria (Sphaerotilus natans, Beggiatoa alba or Thiothrix nivea) or fungi development. Non-fibrous swelling is caused by bacterial development, which produces excess amounts of mucous.

Active sludge biocenosis is made up of not only bacteria but also protozoa, nematodes and rotifers. Even though these microorganisms do not play a major role, their presence is equally important. Protozoa feed upon bacterial cells forcing them to reproduce quickly, which essentially make them an important renewal and reactivating factor of the activated sludge.

The most common protozoa are: Vorticella, Carchesium and Opercularia as well as Anthophysa, Oxytricha,Stylonychia and Lionotus. There is an inverse relationship between flagellates and ciliates within activated sludge. While a large number of flagellates indicate an overload of sludge, the presence of ciliates goes to show it is functioning properly. During the course of sewage purification with activated sludge a characteristic succession of biocenosis is observed.

32.6.2.2 Activated sludge process (Fig. 32.4)

Sewage is directed to aeration tanks filled with activated sludge (thick suspension of microorganisms) after its mechanical purification. The content of the aeration tank is constantly aerated in order to provide an adequate amount of oxygen, to keep the activated sludge in a suspended state and to ensure its constant mixing. The aeration tank is a device, in which the development of the activated sludge results from continuous cultivation. There is a state of equilibrium between the rates of sewage inflow, concentration of nutrients, bacterial reproductive rate, and the rate of the sewage outflow containing some activated sludge in it. During the time of contact of sewage with the activated sludge, the decomposition processes occurring simultaneously enable the development of activated sludge biomass. Separation of purified sewage is done in a secondary settlement tank. Both sedimentation and clarification of the purified sewage, which is then carried off to a receiving body of water, occurs in the device. Activated sludge may be used again for purification; it is then recycled into the aeration chamber. However, quite often, before reuse, the sludge is directed to a regenerative chamber, where it is aerated in order to bring back its particular physiological properties. When the sludge collected in a secondary settling tank is not

recycled, then, as an excess sludge, it is removed and subjected to additional processing.
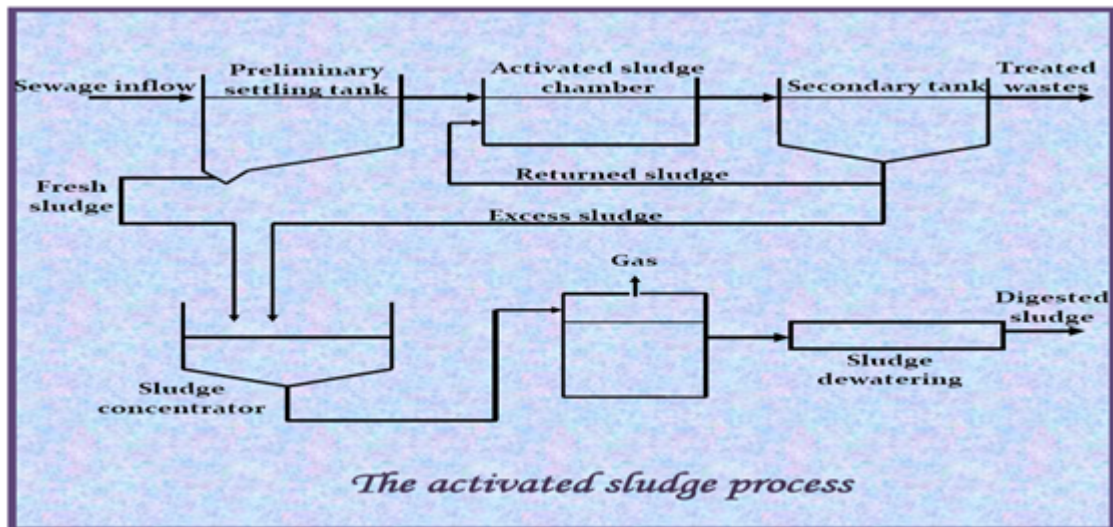
Fig. 32.4 Activated sludge method

32.7 Chemical Wastewater Treatment

Purification of industrial sewage that contains mineral and organic compounds, and heavy metals, utilizes physical-chemical and chemical methods. They include the following processes: neutralization, coagulation, oxidation, reduction, sorption, flotation, membrane processes, extraction, electrolysis, distillation.

32.7.1 Neutralization

It is a process of chemical neutralization of sewage in relation to the pH. Depending on the make-up of sewage and the type of the reacting substance used, neutralization may be accompanied by a chemical process of precipitation and coprecipitation. Neutralization may be conducted by mixing acidic sewage with bases. Hydroxides are substances most often used in the process of neutralization: NaOH in the form of 20-30% solution, $Ca(OH)_2$ in the form of 5-15% milk of lime, Na_2CO_3 in solution form $CaCO_3$, $MgCO_3$, MgO, dolomite in the form of a grainy filter. Mineral acids are used for the neutralization of basic sewage: H_2SO_4, HCl, H_3PO_4 in the form of solutions as well as CO_2 in the form of a clear gas.

32.7.2 Coagulation

Coagulation is a process of binding colloidal particles and the suspension into clusters of particles called the agglomerates, which results in precipitation of the sediment in the form of coagulate. The factors which most often cause coagulation are: addition of an electrolyte solution to lower the electrolytic potential, addition of colloids of an opposite charge into the colloidal particles, creation of metal hydroxides that absorb ions, colloids and suspensions.

32.7.3 Oxidation

An oxidation process is conducted in order to remove organic compounds, non-organic compounds and microorganisms from sewage. The reacting substances used in oxidation are: chlorine, chlorine oxidizing compounds; NaOCl, CaOCl$_2$, chlorinated lime, chlorine dioxide, ozone.

32.7.4 Reduction

The process of reduction used in sewage purification mainly concerns chromium. Chromium salts (VI) are toxic, carcinogenic, bacteriocidal and are irritants to skin. Its bacteriocidal properties slow down the process of water self-purification.

Reduction of chrome from oxidation state of 6^+ down to 3^+ is conducted through reduction and precipitation of hydroxide, which belongs to a group of barely soluble compounds. Reduction is conducted either chemically or electrochemically.

32.7.5 Sorption

Sorption consists of binding liquid soluble substances to the surface of solids. Depending on the characteristics of the process it may be irreversible (chemiosorption), or reversible - adsorption. The characteristic of the process of sorption is determined by one of the components of force:

- Physical sorption - the result of van der Waals forces
- Chemical sorption - the result of valence forces
- Ion sorption - between groups of cations and anions in the structure of the substrate
- Sieve sorption - at the molecular level according to the mechanism of a molecular sieve

32.7.6 Flotation

A process of structural separation consisting of raising the hydrophobic impurities into the foam along with the rising gas bubbles. As a result, the foam formed has a much higher concentration of pollutants than the rest of the sewage.

32.7.7 Membrane processes

These processes consist of separation of particles by flowing through a porous layer (membrane). The following are the types of membrane processes: reversed osmosis, nanofiltration, ultrafiltration, electrodialysis.

32.7.8 Extraction

This consists of transfer of components from one phase of the solution into the second liquid phase (dissolvent). Consequently a solution of the component in the dissolvent is obtained. The required condition for the process is the presence of two liquid phases.

32.7.9 Electrolysis

It is the process in which electrical energy invokes chemical changes of the electrolyte. As a result

of the electrical field the movement of ions toward the electrodes (upon which the process occurs) occurs

- Cathode $Me^{++} e^- \rightarrow Me$ (reduction)
- Anode $X^- \rightarrow X + e^-$ (oxidation)

32.7.10 Distillation

Process that utilizes the difference between the composition of a liquid and vapor in the state of equilibrium.

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