1 Understanding the Small World: The Microbes

Pompee Chanda and S. R. Joshi

Abstract

One cannot see the microbes which, in fact, are the leading entity of life on Earth. Other than their widespread occurrence in natural habitats such as the soil and the aquatic environments, microbes also thrive in extreme environments like arctic waters and hot springs. They add up to half of the world's biomass. The natural habitats are the source of microbes. They inhabited these habitats billions of years ago even before the humans came to their existence and are expected to remain and dominate the Earth even after humans are gone. Without their presence, life, as we know it, would not have been possible. Major part of our body mass contains more bacterial cells as compared to cells in the human body. Undeniably, the relationship between microbes and humans is delicate and intricate. Understanding the small world—the microbes—provides a clear and accessible introduction on how microbes impact our lives and are involved both in good health and sickness through their benefit and harm. This chapter provides content on how microbes are isolated and cultured, and strain improvement techniques are undertaken to extract useful or novel products which have extensive applications in biotechnology industry. This interactive and engaging chapter provides the basics of microbiology, in a contemporary context. It will be equally useful in the fields across the biological, environmental and health sciences, and for the interested reader wanting to explore more about these ubiquitous microbes.

Keywords

Microbes · Habitats · Ubiquitous · Distribution · Applications

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Abbreviations

70S	70 Svedberg units
80S	80 Svedberg units
AFM	Atomic force microscope
AIDS	Acquired immunodeficiency syndrome
ASBVd	*Avocado sunblotch viroid*
BSE	Bovine spongiform encephalopathy
CCCVd	*Coconut cadang-cadang viroid*
CEVd	*Citrus exocortis viroid*
cfu	Colony-forming units
CJD	Creutzfeldt-Jakob disease
COVID	Coronavirus disease
CPrP	Cellular prion protein
CSVd	*Chrysanthemum stunt viroid*
DICM	Differential interference contrast microscopy
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
EM	Electron microscope
GI	Gastrointestinal
GPI	Glycosyl phosphatidyl inositol
HDV	Hepatitis delta virus
HMP	Human Microbiome Project
HSV-1	Herpes simplex virus type 1
ICTV	International Committee on Taxonomy of Viruses
IR	Infrared
mBRC	Microbial biological resource centre
MERS	Middle East respiratory syndrome
MPN	Most probable number
mRNA	Messenger ribonucleic acid
NMR	Nuclear magnetic resonance
PAMP	Pathogen-associated molecular pattern
PSTV	Potato spindle tuber viroid
RNA	Ribonucleic acid
SARS	Severe acute respiratory syndrome
SEM	Scanning electron microscopy
SPM	Scanning probe microscopy
STM	Scanning tunnelling microscope
TEM	Transmission electron microscopy
TMAO	Trimethylamine N-oxide
TMD	Tobacco mosaic disease
TPMVd	Tomato planta macho viroid

1.1 Introduction to Microbiology and Microbes

Microbiology is the study of the structure, activity, and categorization of bacteria with the goal of manipulating and regulating their behaviour (Gross et al. 1995). It all began with Antonie van Leeuwenhoek's pioneering study on microbes in the seventeenth century, which he conducted with a self-designed microscope (Gest 2004). A microorganism can be categorized into two groups—one that contains cells termed as cellular and the other without cells termed as acellular. The first category of microbes comprises bacteria, archaea, fungi and protists. The protists belong to the kingdom Protista. Algae, protozoa, slime moulds, and water moulds are among the microbes that belong to the kingdom. This type of microbes can be either single celled (unicellular) comprising the complete organism, or they can be aggregates of many number of cells which usually range from hundreds to billions of cells (multicellular), which constitute the entire organism. Microbes such as viruses, prions, and viroids, are found in the acellular category and are considered infectious agents (Pepper et al. 2014). Our everyday existence is intertwined inseparably with microbes. Microbes not only inhabit the human body; they are also found to thrive in soil, in hot springs, in deep oceans, in high altitude atmosphere and also deep in rock bed (Orcutt et al. 2011). Microbes are invisible, yet seem to be abundant on Earth; they provide sufficient evidence of its existence in both negative and positive ways. They can cause spoilage when they cause decomposition and diseases. And at the same time of various advantages to mankind., which include its application in various industries such as fermentation, food, pharmaceuticals, etc. (Sharma et al. 2020). Most importantly, microbes are vital in maintaining the ecological balance of the Earth. They decompose animal and plant remnants and convert them to biodegradable and recyclable substances. Thus, they have an incalculable contribution to the Earth's biogeochemical cycle—the carbon cycle and the nitrogen cycle (Rousk and Bengtson 2014).

1.1.1 Microbes

The term *microbe* was used for the first time in the late nineteenth century to describe all the invisible life forms existing on Earth. Professor Charles Sédillot (1804–1883) the pioneer of modern medicine, surgery, anaesthesiology, histopathology and infectiology was the one to first use the term microbe. Long before the explanation on infections was put forth by Semmelweis (1818–1865), Professor Charles Sédillot predicted the existence of microbes and understood their action mechanism, which he found to develop as postoperative infections (Billmann 2012). As microbiology in due course of time developed into a specialized field of study in biology, it was discovered that microbes are congregation of varied organisms which are microscopic, suggesting the use of an external support, the microscope, in order to see them. Of course there are exceptions. Some unicellular organisms studied by microbiologists are macroscopic. These include *Valonia ventricosa* (Guiry and Guiry 2021), for example, can grow up to 5 cm in length. They're microscopic

algae that go by the names “bubble algae” and “sea pearl.” Each bubble is made up of a single cell and is considered one of the world’s largest single-celled organisms. They are unique sorts of algae species that are widely distributed and have the ability to survive low light. They live in mangrove forests and are considered pests because they grow on their roots. *Thiomargarita namibiensis*, another variety of macroscopic organism with diameters ranging from 100 to 750 micrometer (Schulz and de Beer 2002), was also discovered.

1.1.2 The History of Uncovering the Mystery of Microorganisms

The existence of microbes was first reported during the late sixteenth century by two most distinguished fellows of the Royal Society, Robert Hooke and Antonie van Leeuwenhoek. Antonie van Leeuwenhoek, a cloth draper by profession was the first to watch microbes, with the aid of his self-designed microscope, and thus made one of the most remarkable contributions in the field of biological sciences. Seemingly, he found pleasure in screening of microbes in samples from different sources such as water from the pond, faecal wastes, scrapings from the teeth, etc. Antonie van Leeuwenhoek made comprehensive diagrammatic illustrations to document his findings in detail. Which was later conveyed to the scientific society of that time the Royal Society of London. Robert Hooke published a book *Micrographia* in the year 1665. In this book for the first time, Robert Hooke formally introduced his published illustration of a microorganism, which is a microfungus termed as *Mucor*. Such breakthrough discoveries led to the conception and using of simple microscopes that could magnify objects from about 25- to 250-fold. As a result, about a century and a half after its discovery, the microscope has become the most significant aspect of human consciousness for comprehending the role that bacteria played in generating infections in many life forms and organisms (Gest 2004). Other eminent scientists also made their contributions to microbiology.

- Lazzaro Spallanzani, an Italian physiologist and biologist, was not far behind in contributing towards the investigation of microbes. His detailed research on biogenesis debunked the hypothesis of spontaneous generation. He discovered that boiling the broth sterilised it and killed any germs contained in it, but that leaving it out in the open may lead to infection (Mancini et al. 2007). His findings paved the way for the future research of Louis Pasteur who had ultimately put a dead end to the doctrine of biogenesis and supported germ theory instead (Sharma et al. 2020). Thus, with the discoveries of Van Leeuwenhoek, Lazzaro Spallanzani, and others, the hypothesised notion that life arose spontaneously from non-living objects during the decay process was disproved.
- Ferdinand Julius Cohn, a biologist from Germany and a pioneer in developmental biology of lower plants, was the first to categorize algae as plants and to give explanation as to what property discriminates algae from green plants. He significantly encouraged the taxonomical and physiological classification of bacteria. He divided the bacteria into four different groups on the basis of shapes, namely,

spherical (coccus), rod-shaped (bacillus), comma-shaped (vibrio) and spiral (spirilla). This type of bacterial nomenclature is being used till date. Also Cohn has proposed that Bacillus has the ability to change its state from vegetative form to an endospore when subjected to an unfavourable environment (Drews 2000).

- A Prussian physician and microbiologist Heinrich Hermann Robert Koch, the pioneer of contemporary bacterial study, was the first one to identify the infectious agent behind the diseases like tuberculosis, cholera and anthrax which killed humans as well as animals in the past. He also provided the experimental evidence for the same. He later established Koch's postulates, which consist of a sequence of four universal principles that states specific organisms are causing specific diseases (Segre 2013). Koch was the recipient of the Nobel Prize in Physiology or Medicine in the year 1905. The Robert Koch Institute is named to honour his contribution in the study of bacteriology.

1.1.3 Types of Microorganisms

Microbiology came into being with the studies and investigations of bacteria. As already stated the research of Louis Pasteur and other scientists in the late eighteenth century helped in establishing the fact on how important microbes are in our lives. It was due to their unwavering effort which led to the invention of new techniques for the thorough examination of bacteria using microscopes. However, the technique was later modified to study other microbes other than bacteria, therefore the expansion of bacteriology to microbiology. Microorganisms can exist as unicellular, multicellular or cell constellations. They can be divided into six major types: bacteria, archaea, fungi, protozoa, algae, slime mould and viruses.

1.1.4 The Two Types of Cells: The Eukaryotes and Prokaryotes

Microscopic examination of microbial cell has affirmed the existence of two types of cellular structure: larger cells having complex interior and smaller cells with simple internal organization. Larger cells are known as eukaryotes and the smaller cells are known as prokaryotes. At the ultrastructure level, eukaryotes have an internal structure composed of membranes that divide the cytoplasm into a number of different compartments such as nucleus containing DNA molecule wrapped up in filamentous or thread-like structures termed as chromosomes, whereas prokaryotes lack proper membrane-bound nucleus. The DNA of the prokaryotes is scattered as a long, folded strand randomly within the cell. Despite being small and simple, prokaryotes are considered to be highly organized structure, though the organization is at molecular level and not easily visible through the naked eyes. Prokaryotes grow rapidly by utilizing the nutrients dissolved in the liquid surrounding them. Thus prokaryotes have evolved with a permeable structure, highly efficient and having tightly regulated metabolic system to support their survival. On the other hand eukaryotes depend on other species for their survival. They are predators. Example

of such eukaryote are protozoans. Protozoans are free-living single-celled eukaryotes. They live by feeding on smaller prokaryotes for which they occupy the higher hierarchical level in the ecosystem. As such their size has increased and has become more complex in their structure, and over the years they have evolved into multicellular species like algae, fungi and plants (Wheelis 2008). Together algae, protozoa and some lower fungi are grouped as protists (Corliss 1984).

1.1.5 The Three Lineages of Microbial Life

Life forms on Earth are thought to have developed from a common ancestor roughly 3.8 billion years ago. It is thought that the first organism to exist was prokaryotic in nature, with a much simpler structure, followed by the current prokaryotes, which endured hard settings such as hot springs and oxygen deficiency, and had to split into three lineages to adapt further to such circumstances. Accordingly, they were divided into three groups: eubacteria, archaebacteria, and eukaryotes. Approximately around 20 sublineages of plant and animals have been added as new members of the microbial family (Wheelis 2008, Fig. 1.1).

1.1.6 The Prokaryotes (Bacteria)

Bacteria or eubacteria or "true" bacteria form the kingdom (Woese and Fox 1977). They belong to the kingdom Monera. All members of the Monera kingdom are prokaryotes without any nucleus or organelles. Bacteria are unicellular organisms, present everywhere in the biosphere (air, soil, water). They are found in extreme variety of habitats such as boiling thermal springs or in ponds which are either acidic

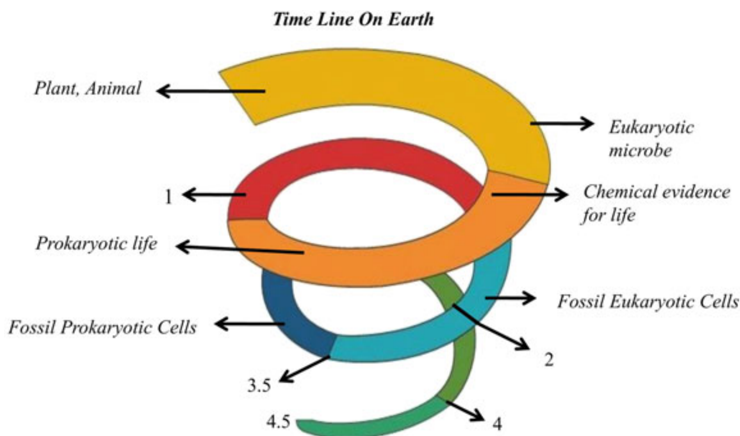

Fig. 1.1 Timeline of life on Earth. The numbers in the figure depict years in billion. (Adopted and modified from Wheelis 2008)

or alkaline. Bacteria have specialized cellular structure. Each bacteria is encased in a rigid cell wall formed of peptidoglycan, a protein-sugar (polysaccharide) polymer. The cell wall protects the cytoplasmic membrane from the environment and provides the cell its form. The cytoplasm, or protoplasm, of bacterial cells is where cell growth, metabolism, and replication take place. It's a gel-like matrix that contains ribosomes, chromosomes, and plasmids (it is distinct from the bacterial chromosome and capable of replication independently). The cell envelope encases the cytoplasm and all of its constituents. Although it is not contained in the nucleoid, the chromosome, which is a single continuous strand of DNA, is positioned there. The cytoplasm is populated with the rest of the cellular components. Some bacteria may have a capsule which is an additional layer that gives protection and have flagella for movement. Bacterial cells can exist in different shapes. They are divided into five types based on their basic shapes: spherical (cocci), rod (bacilli), spiral (spirilla), comma (vibrios), and corkscrew (corkscrew bacteria) (spirochaetes) (Fig. 1.2). Bacteria have many special characteristics. Bacteria have unique way of reproducing. They multiply by the process of binary fission. Bacteria have properties that classify them into different categories. One such feature is the structure of cell wall which classifies them into two groups—gram-positive bacteria and gram-negative bacteria. Gram-positive bacteria have a thick layer of peptidoglycan and lack outer lipid membrane, while gram-negative bacteria have a thin peptidoglycan layer surrounded by lipid membrane. Based on this property, the bacterial cells decide how they take up the stain, hence the names gram-positive bacteria and gram-negative bacteria. Bacteria can be further classified based on how they respond to the

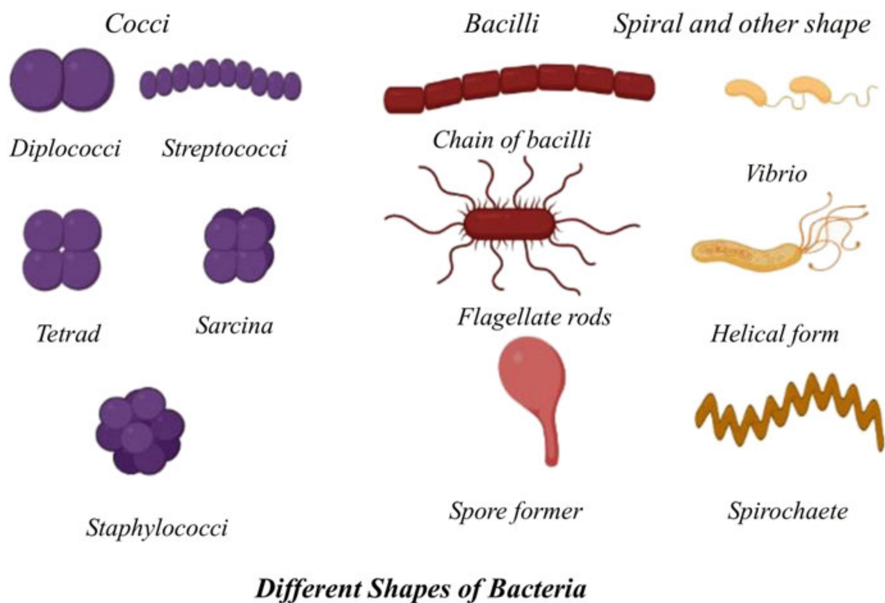

Fig. 1.2 Different shapes of bacteria

presence or absence of atmospheric oxygen into the following groups: They can be aerobic surviving with the available oxygen, anaerobic surviving without oxygen and the one which can live both in presence and absence of oxygen called facultative anaerobe. Bacteria can be further divided on their mode of nutrition. They can be classified as heterotrophs or autotrophs. Autotrophs are able to make their own food by either utilizing the energy from the sun termed as photosynthetic bacteria or using chemical energy, in which case they are called chemosynthetic bacteria. Furthermore according to their habitat, source of food and their association with other organisms, heterotrophic bacteria can be saprophytic which feed on decaying organic matter or parasitic, living on other organisms. They may further be categorized according to the type of environment they live in. Hyperthermophiles grow optimally between temperatures above 80 °C and higher (Uehara et al. 2017), and microbes that can survive extremes of pH are grouped as either acidophiles, which grow optimally below pH 3, or alkaliphiles, organisms that grow at an optimum pH between 8 and 10 (Tiquia-Arashiro and Rodrigues 2016).

1.1.7 The Prokaryote (Archaea)

Archaea form the considerable part of the microbial biomass on Earth. Around the late 1970s, it was reported, on the basis of rRNA phylogenetic studies, that archaea (archaebacteria) were recognized as a separate domain of biological life in addition to bacteria (eubacteria) and eukaryotes. Archaea exhibit a huge variety of lifestyles and metabolic activities. Many archaeal species defy all odds to tolerate the harshest condition from extreme salinity to high temperatures such as hot water vents, hot springs of the land and extreme saline, acidic and anaerobic situations (Schäfer et al. 1999). For example, *Pyrolobus fumarii can stay alive in* hydrothermal vents *at* a maximum of 113 °C (235 °F) temperature. *Picrophilus*, an acidophilic species, is found in the acidic hot springs and dry hot soil in Hokkaido (Japan) capable of survival around pH of −0.06 (Schulze-Makuch and Irwin 2008). Methanogens are anaerobic microorganisms that create methane as a metabolic byproduct. They live in wetlands, where they create marsh gas, and marine sediments, where they make methane (Kristjansson et al. 1982), as well as other severe environments such as hot springs, subsurface hydrothermal vents, and the Earth's crust. They are also responsible for the presence of methane in the digestive tracts of ruminant animals and humans, causing ruminant burping and human flatulence (Lengeler et al. 1999). Though archaea have some characteristics in common with bacteria, they also have several characteristics that set them apart (Niederberger 2020). In archaea, unique ion-transduction processes, the presence of protein complexes in membranes, and the application of novel cofactors in bioenergetics of methanogenesis have all been discovered (Schäfer et al. 1999). Archaea are critical to maintaining the Earth's geochemical cycles, such as the carbon and nitrogen cycles (Offre et al. 2013). Some archaea, such as Thaumarchaeota, are engaged in the nitrogen cycle's conversion of ammonia to nitrite (Kimble et al. 2018). They actively contribute to greenhouse gas emissions (Welte 2018).

1.1.8 The Eukaryotes (Algae)

Algae constitutes a consortium of different primordial plants which comprise diverse evolutionary lineages and are photoautotrophic in nature. All the different groups are of polyphyletic origin and represent the majority types of existing plants. Algae are thallophytes. Their vegetative body unlike plants does not differentiate into roots, stems or leaves. They are non-vascular, photosynthetic and chlorophyll-bearing organisms. Many algae live in lonely cells, primordial plant bodies. Algae are cryptogams that proliferate by using secret reproduction plan. They are mostly inhabitants of humid and aquatic environment (Likens 2009). Through the evolution, algae have formed a number of symbiotic relationship with different types of organisms ranging from other microbes to animals and have integrated their chloroplasts into other eukaryotes. Green algae show many complexity in their organization; they can be both unicellular and multicellular thalli having the length around 120 m. Algae also comes in different shapes. Algae which are single-celled can be spherical, rod-shaped, club-shaped or spindle-shaped. Some algae are motile in nature. They have wide variety of applications. Commercially, they are employed as feed as well as in the food and pharmaceutical industries (Lewis et al. 2011; Pelczar 2020).

1.1.9 The Eukaryotes (Fungi)

Fungi are a branched mass of tubular filaments surrounded by a solid cell wall. The thallus, or undifferentiated section of a typical fungus, is made up of hyphae, which are filament-like structures that create a branched and repeating network termed the mycelium. Organelles and nuclei of the fungi are membrane-bound. Because fungus lacks chlorophyll, it is unable to perform photosynthesis. Fungi, like animals, are heterotrophs, meaning they get their nourishment by absorbing dissolved chemicals and secreting digestive enzymes into their surroundings. The growth of the mycelium depends on the nutrients from the surrounding environment, and at maturity they form reproductive cells called spores. Fungi are eukaryotic organisms that include microbes like yeast, rusts, smuts, mildews and moulds, as well as the mushrooms. There are certain species which are fungi like. They are slime moulds and oomycetes (water moulds). Fungi are both microscopic and macroscopic in size. Macroscopic fungi include mushrooms and bracket fungi that are found to grow in places which are damp. Multicellular fungi include filamentous structure such as moulds, and unicellular fungi include yeasts. Moulds have cylindrical cells that bind from one end to the other to form a thread-like filamentous structure called hyphae which again intricately intertwine to form mycelia. Yeasts on the other hand may be spherical or oval-shaped to filamentous. Though fungi are free-living terrestrial and aquatic bodies, they still can be in symbiotic relationships with other flora and fauna. The mode of nutrition of fungi is heterotrophic as mentioned above. When fungi feed upon a living plant or animal, they are known as biotrophs, and when they obtain their nutrients from dead plants or animals, they are known as saprotrophs

(saprophytes, saprobes). They help in the breakdown of organic compound to release carbon, oxygen, nitrogen and phosphorus into the atmosphere. When a fungi feed on a host by killing, it is known as necrotrophs. Fungi have many important commercial applications also (Hyde et al. 2019). Fungi are used in industry to raise bread dough and produce wine, beer and cheese by fermentation. Some fungal species such as mushroom and Fusarium *venenatum* are used as food grade protein source (González et al. 2020). The latter produces a protein known as mycoprotein (Gilani and Lee 2003). Also fungi are not far behind in contributing to medicine. The discovery of penicillin by Alexander Fleming was a breakthrough in the field of medical history (Tan and Tatsumura 2015).

1.1.10 The Eukaryote (Protozoa)

All protozoans have a membrane-bound nucleus. They are nonfilamentous and inhabit moist or aquatic environments. They are found everywhere from South Pole to the North Pole. Cell size of protozoans ranges in size from 1 μm up to 2000 μm in diameter. They have both symbiotic and parasitic relationship. Protozoans are complex in their internal structure and therefore carry out complex metabolic processes. Protozoa have different structures which are used for their movement. For the process protozoans possess flagella, cilia or pseudopodia. Protozoans may have different shapes such as oval, spherical and elongated during their different phases of life cycle. One such stage is the trophozoite stage. This is the parasitic stage of protozoans that feed and reproduce vigorously, and when it has a tough membrane surrounding, it is known as cysts. They reproduce asexually by binary fission. All parasitic protozoans have holozoic mode of nutrition (depend on preformed organic substances). Protozoans are agriculturally important species. They feed on soil bacteria and enhance the fertility of soil. They also help bacteria in decomposing dead organic matter at a faster rate. Protozoans produce nitrogen as ammonium and phosphorus as orthophosphate as a metabolic byproduct, which helps in plant growth. Protozoans also have an indispensable role in wastewater treatment. Some commonly known protozoans include members like dinoflagellates, amoebas, paramecia and the malaria-causing *Plasmodium* (Diaz et al. 2019).

1.1.11 The Eukaryote (Viruses)

A virus is a miniature compilation of genetic code, either DNA or RNA, enclosed in a protein cover called capsid. It is submicroscopic particle not visible under a microscope. Their genetic material may be single or double stranded. A virus cannot reproduce on its own. It must infect cells and make use of replication system of the host cell it infects, to make copies of themselves. Often, in the process they kill the cell and cause harm to the host. An entire infectious virus particle is known as a virion. Viruses are everywhere. They have been discovered to be in greater abundance than other microorganisms (Lodish et al. 2000). Viruses are difficult to

eradicate. Antibiotics are ineffective against viruses because they lack the same machinery as bacteria; antiviral drugs or vaccinations, on the other hand, can eradicate or reduce the severity of viral infections, which includes AIDS, measles, smallpox, SARS-CoV (severe acute respiratory syndrome coronavirus), MERS-CoV (Middle East respiratory syndrome coronavirus), chikungunya virus, Ebola virus, Zika virus (Colson and Raoult 2016) and the present Covid-19. More details of virus are discussed in later section of this chapter.

1.2 How We Look at the Small World of Microbes

The use of light to observe the little and unseen world which otherwise is difficult to see with the human eye has become prominent in recent ages. The discovery of the microscope has been a breakthrough in this, to observe and study the different aspects of microbial cells. The use of microscopy techniques has enabled us to manipulate the different aspects of the cells like the cell colour and cell size that helps us to determine the specific type of an organism and also make a diagnosis about the diseases caused by them (Fleming 2019). To do so we need to have a clear understanding of the features that differentiate the prokaryotes from the eukaryotes. Also, this section travels to the period when and how various types of microscopes were discovered to use light as an insight into the invisible world of microbes. With the understanding of how different types of microscopes work, scientists can now explore microbes in depth by studying their images that may prove to be useful for both research work and clinical diagnosis. Microscopy and its working principles are better understood from their historical perspectives. Girolamo Fracastoro an Italian scholar, in his book *De contagione et contagiosis morbis* (1546), officially put forwards a postulate that diseases were caused by small invisible seminaria, or “seeds of the contagion”, which could be transmitted through any possible types of contact (Iommi Echeverría 2010). However, during his time, technology for viewing tiny objects was not at an advanced stage; therefore the existence of the seminaria remained hypothetical for long—an invisible world remained unexplored till the discovery of microscope. In principle, it is believed that Girolamo Fracastoro was the first one to propose the germ theory even before Louis Pasteur and Robert Koch.

1.2.1 Difference Between Prokaryotes and Eukaryotes

The distinction between prokaryotes and eukaryotes gives us a clear picture of the different features in every group of organisms. Prokaryotes were the very first form of life on Earth. They appeared on Earth billions of years ago even before the eukaryotes came into existence. It is a well-accepted fact that differences in cell structural design between prokaryotes and eukaryotes are that prokaryotes do not possess membrane-bound organelles, whereas eukaryotes contain membrane-bound organelles including the nucleus (Fig. 1.3).

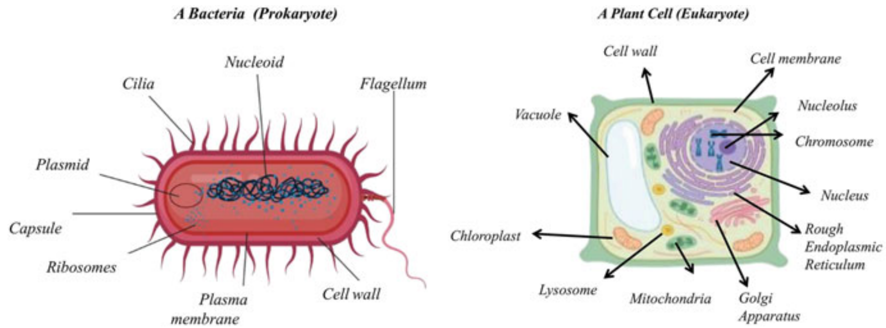

Fig. 1.3 Prokaryote and eukaryote

1.2.2 Microscopic Analysis of Microorganisms

Scientists made contributions in the field of microscopy to offer us images of small species that would otherwise have remained unknown, as described in the previous section. This may be seen in Robert Hooke's book Micrographia (1665), when he first described the structure of a cell by looking at a piece of cork under a compound microscope (Davidson 2010). We'll learn about the several types of microscopes used in the laboratory to investigate bacteria in this part.

1.2.2.1 Types of Microscopy

- *Light microscopy (bright-field microscopy)* is also popularly known as the compound light microscope. It is the basic microscope that is used in biology laboratories. It is an optical microscope that makes use of light rays to illuminate the specimen to produce a dark image against a bright background. This microscope is applied to observe fixed and live specimens, stained with basic stains producing a contrast between the image and the background. It has lenses that are modified versions of magnifying glasses that modify the specimen to produce an image to be seen through the eyepiece (Malacara 1988).
- *Darkfield microscopy* is used to create images of the live, unstained and transparent specimens which are high-contrast and high-resolution images. This microscopy offers an advantage as it uses live specimens which otherwise are killed by the use of stains. It is a bright-field microscope with a modified form of a condenser that makes use of cone-shaped hollow light. The condenser scatters light source causing it to reflect off the specimen at oblique angles making the specimen appear bright in contrast to a dark background (Omoto and Folwell 1999).
- *Phase-contrast microscopy* is an optical contrast method that allows unstained structures in biological specimens to be seen. It converts phase shifts in light to brightness in the image as it passes through the specimen. The structures of the specimen cause refraction and interference to create a high-contrast, high-resolution image of an unstained, transparent live sample. The image is formed by

altered wavelengths of light rays that pass through the specimen. For altering the wavelength of the light, an annular stop is fitted in the condenser. The annular stop produces a cone-shaped hollow light that is used to focus on the specimen. The organelles in eukaryotes and endospores in prokaryotes are best observed with phase-contrast microscopy (Zernike 1942; Barer 1947). Similar to phase-contrast microscopy, differential interference contrast microscopy (DICM) enhances the contrast of an image by using polarized light which converts phase shifts to light intensity changes. This produces an image of living organisms with a three-dimensional appearance making the technique useful in electrophysiological experiments. As an infrared (IR) light source of longer wavelength is generally used in DICM, therefore it is best suited for imaging thick specimens such as brain slices (Shribak et al. 2017).

- *Fluorescence microscopy* is an imaging technique. It visualizes the fluorescence emitted from the material to be analysed. The microscope transmits ultraviolet light of shorter wavelength, into the specimen; the fluorescent chromophores also called fluorochromes absorb the excited light and emit visible light with longer wavelengths that produces the image (Dallas 2020). The fluorochrome material is naturally occurring fluorescent substance, e.g. chlorophylls and fluorescent dyes which stain specimen creating the contrast. This technique is highly sensitive and provides information about the dynamic structure of polymers and nanomaterials where lights are adsorbed at liquid-liquid interfaces. Immunofluorescence, an important technique in fluorescence microscopy, is used to recognize pathogens by identifying antibodies bound to the pathogens if any (Sanderson et al. 2014).
- *Confocal microscopy* (also known as widefield fluorescence microscopy) views image by scanning and illuminating at different points of a sample using a pinhole (Rigby and Goldie 1999). It uses a laser light for fluorescence. It produces a high-resolution two-dimensional image which is converted to a three-dimensional image on a computer. This technique is frequently used in biomedical research to repair and fix damaged cells and to know about the various types of activities going inside a living cell. The confocal microscope has an advantage over other microscopes in that it can do thin optical sectioning of the tissues for viewing, and doesn't need any physical sectioning of the tissue (Rigby and Goldie 1999).
- *Two-photon fluorescence microscopy* is a breakthrough invention for imaging biological samples. It makes use of fluorochromes, infrared light of longer wavelength and scanning technique to visualize the specimens. The long-wavelength and low energy light allows photons to strike the same position in the specimen simultaneously exciting the fluorochrome. Viewing images with this microscope does less damage to biological specimens. The long-wavelength light examines the sample noninvasively from all dimensions with resolution less than a micrometre and enhanced diffusion depth. Two-photon microscopy is used to examine live cells in intact tissues such as the brain, embryos, complete organs and even animals (So et al. 2000; Helmchen and Denk 2005; Rosenegger et al. 2014).
- *Electron microscopy* (EM) An electron microscopy is a microscope that uses an accelerated electron beam as a light source. It allows researchers to view a

specimen as small as that of nanometre size. It uses short-wavelength electron beams with increased magnification and resolution (100,000×). EM is best used for viewing extremely minute characters of viruses, cell organelles, etc. (Savile Bradbury and Joy 2019). It is of two types:

  - Transmission electron microscopy (TEM) is a technique for studying the characteristics of extremely small specimens. TEM uses a high accelerated ray of electrons that passes through a very thin sample and gives an in-depth internal view of the specimen. TEM makes use of electrons that have a shorter wavelength than photons, thus enabling the formation of an image of higher magnification and resolution.
  - Scanning electron microscopy (SEM) creates an image by scanning a focussed electron beam across a surface. It is less powerful than TEM. SEM doesnot provide coloured image but creates black-and-white photographs of the specimen's surface (Golding et al. 2016).

- *Scanning probe microscopy* (SPM) is a relatively new method for examining structures at the molecular level. It is a conglomeration of related surface-sensitive techniques, applied to interrogate the specimen at an atomic scale with the aid of a sharp probe. This produces magnified images of up to 100,000,000 such that it allows viewing of every atom at an individual level. It uses no light source. It has the exceptional advantage, from a biological perspective. It not only allows high-resolution imaging of cellular structures below the optical limit but evaluates the micromechanical properties of the cell. It can examine cell dynamics and processes running even in real time in their most native physiological media such as aqueous solutions (Roberts et al. 1999).

  There are two types of scanning probe microscope:

  - *Scanning tunnelling microscope* (STM) produces an image by a fast-moving sharp metal wire tip over the surface of a specimen. The tip comes very near to the surface and applies an electrical voltage between the tip and the sample. The microscope scans every individual atom (Bottomley et al. 2005).
  - *Atomic force microscope* (AFM) works in the same way as STM. A sharp tip is scanned over a surface with a feedback loop to regulate the parameters desired to form the image of the surface. AFM uses atomic forces to record the interactions between the tip and surface of the sample to obtain the image (Bottomley et al. 2005).

1.3 Prokaryotes Diversity

The prokaryotes are the most abundant microorganism found on this Earth. Prokaryotes are the most diversified organisms, both metabolically and phylogenetically. Primarily prokaryotes were classified into single largest kingdom. However recent classification has divided prokaryotes into two categories, the bacteria and archaea, which believed to have evolved from distinct lineages (Oren

2004). Prokaryotic cells are small, single-celled and simple in architectural structure. Prokaryotic cells are surrounded by a plasma membrane and do not have membrane-bound organelles in their cytoplasm. And it is the absence of a nucleus and other membrane-bound organelles that differentiates prokaryotes from eukaryotes. Most prokaryotes contain a single chromosome bounded by a double-stranded circular DNA present in the nucleoid. The DNA in prokaryotes is present in the central area of the cell called the nucleoid. Prokaryotes have a circular DNA molecule called a plasmid, which is distinct from the chromosomal DNA and can replicate autonomously (Clark et al. 2019).

With the evolution going with time, archaea and the bacteria have split into many groups and species. Bacteria are divided into two major groups on their ability to retain stain: gram-positive bacteria and gram-negative bacteria. All the gram-positive bacteria belong to one phylum (Proteobacteria, Chlamydiae, Spirochaetes, Cyanobacteria), and the rest belong to gram-negative. The gram-staining method is named after its inventor, Danish scientist Hans Christian Gram (1853–1938) (Chinen et al. 2009). The property of different bacterial staining procedures is due to the presence or absence of a cell wall. Gram-positive bacteria lack the outer membrane, whereas the same is found in gram-negative bacteria. A major portion of the cell wall in gram-positive bacteria is composed of peptidoglycan layer, and the rest is made up of acidic substances called teichoic acid which covalently link the lipids in the plasma membrane to form the lipoteichoic acids. Lipoteichoic acids aid in anchoring the cell wall to the cell membrane. Gram-negative bacteria on the other hand have a thin cell wall composed of a few peptidoglycan layers bounded by an outer envelope of lipopolysaccharides (LPS) and lipoproteins.

The proteobacteria are further divided into alpha, beta, and epsilon groups. All of the animals in this category eat in a distinct way. Some members of the genus are photoautotrophic, some are symbiotic, and still others are human pathogens. *Rhizobium*, a nitrogen-fixing symbiont, *Rickettsia*, an internal obligatory parasite that causes diseases like typhus, *Helicobacter pylori*, which causes stomach ulcers, and *Salmonella typhi*, which causes food poisoning, are all members of this group.

The four other groups of bacteria are also diverse in their lifestyle (Ward 2002). Chlamydiae are pathogens that are intracellular obligate parasites found in animals causing sexually transmitted disease, e.g. *Chlamydia trachomatis*, while Cyanobacteria are photosynthetic bacteria, e.g. *Prochlorococcus*, that contribute half of the oxygen available on this planet. Spirochaetes include both free-living anaerobes which are harmless in nature and pathogenic ones, like the *Treponema pallidum* causative agent of syphilis. The gram-positive bacteria are also not behind in contributing to both health and diseases. They include antibiotic-producing bacteria *Streptomycin*, probiotic bacteria such as *Lactobacillus* sp. present in yogurt and *Bacillus anthracis* causing the disease anthrax.

1.3.1 Archaea

Archaea are single-celled creatures that belong to the Archaea domain. Like prokaryotic cells, they also do not have cell nuclei. Archaeal cells have distinct characteristics that distinguish them from Bacteria and Eukaryota. Archaea are further subdivided into several distinct phyla. Euryarchaeota, Crenarchaeota, Nanoarchaeota, and Korarchaeota are the four primary groups in the domain archaea. Among them the Crenarchaeota and Euryarchaeota are the two phyla that have been examined the most. Methanogens, halobacteria, Sulfolobus, and Nanoarchaeum equitans are all members of this group. Till now there are no reports of the discovery of archaea as human pathogens. Classifying the archaea is somewhat difficult, since the majority of its phylum has never been studied and has mainly been identified by nucleic acid sequencing of the samples from the environment. Archaea mostly inhabit the gut of the humans and animals and all seem to be harmless and render more benefits to the gut. The archaea are also extremophile species; they can live in inhospitable conditions such as radioactive environment, high saline aquatic bodies, etc. Archaea are thought to be significant in global biogeochemical cycling, since they comprise a probable 20% of the world's total biomass. Archaea reproduce asexually by binary fission (Michael Hogan and Monosson 2010) (Fig. 1.4).

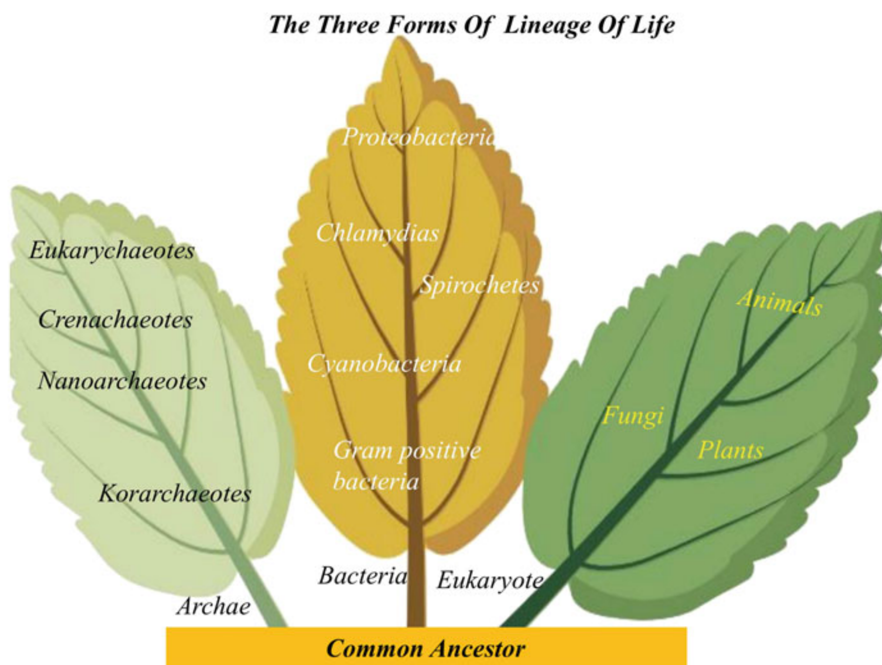

Fig. 1.4 Showing the lineages of three forms of life from a common ancestor. (Adopted and modified from *OpenStax College*, *Biology*)

1.4 Viruses, Viroids, Virusoids and Prions

The virus is a submicroscopic particle that is too small to be seen by an ordinary light microscope. Viruses infect all forms of life, which include animals, plants and microorganisms, such as bacteria and archaea (Koonin et al. 2006). Viruses use the replication machinery of the host cell to make copies of themselves (Lodish et al. 2000). In the process, they kill the host cells and cause serious damage to the host organism. Viruses are found everywhere on Earth (Edwards and Rohwer 2005; Lawrence et al. 2009). Viral diseases have been here on this planet since the onset of life but were not studied until the twentieth century. In 1886, the German chemist Adolf Mayer showed that tobacco mosaic disease (TMD) was an infectious agent and could be transmitted from a diseased plant to a healthy plant. Following this discovery, the Russian bacteriologist Dmitrii Iwanowski (1892) made an effort to isolate the agent of TMD (Artenstein 2012). He observed that the sap of diseased plants which he filtered using his self-designed porcelain filter to retain bacteria had instead filtered out through the tiny pores of the filter. Hence, it was Martinus Beijerinck in 1898 who finally reported the tobacco mosaic virus (Kammen 1999). Since then around 200,000 virus species have been reported (de Jesus 2019). Viruses are an interesting organism. They are made up of two or three components. They are a minute aggregation of either single-stranded or double-stranded RNA or DNA, enveloped by a protein coat capsid (Gelderblom 1996). Viruses are a different entity and don't have the same components as bacteria, so any antibiotics are ineffective against them except antiviral drugs or vaccines which can eradicate or reduce the severity of viral diseases already discussed earlier (Graham 2020). Viral diseases such as measles and smallpox have been completely eradicated, yet research is in full swing to find complete cure for diseases like AIDS, Covid-19, etc. Advancement in the biological science in the later part of the nineteenth century led to the identification of several new virus causing diseases such as human immunodeficiency virus (HIV), SARS, MERS-associated coronavirus, H5N1 influenza A and H1N1 to name a few (Abdelrahman et al. 2020). Outbreak of diseases, such as Israeli acute paralysis virus (2006) (Chen et al. 2014), Ebola virus (2018) of Eastern Democratic Republic of the Congo (Aruna et al. 2019) and Nipah (2018) in India (Thomas et al. 2019), was also reported which had become a national concern in each of these countries. The different types of viruses are represented in Fig. 1.5.

Viroids

In 1971, pathologist Theodor Diener, at the Agricultural Research Service, isolated an agent from diseased higher plants, which lacks the property of an intact cell particle that he named as viroid, meaning "virus-like". Viroids are the smallest pathogens known, single-stranded non-coding RNA molecules that do not code protein yet reproduce independently via rolling-circle mechanism when introduced into host plants (Owens 2008). Viriod consists of low molecular weight RNA. Viroids are tiny pathogens that cause disease. They are made up entirely of a single-stranded circular RNA strand. They do not have a protein covering, unlike viruses. They have a short genome size, which protects them from disaster caused by

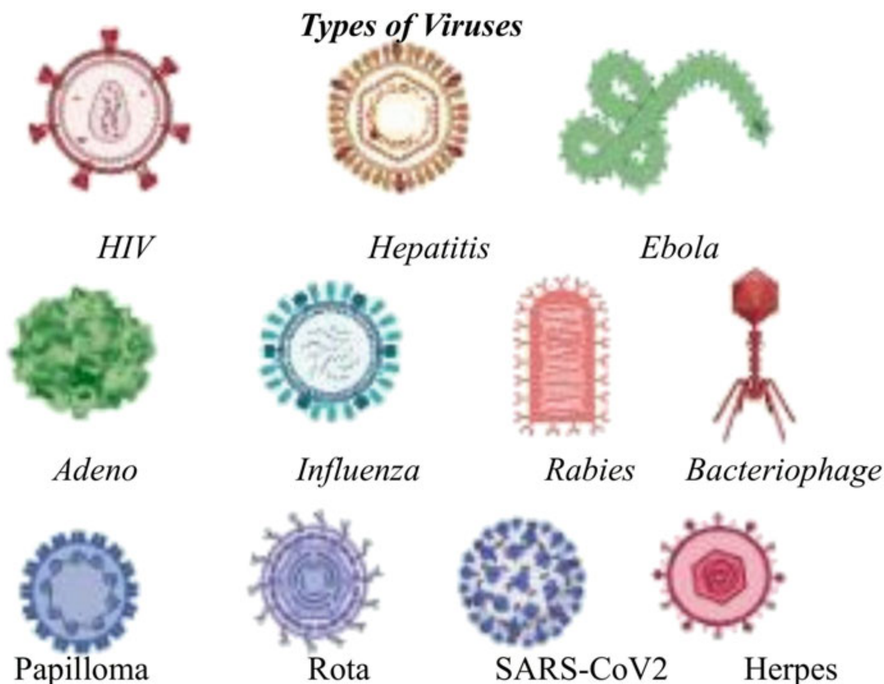

Fig. 1.5 Schematic representation of different types of viruses

error-prone replication, a high GC content, which provides thermodynamic stability, and circular genomes, which prevent information loss. Some viroids are ribozymes, which have catalytic capabilities that allow unit-size genomes to be self-cleaved and ligated from bigger replication intermediates. Viroids reproduce independently with the help of the host's machinery (Kovalskaya and Hammond 2014). The viroid was found to cause the disease potato spindle tuber viroid, which leads to late sprouting and various defects in potato plants. Viroids can be found in monocot and dicot plants, herbs and woody plants, agronomically and ornamentally important plants. Viroids are globally distributed, and their distribution is attributed to the exchange of infected germplasm and transmission through seed and insects. This has resulted in scarcity of food availability and collapsing of the economy (Hadidi et al. 2003). Other diseases caused by viroids include tomato planta macho viroid (TPMVd) in tomato, avocado sunblotch viroid in avocado, peach latent mosaic viroid in peach, *chrysanthemum stunt viroid* (CSVd) in chrysanthemum, *citrus exocortis viroid* (CEVd) in citrus, *coconut cadang-cadang viroid* (CCCVd) in coconut palm and *avocado sunblotch viroid* (ASBVd) in avocado, to name a few (Diener 1987; Hadidi et al. 2003) which lead to loss of chlorophyll content, fragile leaves and small-sized fruits, causing fungal and bacterial infection ultimately resulting in loss of productivity in these field crop. At present around 34 viroid species have been recognized,

length ranging from 120 to 475 nucleotides, and are categorized into two families, *Avsunviroidae* and *Pospiviroidae* (Di Serio et al. 2014). The members of *Avsunviroidae* (*avocado sunblotch viroid*) replicate in the chloroplasts or plastids via symmetric rolling-circle replication using the nuclear polymerase enzyme of the host. Members of *Pospiviroidae* (*potato spindle tuber viroid*) replicate in the nucleus via asymmetric rolling-circle replication using RNA polymerase II (Pol II) enzyme of the host. Their RNAs are rod-shaped having secondary structures without the ribozyme activity (Flores et al. 2014; Ding 2009). The exact mechanism of viriod infection is not fully understood, although identification of viroid-infected plants is accomplished by either symptomatology on indicator hosts, a classic method still used in many certification programmes, or molecular methods such as nucleic acid hybridization or polymerase chain reaction. Flores et al. 2015; Hammond and Owens 2006).

Virusoids

Virusoids are subviral particles, which are part of a specific group of satellite RNAs, connected with the sobemoviruses, and share several common structural and functional characters with viroids. The genome of the virusoid is small, having about 246–371 nucleotides long (Shrestha and Bujarski 2020). Only the satellite RNA of virusoid codes for protein and replicates via rolling-circle mechanisms. Despite these similarities, virusoids differ in certain features from viroids. Virusoids replicate in the cytoplasm with the help of both helper viruses and hosts (Ding 2010). Also, virusoids are coated by their helper virus coat proteins. One example of helper virus is the hepatitis B virus (HBV) found in humans to infect liver along with the satellite RNA hepatitis delta virus (HDV) or hepatitis delta virusoid (Taylor 2015), and the other found in plants is the subterranean clover mottle virus. Once the helper virus enters the host cell, the virusoids are released to be set free in the cytoplasm of the plant cell, having ribozyme activity (Francki et al. 1983). HDV is much larger (1700 nucleotides) than a plant virusoid, with a circular, ssRNA genome, and actively involved in the biosynthesis of HDV-associated proteins (Taylor and Pelchat 2010). There are reports that viroid RNAs are involved in trafficking activity intracellularly, replication and infection (Ding and Itaya 2007). The biological functions of virusoids remain vague.

Prions

Stanley Prusiner Nobel Prize-winning biologist discovered prions or cellular prion protein (CPrP). It is a glycoprotein of mammals which is found attached to the plasma membrane through a glycosylphosphatidylinositol (GPI) anchor (Sarnataro et al. 2017). It is smaller in size compared to virus, with no nucleic acids (neither DNA nor RNA) (López-Pérez et al. 2020). The molecular structure of CPrP has been determined by nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography. The normal prion protein is made up of mutated alpha helices, and beta sheets (Kupfer et al. 2009). Prion-like features have been found in some types of fungi. These fungal prions have been used to study how prions causes infection (Wickner et al. 2007). Prions cannot be destroyed normally as other pathogens as

they do not have nucleic acid. Since prions are the mutated form of a normal protein that is encoded in the body, they do not trigger a host immune response, as other pathogens do (Zabel and Reid 2015). Cellular prion protein is present on the cell surface of the central nervous system, and also in other tissues in the body (Wulf et al. 2017). A prion is responsible for progressive neurodegenerative conditions. The names of two such diseases are bovine spongiform encephalopathy (BSE or mad cow disease) found in cattle and other farm animals and kuru in humans. These misfolded proteins do not infect by replication in the host. Instead, they behave as an inducer adversely affecting the brain by changing the structure of normally folded proteins to the abnormal prion form ($CPrP^{SC}$), which further acts as a template accumulating the abnormal prions in the tissue of the central nervous system. They develop plaques which lead to cluttering of neurofibrils and interfere with the functioning of the synapse. As a result of the process, the nerve cells are damaged and forms tiny pores in the brain, thus giving it a spongy appearance under the microscope and the name spongiform disease (Cobb and Surewicz 2009). Kuru was found to originate in humans in Papua New Guinea, transmitted to humans by means of ritualistic cannibalism. BSE was first discovered in the United Kingdom, by including cattle nervous tissue in the feeds for other cattle which had led to the spread among other cattle. Patients suffering from kuru and BSE showed clinical symptoms like failure of motor function and behaving abnormally such as gelastic epilepsy (sudden burst of laughter) ultimately causing death. Another disease was discovered during an outbreak of BSE, a similar type of encephalopathy in humans known as variant Creutzfeldt-Jakob disease (CJD) which had spread due to consuming beef meat from animals infected with BSE (Cobb and Surewicz 2009). The disease is transmitted via blood transfusion among humans. There are other prion disease that might develop in the complete absence of infectious $CPrP^{sc}$, suggesting the presence of a transmembrane form of the protein tmCPrP which is inherited from the human prion disease (Kovacs and Budka 2008). The different structure of prion mutants and the presence of the GPI anchor have immense power both on the function and the intracellular transport of the prion protein which ultimately causes the disease (Puig et al. 2019).

1.4.1 Classification of Virus

Bennett proposed the first systematic classification of viruses in 1939 on behalf of the newly formed Committee for Virus Nomenclature of the Council of the American Phytopathological Society (Kuhn 2021). Viruses were classified according to the type of morphology and the disease they cause in their hosts, their host tropisms, their modes of transmission and the antigenic, chemical and physical properties of their particles. Bawden, in 1941, suggested a classification based solely on chemical, antigenic and morphological properties of virus particles which was followed by others for many years. In 1948, Francis O. Holmes proposed systematic taxonomic classification that was used for animals and plants. He suggested a top-rank taxon, the order "Virales", that would include three suborders for viruses that infect bacteria

("Phaginae"), plants ("Phytophaginae") or animals ("Zoophaginae"). However, this system did not gain much popularity. There were many classifications put forwards by several taxonomists, but ultimately the taxonomic classification that received broad attention is that of Lwoff, Horne and Tournier (the LHT system), which got approval in the year 1966. The LHT system classified viruses into one phylum ("Vira") with two subphyla based on the presence of RNA or DNA in virions ("Deoxyvira" and "Ribovira"). Each subphylum was further classified based on the symmetry of virion capsids (e.g. the classes "Deoxyhelica" for "helical DNA viruses" and "Ribocubica" for "cubical RNA viruses"). These classes were further classified into orders ("virales") with suborders ("viridales"), families ("viridae"), subfamilies ("virinae"), genera ("virus") and subgenera ("virus") and described species type and common names for taxon members. Even though categorization of the higher taxa was outdated, several family names are still being used today (e.g. "Poxviridae", "Adenoviridae" and "Paramyxoviridae" became the present Poxviridae, Adenoviridae and Paramyxoviridae, respectively), along with the suffixes for order, family, subfamily, genus and subgenus names, which are now italicized ("virales", "viridae", " virinae", "virus"). Viruses are classified on the basis of morphology, chemical composition and mode of replication. The viruses are classified according to the infection they cause in humans. They are at present grouped into different number families, whose host ranges from vertebrates to protozoa and from plants and fungi to bacteria (Gelderblom 1996). Here we will discuss the classification of virus on different aspects.

1.4.1.1 Morphology: Helical Symmetry and Icosahedral Symmetry

Every virus has a protective layer of protein capsid to give protection to its nucleic acid genome. Viral capsids are of two kinds: helical and icosahedral (Louten 2016). During replication of virus with helical symmetry protomers, the protein structural subunits self-assemble into a helical arrangement covering the nucleic acid, forming a spiral shape that twists cylindrically around an axis. In the case of a helical virus, the viral nucleic acid coils into a helical shape, and the capsid proteins wind around the inside or outside of the nucleic acid, forming a long tubular or rod-like structure (Gelderblom 1996). The nucleic acid and capsid together constitute the nucleocapsid which form stiff, highly extended rods and flexible filaments. When inside the cell, the helical nucleocapsid uncoils and the nucleic acid is released making itself accessible. Viruses of the families Paramyxoviridae, Orthomyxoviridae (Couch 1996) and Coronaviridae show helical symmetry. The most extensively studied helical virus is tobacco mosaic virus. Of the two capsid structures, the icosahedron is the most widespread than the helical viral structure. An icosahedron is a polyhedron having 20 equilateral triangle with 12 vertices or faces. An icosahedron has 2-3-5 symmetry (which implies it has twofold, threefold and fivefold axes of symmetry).

1.4.1.2 Morphology: Complex Viral Structure

Viruses have a symmetry coated with a proteinaceous compound called capsid. This capsid has many roles. These include virus attachment to cells, entry into cells,

release of capsid contents into cells, and packaging of newly generated viral particles are all roles played by the capsid during viral infection. The capsid is also in charge of viral genetic material transfer from one cell to another. These structures also influence the viral particle's stability, such as resistance to chemical or physical inactivation (San Martín 2013). Complex viruses create a range of symmetrical mismatches in icosahedral shells to completely asymmetric or pleomorphic capsids (San Martín 2013). This structural complexity of the virus is due to large size genome for larger capsid is the requirement. They may also contain accessory proteins with specific structural or functional roles. For example, HIV have genome size of 7–10 kb of single-stranded (ss) RNA (Goff 2007). Examples of other complex viruses are poxviruses, geminiviruses and many bacteriophages. Poxviruses include the viruses that cause smallpox or cowpox. They are large oval or brick-shaped particles sized 200–400 nm long. Bacteriophages, also known as bacterial viruses or prokaryotic viruses, are viruses that infect and reproduce within bacteria (Louten 2016). The viral structure based on morphology is depicted in Fig. 1.6.

1.4.1.3 Morphology: Presence and Absence of Envelope

Some virus families have an additional extra layer, termed as envelope, which is the extra growth of the modified cell membrane of the host. This envelope is composed of lipid bilayer whose exterior is embedded with virus-encoded, glycosylated (trans)membrane proteins which are like spikes or knobs also known as peplomers. These glycosylated proteins determine the host range and antigenic property of the virion. Virus envelopes also provide an additional protective coat (Firquet et al. 2015).

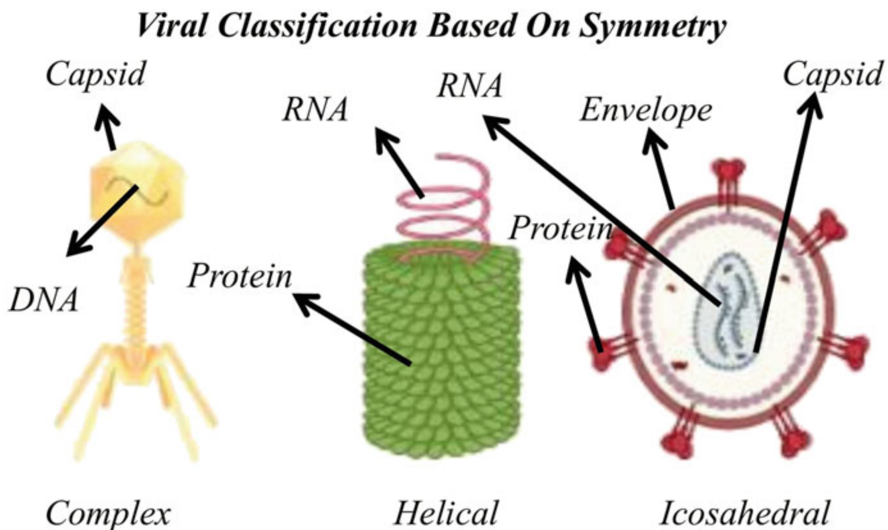

Fig. 1.6 Schematic representation of viral symmetry

- Some members of enveloped virus include the following:
  Influenza virus type A (H1N1), Herpes Simplex Virus Type 1 (HSV-1), Human Coronavirus
- Some members of non-enveloped virus include:
  Coxsackieviruses, Rotavirus, Poliovirus

1.4.1.4 Chemical Composition and Mode of Replication

Viruses are submicroscopic species considered to be non-living particles which cannot replicate independently outside the living host. A virus consists of either DNA or RNA as their genetic material covered by a protein coat. The genome of a virus may contain DNA or RNA, which may be single stranded (ss) or double stranded (ds) or linear or circular. The entire genome is either unsegmented/ monopartite (having one nucleic acid molecule) or segmented/multipartite genome (several nucleic acid segments). It is therefore this variety of genome that leads to different replication strategies. Accordingly, they are classified as DNA viruses and RNA viruses and according to mode of replication.

DNA Viruses

DNA viruses have DNA genomes that are replicated inside the host with the help of its DNA polymerases and deoxynucleotide triphosphate precursors. It is larger than RNA virus. DNA viruses are divided into three major categories: double-stranded DNA viruses (e.g. poxviruses), single-stranded DNA viruses (e.g. parvoviruses) and pararetroviruses (e.g. hepadnaviruses) (Kovalchuk and Kovalchuk 2016). DNA viruses mostly contain a single genome of linear dsDNA. dsDNA serves as a template both for mRNA and for self-transcription process (Payne 2018). DNA viruses have different replication system. They need prior de novo synthesis of proteins to provide the protein products needed for DNA replication. These are generally called “early genes” (White and Spector 2007). After DNA replication, forms the expression proteins, they are required to be packed which later form virions. These are generally called “late genes”. DNA viruses consist of important pathogens such as herpesviruses, smallpox viruses, adenoviruses and papilloma viruses, among others.

RNA Viruses

RNA viruses have RNA as their genetic material. It may be a single-stranded RNA or a double-stranded RNA and uses RNA-encoded RNA polymerases for replication of their genomes. Retroviruses, on the other hand with two copies of single-stranded RNA genomes, use reverse transcriptase which produces viral DNA that can be integrated into the host DNA using its integrase function (Poltronieri et al. 2015). During replication of RNA viruses, three types of RNA must be formed: the genome, a copy of the genome (copy genome) and mRNAs. The RNA polymerases and other proteins required for the synthesis of viral genome are called the replicase complex. The replicase complex comprises the set of proteins required to produce infectious genomes. The RNA virus is subdivided based on the type of RNA they contain. Positive- or plus (+)-strand RNA viruses are those whose genomes have functional

mRNAs that are translated to produce RNA-dependent RNA polymerase which in turn is used to produce RNA virus. The other three groups of RNA viruses are those whose genomes are not mRNAs. They are the negative- or minus-strand RNA viruses, the closely related ambisense RNA viruses and double-stranded RNA viruses (Payne 2017). Human diseases caused by RNA viruses include orthomyxoviruses, hepatitis C virus (HCV), Ebola disease, SARS, influenza, polio, measles and retrovirus including adult human T-cell lymphotropic virus type 1 (HTLV-1) and human immunodeficiency virus (HIV) (Poltronieri et al. 2015).

DNA-RNA Viruses

The RNA tumour viruses called leukoviruses and Rous Sarcoma Virus unusually contain both DNA and RNA as genetic material (Bader et al. 1970).

ICTV Classification

As viruses are non-living entities, they are classified using order, family, genus and species unlike living organisms which are classified using domain, kingdom, phylum, class, order, family, genus and species taxa. Therefore to deal with the challenge, the International Committee on Taxonomy of Viruses (ICTV) was formed and has been responsible for classifying viruses since 1966 (Louten 2016). ICTV utilizes taxonomical system of classifying the viruses. ICTV while classifying viruses takes into consideration the size of the virus, capsid structure, type of nucleic acid, physical properties, host species or disease. A complete catalog of known viruses is maintained by the ICTV at ICTV database known as Virus Metadata Repository. The first ICTV report included only 2 families, 27 genera, 10 subgenera and 18 "virus groups", but the classification system has been extended since then. For instance, the first order (*Mononegavirales*) was established in 1991, and in 2019 the first phylum (*Negarnaviricota*) and realm (*Riboviria*) were made official. *Riboviria* contains nearly all RNA viruses and reverse-transcribing viruses; three separate proposals were approved to establish three realms for viruses with DNA genomes (Walker et al. 2020). By that time, 5560 species had been established (Kuhn 2021). The latest report on containing updated data on new species ratified can be accessed in Virus Metadata Repository: version August 1, 2020; MSL35 (https://talk.ictvonline.org/taxonomy/vmr/m/vmr-file-repository/10312).

Baltimore Classification (1971)

Nobel laureate David Baltimore proposed a classification of virus in the early 1970s that is still today parallelly used with official virus taxonomy. Rather than grouping the viruses to taxa, Baltimore grouped all viruses into six disconnected groups without any subdivisions, now commonly referred to as Baltimore classes (BCs). The Baltimore classification system classifies viruses based on the type of nucleic acid genome they have and replication system of the virus. The system also divides single-stranded RNA viruses into two groups: those that have positive strand (+) and negative strand (−). Positive-strand (also positive-sense or plus-strand) RNA virus is the one which translates into proteins, such as messenger RNA (mRNA) in the cell is positive strand. Negative-strand (also negative-sense or minus-strand) RNA virus is

the one which is not directly translated into proteins. It is first transcribed into positive-strand RNA. Baltimore also included viruses that are able to do reverse transcription, or form DNA from an RNA template, which is something that cells are incapable of doing. The seven classes of Baltimore classification are as follows:

- BC I: double-stranded DNA genome virus that has a replication-expression strategy highly suggestive for that of cellular organisms (e.g. *Adenoviridae*)
- BC II single-stranded DNA genome virus (e.g. *Geminiviridae*)
- BC III: double-stranded RNA genome virus (e.g. *Reoviridae*)
- BC IV: positive-sense RNA genome virus (e.g. *Picornaviridae*)
- BC V: negative-sense RNA genome virus (e.g. *Paramyxoviridae*)
- BC VI: positive-sense RNA genome virus that replicates using DNA intermediates produced by reverse transcription of the genome (e.g. *Retroviridae*)
- BC VII (which was added later to the original system): double-stranded DNA genome virus that packages a double-stranded DNA form or an RNA-DNA hybrid into virions and replicates via reverse transcription (e.g. *Hepadnaviridae*)

1.5 Tools and Techniques of Microbiology

Microbes are universal, and to have a better knowledge of microbes, it is important to know the laboratory and the experiments carried out. Our present knowledge of physiology and regulatory activity of microorganisms has arisen from our ability to isolate them from their natural ecosystems into pure form in the laboratory cultures (Palková 2004). The microbial techniques used in the laboratory are the ones used to study microbes such as bacteria, fungi and protists. They incorporate techniques such as surveys, isolation, culture and manipulation of microorganisms. The first artificial liquid culture medium was developed by Louis Pasteur in 1860 (Bonnet et al. 2020). The study of microbiological tools and techniques is essential as microbes are an important part of our life and are both beneficial and harmful. The present-day threat of an outbreak of pandemics or use of bioweapon in warfare led to public consciousness towards the importance of applying proven precautionary procedures and healing (Riedel 2004). Laboratory culture remains an indispensable part of science. The research work carried out provides a solution towards the issues in society related to health and diseases. Microbiology techniques are very useful in agriculture, evolutionary studies, pharmaceutical industry (Vitorino and Bessa 2017). Modern-day use of biotechnology tools and techniques has encouraged the use of new methods to make further advances in microbiology as a scientific discipline. Microbes are present everywhere around us. To identify and purify specific microbes, they have to be cultured in a controlled and optimized laboratory setup in aseptic conditions. Aseptic techniques are performed under sterile conditions, a method that prevents the entry of contaminants into the working space. This process is executed by strictly adhering to guidelines laid down in the laboratory manual. Culture techniques were developed from the need to grow and identify potential infectious agents for the diagnosis and treatment of any diseases.

Before going into the details of microbial culture techniques, let us first discuss about some basic technique requirements.

- Growth medium is a nutrient medium where microorganisms grow in any type of organic compound that contains carbon and nitrogen in the nutrient medium. Carbon is used for respiration and nitrogen is used for growth. Media can be both solid and liquid. Depending on growth, nutrient media may be enriched with minerals and vitamins.
- The killing or elimination of all microorganisms is known as sterilization. Sterilization can be done using both physical and chemical methods. Physical methods make use of heat, radiation and filtration. To validate the sterilization result via chemical and physical methods, biological indicators may be used. The sterilization processes should be done under strict control measures to get the appropriate results as the frequency of resistance varies. It ranges from highest in the case of endospores to moderate to least resistance in the case of vegetative cells. The most frequently used instrument in the laboratory for the sterilization of media and glassware is the autoclave.
- *Disinfectants* are chemical agents applied to non-living objects to kill microbes except endospores. *Antiseptics* are antimicrobial substances that are applied to living tissue to decrease the prospect of an infection. Disinfectants are of two types—bactericidal one that kills the bacteria and bacteriostatic that hinders the growth of the cell and reproduction.
- Sanitization is done by using a cleansing technique by removing microbes to reduce the contamination. The sanitizers are soap or detergent.
- Inoculation is the technique by which microorganism is introduced into the media.
- Incubation is the process of keeping the inoculated media in an incubator for the growth of the organism under optimal temperature and pressure.

1.5.1 Isolation of Microorganisms

Microorganisms occur in the natural environment in association with each other. Among them some are pathogenic and some are non-pathogenic. The pathogenic ones might cause lethal diseases. Therefore it is necessary to isolate and culture them in pure form according to their functional properties, to identify and control the infectious agents. The primary culture from a natural source will normally be a mixed culture containing microbes of different kinds. In the laboratory, these mixed cultures may be isolated into single or pure culture.

1.5.1.1 Methods of Isolation

There are many specialized techniques used to get pure cultures of microorganisms. A pure culture contains a single bacterial species. An isolated pure culture may be obtained using enriched media containing definite chemical or physical agent components that allows the isolation of the target organism. The basic methods for

isolation of a pure culture include (1) spread plating on solid agar medium using a glass spreader and (2) streaking with a metal loop. The purpose of these two techniques is to isolate individual bacterial cells (colony-forming units) on a culture medium. Both procedures should be executed aseptically (Mara and Horan 2014; Sanders 2012).

- *Streaking*: The streaking technique isolates individual bacterial cells on the surface of an agar plate with the help of a metal wire loop. It is sterilizable and can be reused. While working with Bio-safety Level-2 microbes in a biosafety cabinet, disposable plastic loops may be used. Some scientists may prefer using disposable, presterilized wooden sticks for streaking. The inoculum containing the mixed bacterial culture is spread evenly over the semi-solid surface of agar-based nutrient medium in a Petri dish in a zigzag/quadrant pattern followed with incubation, the inoculum which later grows into visible and viable colonies. The inoculums may be taken either from a suspension of cells (5–10 μL with a micropipette) in broth or from existing viable colonies from another agar plate. The cell suspension is vortexed before applying the aliquot for plating. All the steps are to be performed aseptically.
- *Pour plate procedure* (enumeration of bacterial cells in a mixed sample): This method is used to count the number of viable bacterial cells present in a mixed culture. The mixed culture is poured on a molten agar medium before its solidification (cooled at 4 °C). It includes dilution of the mixture of microorganisms in the broth until only a few hundred bacterial cells are left in each millilitre of the suspension/broth. A very little amount of the diluted inoculum is then placed in a sterile Petri plate employing a sterile loop or pipette. The microorganism and agar are properly mixed and incubated till the appearance of the visible colonies. Viable plate count allows scientists to generate growth curves, to calculate the cell concentration.
- *Spread plate procedure* (formation of discrete bacterial colonies for plate counts): This technique is used to isolate microorganisms when the volume of the sample in the inoculum is less. The inoculum is added to the agar plate with the aid of a wire loop or a micropipette which is then spread over the agar surface using a spreader. Separate colonies grow uniformly distributed across the agar surface. The advantage of this experiment is that it not just allows the growth of viable cells but also allows to examine the growth of cells of a particular genotype. This technique is used for the enumeration of viable cell count. There are two types of spread plate methods. The first one is the turntable method where a glass or metal rod is used. The second method is known as the "Copacabana method", which involves shaking the culture media with presterilized glass beads. Both ensure the smooth spreading of cells across the agar surface. There are other techniques used for the enumeration of viable cell count. They are discussed as follows:
  - *Enrichment culture method*: This involves culturing of mixed organisms in conditions that favour the growth of desired microorganisms with desired metabolic properties and activity. This culture also allows the growth of

other undesired organisms simultaneously. The colonies that grow show phenotypic properties along with the desired genotype.

  – *Selection experiment*: This allows the growth of cells with a particular type of genotype. This type of experiment is done in molecular biology laboratory where recombinant cells are cultured. For example, bacterial strains with plasmids containing antibiotic-resistance genes when plated on a medium supplemented with a particular concentration of an antibiotic will allow selection for those cells that have resistance to that particular antibiotic.
  – *Screening experiment*: In this experiment mixed culture is plated on a medium that allows the growth of those viable cells with the desired genotype expressing a particular phenotype. This experiment again is prevalent in molecular biology laboratories where cloning experiments are performed. One such example is the blue-white screening technique for the recognition of recombinant bacteria. The activity is performed by β-galactosidase, an enzyme occurring in *E. coli*, which cleaves lactose into glucose and galactose.

- *Soft agar overlay procedure* (formation of plaques for isolation and enumeration of phage (plaque assay)): This method is used to identify and quantify microbes and bacteriophage (phage) in culture. Although an electron microscope is employed to observe individual phage particles, they can be detected by the formation of plaques on an agar plate. The phages cannot replicate independently outside their host bacterial cells, so their multiplication and identification require mixing of both the phage suspension and host cells (bacteria) in a test tube before inoculating in the plate. The resulting mixture is poured over the surface of a nutrient agar plate and then incubated. If the phage infects the bacteria, plaques appear which can be observed either by the naked eye or by microscopy. Each plaque is a single virus or phage, and the number of plaque-forming units (pfu) may be calculated, and the concentration, of the phage suspension, may be determined. This experiment, also known as plaque assay, may be used to generate growth curves and for converting bacterial cells for experiments in genetics.
- *Replica plate procedure* (transfer of cells for screening mutants and auxotrophs): Replica plating is a microbiological approach during which one or more secondary Petri plates containing selective growth solid media (missing nutrients or growth inhibitors like antibiotics) are inoculated with an equivalent colonies of microorganisms from the primary plate (or master dish), reproducing the first spatial pattern of colonies. The technique involves producing the exact copies of the colonies within the same spatial pattern in a secondary plate by simply pressing a small piece of velvet to the first. This system is advantageous because it allows a comparatively sizable amount of colonies to be screened simultaneously for several phenotypes during a single experiment.

1.5.2 Types of Nutritional Requirements and Media

Microbes can be categorized based on how they obtain carbon, energy and electrons or hydrogen. Undeniably, the exact nutritional requirements of microorganisms are used to differentiate microbes from another for taxonomic classification. Microorganisms form groups on the basis of their energy needs. Microbes that obtain energy by oxidizing chemical compounds (either organic or inorganic) are called *chemotrophs*, and those that use light energy are called phototrophs (Chan 2003). Therefore to mimic the natural environment in the laboratory to grow the microbes, the culture media were used. Culture media are the foundation of microbiological tests. They are applied to isolate and cultivate pure microbial cultures. A microbiological culture is the medium for a microbe to grow and survive from where it acquires nutrients, factors that promote growth, buffer salts, minerals and gelling agent. Culture media have been in use since the onset of the nineteenth century. The first culture medium prepared was meat-infusion broth by Robert Koch and his colleagues (Sandle 2010). Even with the technological development, majority of techniques used in microbiology laboratory require growth culture media. Such is the importance to have a basic understanding and knowledge of culture media (Bridson and Brecker 1970; Sandle 2010). Media can be differentiated in line with the content material of the nutrients and consistency and may be classified consistent with their physical nature, chemical composition and the function they perform.

1.5.2.1 Classification of Media on Physical State

- Liquid media are useful in the isolation of small numbers of microorganisms. It has no solidifying agent added, for example, nutrient broth.
- Solid media are defined as the media formed by adding of agar to the culture media as a solidifying agent. The concentration of agar can be changed accordingly to make the medium solid or semi-solid. Nutrient agar prepared by adding 2% agar to nutrient broth forms the solid medium. Examples of solid media include blood agar, chocolate agar, MacConkey agar, etc.
- Semi-solid media use less concentration of agar (0.2–0.4%) to make the medium semi-solid.

1.5.2.2 Classification of Media on Composition

- Complex media are undefined and non-synthetic. In undefined media chemicals present are in unknown proportions. Examples of complex media are chocolate agar and MacConkey agar.
- Chemically defined medium is synthetic, containing known quantities of the ingredients. It contains no natural ingredient. Dubos medium with Tween 80 is an example of this medium.

1.5.2.3 Classification of Media on Function

- Enriched media are solid in consistency. It allows fastidious bacteria (organisms that are incapable to synthesize certain nutrients on their own and have to be

supplemented to the medium) to grow. These media are prepared by adding blood, serum and egg to the basal media (Bonnet et al. 2020).

- Enrichment media are liquid media. It permits the growth of certain pathogenic bacteria and simultaneously suppresses the growth of other bacteria. Examples of enrichment media are Selenite Cystine, Tetrathionate Brilliant Green and Rappaport Vassiliadis used for the separation of *Salmonella* spp. (Rall et al. 2005).
- Selective media are solid media (Prinzi and Rohde 2020) containing inhibiting substances to facilitate the growth of desired bacteria and inhibiting the growth of unwanted bacteria. Some examples of selective media include MacConkey agar (Prinzi and Rohde 2020).
- Differential or indicator media are used to differentiate one microorganism from another growing on the same one media by observing their growth pattern. Examples of differential media include MacConkey agar (Prinzi and Rohde 2020).
- Transport media are used when some delicate organisms are needed to be transported. They are used so that the viability of the delicate organisms is maintained during their transport. They only contain buffers and salt and lack all other essential substances such as carbon, nitrogen and organic growth factors. Example of transport media is Stuart's transport medium used for *Neisseria gonorrhoeae* (Ebright et al. 1982).

1.6 Microbial Nutrition

Microorganisms have different types of nutritional needs. Bacteria represent the range of nutritional variety. Bacteria use both complex organic and inorganic substances for their survival. Bacteria represent the boundary between these two types of nutrition (Lundgren 1989). The sources of energy for the microorganisms are carbohydrates, alcohols and amino acids. Most microorganisms are capable of metabolizing simple sugars such as glucose and also complex carbohydrates, such as starch or cellulose, or glycogen in muscle foods to obtain energy, while other microorganisms metabolize fats to obtain energy (Sandle 2015). Metabolism in microbes takes place in two ways—catabolism and anabolism catalysed by enzymes. Catabolism is a process of breaking down of large molecules sequentially in a stepwise manner with the release of energy which is trapped in the chemical bonds, and anabolism is the reverse process which uses up the energy from molecules for biosynthesis (Fani 2012). Also microorganisms obtain their energy by trapping of light energy and ultimately convert it into chemical energy. The kinds of nutrients and how they are assimilated and fed into the various metabolic pathways for energy production and utilization in microorganisms is the subject matter of this section.

1.6.1 Nutritional Classification of Prokaryotes

Microbes are grouped according to their requirements for energy from different sources of food (Chan 2003). Both the prokaryotes and the eukaryotes display exceptional nutritional mode.

1.6.1.1 Modes of Nutrition in Prokaryotes

Prokaryotes are diverse in their way to obtain resources and energy to synthesize organic compounds. The ones that utilize light energy are known as phototrophs, and the ones which use chemicals from the environment are known as chemotrophs. When prokaryotes utilize inorganic carbon source of energy, they are known as autotrophs, and others that utilize organic nutrient as carbon source are known as heterotrophs. A combination of the former two terms along with those used in describing carbon utilization results in the following nutritional types:

- *Chemoautotrophs*: microbes that utilize inorganic chemical substances such as H_2S, NH_3 and Fe^{2+} to obtain energy and carbon dioxide as the main source of carbon
- *Chemoheterotrophs*: microbes that use organic carbon as sources of energy
- *Photoautotrophs*: microbes that use light energy to synthesize organic compounds from CO_2
- *Photoheterotrophs*: microbes that use light energy and organic components as the main source of carbon

The chemoheterotrophs are very diverse and unique in the group—some have very specific and stringent requirements, while others are flexible in their requirements. For example, lactobacillus will grow well only when the medium contains certain other molecules such as amino acids, several B vitamins, ascorbic acid, glucose, acetate and oleate (Guirard and Snell 1964), whereas *Eschericia coli* is a chemoheterotroph which can grow on only sugars or amino acids or mixtures of both (Boncristiani et al. 2009).

Microorganisms are grouped according to hydrogen atoms or electron requirement.

Microorganisms that use reduced inorganic compounds as their electron source are called lithotrophs. And the ones that obtain electrons or hydrogen atoms organically are called organotrophs (Chan 2003). These groupings are further subdivided as follows:

- Photolithotrophic autotrophy
- Photoorganotrophic heterotrophy
- Chemolithotrophic autotrophy
- Chemoorganotrophic heterotrophy

1.6.2 Microorganisms Employ Metabolic Pathways to Metabolize Glucose and Other Biomolecules

- *Nitrogen metabolism*: Prokaryotes can metabolize nitrogen compounds. Nitrogen is an important biomolecules required for survival of the microbes. Hence they play an important role in the nitrogen cycle of the ecosystems (Bernhard 2010).
- *Oxygen metabolism*: Bacteria can be grouped on the basis of their requirement or sensitivity to oxygen (Coleman and Smith 2007).
  – Obligate aerobes obtain most of their energy by oxidative phosphorylation using O_2 as the terminal electron acceptor in the process.
  – A facultative anaerobe survives via anaerobic respiration. They can tolerate both the presence and absence of oxygen.
  – Obligate anaerobes survive without the requirement of molecular oxygen. They are highly sensitive to O_2 which leaves them unfit to grow in its presence.
  – Microaerophilic organisms best grow at lower oxygen level concentrations and their growth is inhibited when the concentration increases.
- *The evolution of glycolysis*: Since the evolution cells were able to obtain food from the organic molecules present in the environment. But everything reaches a threshold limit and becomes self-limiting; hence prokaryotes needed to resort to an alternative source and evolve a self-mechanism to generate energy and synthesize the biomolecules required for their survival and growth. The first prokaryotes, probably the chemotrophs which evolved 3.8 billion years ago, absorbed free organic compounds (including ATP) via abiotic metabolic activity. All cells use adenosine 5′-triphosphate (*ATP*) as their source of energy to carry on their cellular activities. However as ATP supplies were low, prokaryotes resorted to an alternate way to regenerate ATP from ADP, leading to evolution of glycolysis (Fani 2012).
- *The evolution of electron transport chains and chemiosmosis*: The process tells us how microbes can obtain energy by some other methods instead of from high-energy molecules. The chemiosmotic theory states that energy-generating membranes, such as cell membranes, mitochondrial membranes and chloroplast membranes, pump protons across the membrane that generates an electrochemical gradient of protons across the membrane. ATP is used as energy molecules to drive the pumps (Alberts 2002).
- *The evolution of photosynthesis*: With the evolution, free ATP and abiotically produced organic molecules were depleted, and therefore nature resorted to an alternative way where organisms could make their own organic molecules from inorganic resources. Hence, the development of photosynthesis allowed the cell to utilize energy from sunlight, instead of from other organic molecules for driving their metabolic requirements (Blankenship 2010).
- *The evolution of cellular respiration*: Cyanobacteria are the bacteria that can perform aerobic photosynthesis and respiration simultaneously in the same location, and some cyanobacterial species can also fix nitrogen (Vermaas 2001).

1.7 Growth in Microbes

Microbial growth is increase in population which is either the increase in cell number or the mass (Maier and Pepper 2015). Microbial growth is important parameter in microbiology for the examination of activities such as cell growth and biocatalytic functions. There are techniques available to observe cell growth by direct or indirect measurements. Certain factors are considered while calculating microbial growth, such as cell dry weight of the cell, optical density of the cell, turbidity, cell respiration, substrate utilization and product formation (Najafpour 2015). Microbial growth is achieved by cell division which leads to the growth of cell population. They are of two types:

- *Binary fission*—Bacteria divide by fission. The bacterial cell becomes double in size and replicates its genetic material (DNA) present in the chromosomes (Margolin 2014).
- *Budding*—Some bacteria reproduce by budding, forming a bubble-like structure that increases in size and detaches from the parent cell (Hirsch 1974).
- *Bacterial Growth Curve*: Measuring bacterial growth is an important characteristic in microbiology for its various applications in everyday life. It can be done in two ways—closed system or batch culture and continuous culture system (Maier and Pepper 2015). Batch culture is a culture where no nutrients are renewed or wastes removed. Hence the exponential phase is maintained till a limited period of time. Here in this culture, the bacteria grow in a particular pattern leading to growth curve composed of four distinct phases of growth: the lag phase, the exponential or log phase, the stationary phase and the death or decline phase. Growth curve can also tell about the generation time for an organism—the time for the population to double. The details of the growth curve having the different phases will be discussed later in this section.
- *Measuring Microbial Growth*: As mentioned microbial growth can be measured in two ways (O'Toole 1983):
  - *Indirect Measurements*: In this method the property of the mass of cells is measured.

    Turbidity: It involves a colorimeter that measures the cloudiness or turbidity of the culture as cell numbers increase. Results are calculated by comparing the light absorbance with a standard graph plotted against known cell numbers.

    Metabolic Activity: The metabolic activity is observed by calculating the rate of formation of metabolic products, such as gases or acids in the culture medium, and rate of utilization of a substrate such as oxygen or glucose.

    Dry Weight/Wet Weight: Measuring the weight of cells both in dry or wet condition after centrifugation.
  - *Direct Measurements*—This method gives more exact measurements of numbers of microbes.

Direct Counts—It measures only bacterial cells. It measures both live and dead cells. It uses Coulter counter—electronic counter.

Plate Count—It is the indirect way of counting viable cell. When organisms are plated on a suitable medium, each viable cells grows and forms a colony which is counted as a colony forming unit (cfu).

Filtration—It is used for the measurement of very small quantities of bacteria. The pores in the filter do not allow microbial cells to escape. The filter retaining the cells is then placed on a medium and incubated and observed for viable cell growth.

Most Probable Number (MPN)—It is used to measure bacterial cells that are incapable of growing on solid medium.

1.7.1 Factors Affecting Microbial Growth

There are several factors which control the growth of microorganisms. The most important factors that affect microbial growth are the following: (1) "intrinsic factors" which include nutrient content, water activity, pH value and the presence of antimicrobial substances; (2) "extrinsic factors" which include temperature, composition of gases, relative humidity and pH; (3) "implicit factors" related to the microorganisms themselves, which include interactions between the microorganisms, their abilities to utilize different types of nutrient sources and tolerate stress, etc.; (4) "processing factors" which include heating, cooling drying; and (5) "the combined effects" which include interaction between the above stated factors that also affect the growth of microorganisms (Abo-Elenain 2017). Some of the factors are discussed as follows:

- *Intrinsic Factors*: Intrinsic factors are those that are characteristic of the food itself. They include the following:
  - *Nutrients*: Microorganisms require nutrients for its growth and maintenance of metabolic activities. These nutrients include water, energy source, nitrogen, vitamins and minerals (Mossel et al. 1995). Microorganisms primarily obtain carbon and nitrogen from biomolecules such as proteins, fats and carbohydrates. Some microorganisms search for it outside and absorb such food particles. Others may undergo biochemical reactions with surrounding elements such as carbon dioxide to obtain their needs, while others perform photosynthesis and obtain their sugar as a source of energy. On the other hand, microbes obtain nitrogen, required to synthesize proteins, through nitrogen cycle or from other organic matter (Hayatsu et al. 2008). The nutrient that microbes obtain and their growth are closely related. This relation between the nutrient concentration and microbial growth is expressed mathematically by Monod equation which is mathematically related to the Michaelis-Menten equation of enzyme kinetics. It states about the relation between microbial growth and rate-limiting enzyme reaction (Okpokwasili and Nweke 2006). Monod equation is designated by:

$$\mu = \mu_m S/S + K_s \tag{1.1}$$

where:

μ = specific growth rate
μ_m = maximum specific growth rate
S = concentration of limiting nutrient
K_s = saturation constant

– *pH*—Microorganisms, such as bacteria, are responsive to the hydrogen ion concentration. They have to maintain their pH above a significant point at or below which their protein denatures. Certain mechanisms are there that help bacteria to maintain intracellular pH. These mechanisms include homeostasis response, acid tolerance response and synthesis of acid shock proteins. Most bacteria grow best at neutral pH values (6.5–7.4); however some flourish in extreme acidic condition. Based on their property to tolerate different pH, bacteria are divided into the following categories (Keenleyside 2019):

Acidophiles or acid-loving—They grow at a pH of 1–6.8; examples are sulphur-oxidizing *Sulfolobus spp*. (Yellowstone National Park) (Brock et al. 1972), archaean genus *Ferroplasma spp*. (acid mine drainage) (Dopson et al. 2004) and *Lactobacillus spp*. (fermented milk) (Parker et al. 2018).

Neutrophiles—They grow at pH of 5.4–8.5; examples are *Escherichia coli*, *Staphylococci spp*. and *Salmonella* spp. (Keenleyside 2019).

Alkaliphiles or base loving—They grow at pH 7.0–11.5; examples are *Vibrio cholerae* (causes cholera) and the bright pink halophilic archaean *Natronobacterium*, found in the soda lakes of the African Rift Valley (Keenleyside 2019).

– *Redox potential*: A compound's redox potential, or more precisely, its reduction potential, relates to its tendency to receive electrons and hence be reduced (Prévost and Brillet-Viel 2014). The redox potential is measured in terms of millivolts. The redox potential is dependent on the pH of the substrate; redox potential is normally measured at pH 7.0 (Jay 2000). Examples of microorganisms whose growth is dependent on redox potential are aerobes, anaerobes, facultative aerobes and microaerophiles.

– *Water activity*: The water activity of food (a_w) is the measure of the amount of water "available" in food. Water availability (water activity a_w) is the vitality and functional activity of living systems (Stevenson et al. 2015). The water activity is expressed in terms of value ranging from 0 to 1. Pure water is expressed as 1, which signifies presence of all water, whereas a product with no water at all has a water activity of "0". Majority of microbes do not reproduce below 0.900 a_w (Bader et al. 1970; Manzoni et al. 2012; Moyano et al. 2013); except for the most extremophiles, cell division takes place at 0.61 a_w (Pitt 1975; Williams and Hallsworth 2009). The water activity of archaeal and bacterial cell at which their cell divides is between 1 and 0.755 (Anderson 1954; Grant 2004).

  – *Antimicrobial agents*: An antimicrobial agent is a natural or synthetic substance that either kills or inhibits the growth of microorganisms (Burnett-Boothroyd and McCarthy 2011). There are two types of antimicrobials: microbiostatic compounds that stop growth of microorganism and microbicidal compounds that kill microorganisms. There are a number of plant-based antimicrobial constituents which are microbicidal in nature (Jay 2000).
- *Extrinsic factor*: Environmental factors such as relative humidity, temperature, etc. determine the presence of competitor microbes that affect the microbial growth.
  – *Relative humidity*: Different types of microbes require varied amounts of water in the form of vapour for their activity. Microbes require relative humidity (RH) of 60% or more for their survival, whereas some can survive and multiply in >20% RH (Arfalk 2015). Temperature: Temperature and gas composition are the main extrinsic factors influencing microbial growth. Temperature has profound influence on growth and physiology. Temperature has influence on gene expression as well. Cells grown at refrigeration temperature grow differently than those at room temperature. Above and below optimal temperature, the growth rate decreases (Keenleyside 2019).
  – Effect of temperature on the growth of microorganisms:

    Psychrophiles—Psychrophilic are cold loving which inhabit cold environments such as deep sea, mountain and polar regions. They are further classified as the psychrotolerant or psychrotroph. Examples include *Moritella profunda* (D'Amico et al. 2006).

    Mesophiles—Mesophiles grow at moderate temperatures between 20 and 45 °C and at an optimum temperature in the range of 30–39 °C. They grow both in soil and water. Pathogenic microbes infecting animals and humans at temperature (37 °C) are mesophiles; Examples include *Streptococcus pyogenes* (Schiraldi and De Rosa 2014).

    Psychrotrophs grow at 0 °C. Psychrotrophs have a maximum temperature for growth above 20 °C and are widespread in nature. They are responsible for the spoilage of refrigerated food. Examples include the human pathogen *Listeria monocytogenes* (Gounot 1986; Farber and Peterkin 1991).

    Thermophile and hyperthermophile organisms grow at optimum temperatures ranging between 50 and 80 °C ("heat loving"). They are inhabitants of extreme habitats such as deep-sea hydrothermal vents, terrestrial hot springs and other extreme sites including volcanic sites, as well as decaying matters such as the compost (Panda et al. 2019). Examples include photosynthetic bacteria which generally grow up to 70–75 °C and include many cyanobacteria as well as green and purple sulphur bacteria (Holden 2009).
  – *Gaseous content*: Microbes are categorized in terms of their ability to use and tolerate oxygen. On the basis of their requirement to oxygen, microbes are categorized as follows (Singh et al. 2017):

    Obligate aerobes: They can tolerate and grow at full oxygen concentration (greater than 21%).

Microaerophiles: Microaerophiles can use oxygen when present at low levels in air (Rihane et al. 2020).

Facultative anaerobes: Facultative organisms can grow both in aerobic or anaerobic conditions. Example includes *Clostridium* and *Propionibacterium* (Stieglmeier et al. 2009).

Anaerobes lack respiratory systems and thus cannot utilize oxygen. There are two types of anaerobes:

Aerotolerant anaerobes can tolerate oxygen and grow in its presence even though they cannot use it.

Obligate anaerobes are killed by oxygen, for example, *Bacteroides fragilis* (Onderdonk and Garrett 2015).

Competitive microflora: The mutual interactions among different microflora greatly affect microbial growth (Boziaris and Parlapani 2017).

1.7.2 Microbial Growth Kinetics

Growth is a dynamic nature of living organisms. Growth kinetic studies tell about how all activities of microbial life relate to growth, survival, death, etc. (Panikov 1995). Most importantly kinetic model of cell growth predicts about product formation. It establishes a relationship between growth and environmental factors, especially the nutrients (Van Niel 1949). The growth of microorganisms is a highly composite and synchronized process expressed by rise in cell number or cell mass. Growth depends on certain factors such as the availability of nutrients, and other environmental factors. Different microbes have different growth patterns and each of them is unique. For example, bacteria divide by fission, yeast cells grow by budding, and viruses reproduce within a host (Wang and Levin 2009). The microorganisms can be grown in batch (Blaby et al. 2011), fed-batch (Stanbury et al. 2017), semi-continuous or continuous culture (Kuenen and Johnson 2009) systems in a bioreactor depending on nutrient supply (Table 1.1).

- *Batch Culture*: In batch culture the fermentation is performed in a closed system. Here the substrate and nutrients are added immediately after inoculation termed as zero time, and the incubation is carried out under optimal physiological conditions (pH, temperature, O_2 supply, agitation, etc.). Alkali or acid is added to maintain the pH and antifoam agent is used to avoid over-foaming. Batch culture is determined by certain growth patterns or phases (Maier and Pepper 2015):
  - *The Lag Phase*: The period immediately after inoculation is the lag phase. In this period the microorganisms try to adapt to their new environment such as available nutrients, pH, temperature, pressure, etc. This period is characterized by slight increase in cellular weight but no increase in cell number. Multiple

Table 1.1 List of some bioreactors and their application

Serial no	Bioreactor	Function	Reference
1.	Super-high-rate anaerobic bioreactor	Wastewater treatment	Abdelgadir et al. (2014)
2.	Biologically active carbon reactor	Removal of perchlorate and nitrate from contaminated water	Li et al. (2010)
3.	Continuously stirred tank bioreactor	Wastewater treatment	Gargouri et al. (2011)
4.	Air lift	Decolorizing of textile dye using fungi	Mahmood et al. (2015)
5.	Slurry phase	Bioremediation of soil	Quintero et al. (2007)
6.	Fluidized bed	Pharmaceutical industry	Gros et al. (2014)
7.	Packed bed	Bioremediation of chemicals from industries	Liu et al. (2017)
8.	Biotrickling filter	Wastewater treatment	Olivieri et al. (2012)
9.	Woodchip bioreactors	Removal of nitrate from agricultural wastewater	Jéglot et al. (2021)
10.	Membrane bioreactor	Saline wastewater treatment	Tan et al. (2019)

lag phases might occur if the media are supplemented with multiple glucose nutrient, and such type of growth is referred to as diauxic growth.
  - *The Log Phase*: The lag phase is followed by log phase in which there are an exponential growth of cell mass and increase in cell number and at the same time nutrients are exhausted leading to deceleration phase.
  - *The Deceleration Phase*: This phase is characterized by accumulation of toxic compounds. This period is short.
  - *The Stationary Phase*: As the nutrient in the growth medium gets exhausted, and the metabolic end products that are formed in the process inhibit the growth, the cells enter the stationary phase. The microbial growth slows down gradually. The biomass produced remains constant during stationary phase and it is in this phase the secondary product or the antibiotic is produced.
  - *The Death Phase*: This phase is associated with termination of metabolic activity and decrease of energy reserves. The cells die at an exponential rate in this phase. This phase is of huge application in the commercial and industrial fermentations as the cells are harvested in this stage after the cells halt their metabolic activities finally.
- *Fed-Batch Culture*: Fed-batch culture is an improvised version of batch fermentation wherein the substrate is added in instalment at different stages during the ongoing fermentation. The periodical addition of substrate increases the time period for log and stationary phases which results in an increased biomass leading to production of and also increases in production of metabolites such as antibiotics.
- *Continuous Culture*: Continuous culture cultivates microorganisms at submaximal growth rates at different growth parameters in such a way that the culture

conditions remain constant (in "steady state") over long periods of time. Continuous culture principles were first applied in the labs of Herbert, Monod and Novick. Continuous fermentation is carried out in an open system. The steady state is maintained with simultaneous removal and replacement of culture medium to keep the working volume constant in a bioreactor. A bioreactor is a vessel in which a biological reaction takes place. A bioreactor includes all the parameters required for the growth of microorganisms. The bioreactor is a place where an optimum external environment is maintained to support a high yield biological process. A bioreactor is available both for plant metabolites and microbial metabolites.

1.7.2.1 Measurement of Microbial Growth Kinetics

Growth kinetics study tells about the direct proportionality between growth and the concentration of cell (Sakthiselvan et al. 2019). The concentration of cell is measured by both direct and indirect method as already mentioned in previous section. One direct method is to use the spectrophotometer to measure the absorbance of cell suspensions. Using this technique the absorbance is plotted as a standard curve versus cell concentration and thus the cell concentration of an unknown sample determined. A counting chamber can be used to perform direct microscopic counts of cells. For this, electronic counting chambers can be employed. Indirect viable cell counts can be done with plate counts by diluting cultures on nutrient medium. Turbidity is another way of measurement for counting large numbers of bacterial cells in clear liquid media and broths—but cannot count cell densities less than 10^7 cells/mL. Measuring biochemical parameters such as O_2 uptake, CO_2 production and ATP production is another method to count cell culture. Bacterial growth rates or the generation time is measured during the exponential growth, under the influence of standard nutritional conditions such as culture medium, temperature, pH, etc. Bacterial cells multiply exponentially by binary fission and the increase is a geometric progression. The generation time is the time interval required for cells' population to divide and designated by the following equation (Todar 2020):

$$G = t/n \tag{1.2}$$

where:

G is generation time
n is number of generations
t is time in min/h

The equation for growth by binary fission is:

$$b = B \times 2^n \tag{1.3}$$

where:

b is number of bacteria at the end of a time interval
B is number of bacteria at the beginning of a time interval
n is the number of generations (number of times the population doubles in the time interval)
$\log b = \log B + n \log 2\, n$
$n = (\log b - \log B)/\log 2$
$n = (\log b - \log B)/0.301$
$n = 3.3 \log b/B$

$$G = t/3.3 \log b/B \tag{1.4}$$

Growth kinetics can be classified based on relationship between synthesis of product and generation of energy in the cell such as:

- Growth associated: Product formation is related directly to growth of cells.
- Non-growth associated: The products formed are the end product of the metabolic activity of the cell. The byproducts formed are called secondary metabolites. Product formed is not linked to growth rate but it is a function of cell concentration.
- Mixed-growth associated: The formation of product depends on both growth rate and cell concentration.

Product kinetics: Microbial growth kinetics is the relationship between the specific growth rate (μ) of a microbial population and the substrate concentration (s). It is an important tool in microbiology. Of all the different kinetic equation proposed, Monod equation is most accepted. The model specifies that the microbial specific growth rate μ depends on the growth-limiting substrate (S) (Manikandan et al. 2008; Song et al. 2010) such that:

$$\mathrm{d}x/\mathrm{d}t = \mu x \tag{1.5}$$

$$\mu = \mu_{\max} S/(K_{\mathrm{s}} + S) \tag{1.6}$$

where μ is specific growth rate (day^{-1}), $\mu_{\max}$ is maximum specific growth rate (day^{-1}), K_{s} is half-saturation constant (mg/g), x is microbial cells measured as glucosamine concentration (mg/g), and S and t are initial substrate concentrations (mg/g) and time (day), respectively.

Conclusion: Kinetic model studies have wide application in the evaluation of the product formation kinetics. The mathematical model helps in the data analysis and provides a solution to difficulties experienced in fermentation. It also helps in the batch process performance.

1.8 Maintenance and Preservation of Microbial Cultures

Microorganisms are important both commercially and ecologically (Hayat et al. 2010; Senni et al. 2011). Other than that they are considered as the support system of biotech industry (Stahl and Wagner 2006; Vibha and Neelam 2012) and are also natural source of novel therapeutics and biomolecules used in pharmaceutical industry (Zaidi et al. 2009; Hayat et al. 2010). Therefore, proper maintenance and preservation of microbial cultures is a prerequisite to ensure long-term availability and viability of microbes without losing their morphological, physiological and genetic traits (Ward et al. 2001). Agar slant culture, stab culture, saline suspension, glycerol and DMSO preservation, drying on silica gel, drying on soil, sterile water, lyophilization, cryopreservation, etc. are the laboratory techniques used for preservation of microorganism. However, the choice of method to be used depends on type of microorganism to be used and purpose of and duration of preservation. In this regard, culture collections (CCs) and microbial biological resource centres (mBRCs) play an important role in the preservation and circulation of biological resources. Both are ex situ repositories for biodiversity and provide collection of useful microorganisms and related information, for research (Caktu and Turkoglu 2011; Stackebrandt et al. 2014). Preservation of microbes is a very old process (Nakasone et al. 2004). Different types of methods have been regularly employed for the preservation of microorganisms which include repeated subculturing, on agar beads (Winters and Winn 2010), oil overlay of slant-grown cultures (Nakasone et al. 2004), use of silica gel (Liao and Shollenberger 2003), cryopreservation (Gorman and Adley 2004) and lyophilization (Berner and Viernstein 2006). Here in this section, we shall discuss in brief the techniques used in preservation of microbes for long-term and short-term storages.

- *Continuous culture*: In this method cultures are grown in agar for short-term storage and are stored at 4–21 °C to delay the time period required for subculturing. Fresh cultures of the strain can be maintained from the previous stock culture. This method is usually done in an agar slant or agar stab media in a test tube. Medium used is nutrient agar or potato dextrose agar. In this medium the cultures remain viable for weeks to several months (Kumar et al. 2013).
- *Drying method*: It is the most useful method for storing produced spores. Silica gels, glass beads and soil are the common media for drying. For example, fungi have been stored in silica beads in this way for years (Kumar et al. 2013).
- *Refrigeration*: Using this method the microbial cultures in agar slant, stab media or Petri plate are stored at 4 °C. The cultures are properly sealed (Kumar et al. 2013).
- *Mineral oil/liquid paraffin storage*: Fresh grown cultures in slants can be covered in mineral oil or liquid paraffin to preserve the culture for longer period of time and to cut off the access to oxygen to slow down the metabolic rate and prevent the cultures from drying. The most commonly used oil is paraffin oil or Vaseline with a thickness of 1–2 mm (Kumar et al. 2013).

- *Freezing*: This technique is the most preferred technique to keep the cells viable at very low temperatures. Freezers used for this are of three types (Kumar et al. 2013).
- *Freezing bacteria using glycerol*: Though freezing has its advantage, there is certain drawback about it. The ice crystals formed might damage the viability of the culture by dehydration caused by increase in local concentration of soil. To lessen the effect of freezing, cultures can be preserved using 15% glycerol. The microfuge tubes with screw cap and sterile glycerol are used. The glycerol is diluted to 30%, and equal amount of glycerol and broth are mixed along with the culture and then frozen (Kumar et al. 2013).
- *Freeze-drying or lyophilization*: Lyophilization involves removal of water from the sample which serves a purpose for enzymatic reaction. It freezes sample with the aid of a lyoprotectant (usually a sucrose) and pulling out of water using a vacuum helps in the preservation of cultures (Prakash et al. 2013). Lyophilization preserves many bacteria, yeast and sporulating fungi, but does not preserve non-sporulating fungi.
- *Cryopreservation:* Cryopreservation uses preservation of biological materials at cryogenic temperatures, at −80 °C (dry ice) or at −196 °C (liquid nitrogen). Low temperature acts as a protective shield for proteins and DNA from denaturation and damage and slows the water movement in the cell. As a result, biochemical and physiological activities of the cells are kept halted, and cells are preserved for long periods of time (Prakash et al. 2013).

1.9 Strain Improvement

Strain improvement is the technology of genetically changing microbial strains to improve their potential and metabolic activity. Genetically improved strain has several application in numerous biotechnology applications, fermentation techniques and assay. Wild strains are not capable of producing high yield metabolites; however the yield can be increased by optimizing the fermentation process. Metabolite production is genetically determined; therefore new genetic strains have to be developed for increased product formation in a cost-effective way. Successful development of improved strains requires a proper knowledge of physiology and metabolic activity (Parekh et al. 2000). Microorganisms produce new genetic characters ("genotypes") by two processes: mutation and genetic recombination. Mutations are of two types: spontaneous mutation or induced mutation. The benefits of mutation are of application to humans. This has been a breakthrough in the fermentation industry. The main development of microbial genetics began in the late nineteenth century with the onset of penicillin production. The benefits of mutation led to a growing interest to genetically engineer microorganisms. Thus the collaborative "strain-selection" experimental programme was started among researchers at the US Department of Agriculture laboratories in Peoria, the Carnegie Institution, Stanford University and the University of Wisconsin, followed by the extensive worldwide strain improvement programme

in various industrial laboratories throughout the world to develop genetically improved strain (Adrio and Demain 2006).

1.9.1 Applications of Mutation

Mutation has a great role in improving the yield of microbiological product (Vinci and Byng 1999). The spontaneous mutations are of low frequency, not suitable for industrial purposes. Therefore mutations may be induced in the laboratory by mutagenic agents such as ultraviolet light and various chemicals (nitrosoguanidine (NTG), 4-nitroquinoline-1-oxide, methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS) and hydroxylamine (HA)). Site-directed mutagenesis is also important for strain improvement. The strain that survives mutation is plated out, selected randomly for growth in flasks for future prospects (Simpson and Caten 1979). Selection of mutants can be done in two ways (Schwab 1988):

- *Random screening*: The mutated strain is selected for their ability to produce the desired product with a model fermentation unit. The strain with the maximum yield is selected.
- *Selective isolation of mutants*: There are several ways such as:
  - Isolation of antibiotic resistant strain: Both the wild and the mutant are grown in a media containing antibiotic. The wild strain dies out and the mutant strain with the antibiotic strain survives.
  - Isolation of antimetabolite strain: Antimetabolite having structural similarity to metabolite may inhibit the metabolic pathway and kill the cell. Mutant strain having resistant gene to the antimetabolite survives and can be selected for industrial purpose.
  - Isolation of auxotrophic strain: An auxotrophic may be characterized by detecting the defect in one of the biochemical pathways.

1.9.2 Recombination

The strain improvement can be done by exchanging of genetic information between two strains, by a process called genetic recombination. The recombination can be done by transformation, transduction, conjugation and protoplast fusion. Protoplast fusion is in much use.

- *Protoplast fusion*: Since sexual reproduction does not exist in prokaryotes, therefore, genetic recombination in microorganisms is done by parasexual mechanisms which is not of much efficiency. Protoplast fusion techniques are of great advantage to be applied to newly isolated, not fully characterized, strains (Ferenczy 1981).

- *Recombinant DNA*: Recombinant DNA technology (genetic engineering) is the combination of all those newly developed methods that allows for the isolation, characterization, manipulation, expression of specific genes, etc. rDNA technology can transfer specific genes between organisms. Genetic engineering has been instrumental instrument in the production of mammalian proteins by microorganisms; examples include human insulin, interferon blood proteins, peptides of the neurosystem and also proteins used as vaccines which are presently under development (Riggs et al. 1984). Organisms such as *Escherichia coli*, *Bacillus subtilis* and *Saccharomyces cerevisiae* are used as hosts (Schwab 1988). Cloning systems have now been developed for use in genetically engineered strains for commercial purpose.
- *rDNA technology*: rDNA techniques for strain improvement are used for the controlled expression of specific genes. Such genes are obtained either from homologous or from heterologous systems with the aid of cloning using shuttle vectors. Three points have to be considered to have an effective rDNA technology for its application in microbial system (Schwab 1988):
  - DNA transfer systems
  - Vectors' availability with selection systems
  - Efficient functional control elements for gene expression
- *DNA transfer systems*: DNA transfer into living cells is an important requirement. The following strategies may be applied to achieve so (Schwab 1988):
  - Transformation
  - In vitro packaging of DNA into phage heads and transferring to host cells via phage infection
  - Microinjection
  - Conjugative plasmid transfer from an intermediate host

1.10 Microbe-Human Interdependence

It may appear to us that we humans are a single living creature embodying flesh and blood, but that's not the entire truth. We are in fact full of microscopic organisms that are found inside and outside of the body. Such microscopic organisms which are known as microbiome have advantages on our physiological and mental health (Johnson and Versalovic 2012). Today, researchers have realized the need for a more holistic approach towards the microbial world around us: Microbes and their hosts (including humans) are interdependent for survival. All organisms are not harmful; some are beneficial too. Scientists believe that about half of all human DNA genetic material originated from viruses that infected our ancestors' egg and sperm cells. In fact, our bodies contain at least ten times more bacterial cells than human ones (Sender et al. 2016). Microbes present in the human gastrointestinal tract alone comprise at least ten trillion organisms, representing more than 1000 species, which prevent colonization of pathogens in the gut (Drexler 2010). Due to limitations in

technology, the complex and dynamic nature of our microbiota was not fully recognized. Recent endeavours such as the Human Microbiome Project (HMP) (Peterson et al. 2009), hosted by the US National Institutes of Health, and the Metagenomics of the Human Intestinal Tract (MetaHIT) project (Ehrlich and MetaHIT Consortium 2011), through the European Commission, have initiated extensive research aimed at surveying and analysis of the collection of microbial genes and genomes collectively termed the microbiome. The efforts of the HMP have produced >70 million 16S ribosomal gene sequences characterizing the microbial communities across 15 body sites and >3.5 tera-base pairs (Tbp) of whole-genome shotgun metagenomic data encoding >60 million predicted genes (Methé et al. 2012; The Human Microbiome Project Consortium 2012).

1.10.1 Health and Disease

Microorganisms have profound impact on human health via different mechanisms. One such component linked to human health is pathogen-associated molecular patterns (PAMPs). PAMPs are molecules such as endotoxin and lipopolysaccharide (a component of bacterial cell membranes), flagellin (from bacteria) and (1–3)-β-D-glucans (also referred to as triple helical glucan, fungi wall membrane component). These molecules are found in microbes (bacteria or fungi) which affect human innate immune system and epithelial cells in the respiratory tract (Lambrecht and Hammad 2013, 2014). For example, many indoor fungi produce such metabolites that can induce respiratory allergic response (Kuhn and Ghannoum 2003).

The relationship between humans and microbes is profound. Both are dependent on each other through benefits and loss (Hess et al. 2011). For example, connection has been found between the resident gastrointestinal microbiota and atherosclerosis. A metabolic pathway was diagnosed that involves microbial metabolism of dietary phosphatidylcholine, resulting in the synthesis of the metabolite trimethylamine N-oxide (TMAO), a biomarker for cardiovascular disease (Wang et al. 2011). A recent study described the generation of the microbiota among individuals with a genetic link for celiac disease (Sellitto et al. 2012). Interest in proper understanding of the human microbiota consortia in relation to health and disease is fundamental for the development of personalized medicine.

1.10.2 Gut Microbiome

The human gastrointestinal (GI) tract contains complex and dynamic population of microorganisms, the gut microbiota, which has a great impact on the host in health and disease. The microorganisms inhabit the gut during infancy, and factors like diet are responsible for the establishment of gut microbiota within a lifetime (Thursby and Juge 2017). The collection of different types of microbes such as bacteria, archaea and eukaryote colonizing the GI tract is termed as the “gut microbiota”. They have co-evolved with the host over the past years to form a mutually beneficial

relationship (Bäckhed et al. 2005). It is estimated that 10^{14} number of microorganisms inhabit the GI tract (Bäckhed et al. 2005; Gill et al. 2006). However, a recent revision of the survey has reported that the ratio of human-bacterial cells is actually closer to 1:1 (Sender et al. 2016). Growing interest towards the human gut microbiome has increased in recent dates due to the rapidly advanced technology such as sequence-based screening and human gnotobiotic model in analysing the dynamism of commensal microbiota. As mentioned gut microbiome plays a pivotal role in maintaining the immune and metabolic homeostasis of the body. Therefore gut microbial imbalance (dysbiosis) may lead to faulty host machineries, thereby contributing to pathogenesis and associated broad spectrum of illness. Some of the most noteworthy diseases to be mentioned which are associated with gut microbiome dysbiosis include *Clostridium difficile* infection (infectious disease), inflammatory bowel disease (intestinal immune-mediated disease), celiac disease (multisystemic autoimmune disorder), obesity (metabolic disease), colorectal cancer and autism spectrum disorder (neuropsychiatric disorder). Analysis of this mutual relationship of human and microbe has great potential in future research in medical science. Novel therapies derived from microbiome studies such as faecal microbiota transplantation, probiotic and prebiotics have been implemented to correct the dysbiosis, thus revealing a new scientific breakthrough towards disease treatment (Kho and Lal 2018).

1.10.3 Emerging and Re-emerging Diseases

Emerging diseases are infectious disease whose frequency of occurrence in humans has increased within the recent past or threatens to augment in the near future. They may be the outbreaks of earlier unknown diseases that are rapidly increasing in occurrence or geographic area in the last two decades. These may include new or old diseases with new features. These new features may include the introduction of a disease to a new area or a new vulnerable population, with new clinical features, including resistance to available drug (Dikid et al. 2013). Many risk factors add to the emergency and rapid spread of epidemics in the region including severe and long-lasting humanitarian emergencies, resulting in weak health systems, increased population mobility, speedy urbanization, sudden climate change, weak surveillance and less laboratory diagnostic capacity and increased human-animal interaction (Buliva et al. 2017). Emerging diseases include lower respiratory tract infections, diarrheal diseases, HIV/AIDS, malaria and tuberculosis (TB), Middle East respiratory syndrome, extensively drug-resistant tuberculosis (XDR TB) and Zika virus (Morse 1995; Vos et al. 2015). Re-emerging diseases are the ones that make reappearance after they have been on a major fading phase. Re-emergence happens when there is a violation of protocol in public health measures for diseases that were contained. They can also happen if mutant strains of known disease-causing organisms emerge. Also human behaviour may be one of the reasons for re-emergence. For example, overuse of antibiotics has led to emergence of mutant strains that are resistant to existing treatment. It is reported that about 30 new

infectious disease-causing agents have been detected worldwide in the last three decades and most of them are zoonotic in origin, causing the emerging and re-emerging of infections leading to epidemics and pandemics. Besides health issues, emerging infections also affect the economic development. As such in the recent past, the emergence of SARS caused by coronavirus (Dikid et al. 2013) and again the re-emerging of the novel coronavirus infection in the form of Covid-19 have devastated the global economy.

1.10.4 Epidemic and Pandemic Diseases and Microbes

Infectious diseases leading to epidemics, pandemic and endemic have caused devastation to humanity throughout its existence, often changing the course of history. All these three words epidemics, pandemic and endemic are from Greek literature, and the one they have in common is demic (pertaining to people or population) (Bharat Biotech 2020).

- *The term epidemic* is used when an infectious disease affects a substantial number of people in a given city, region or country for a fixed period of time.
- *Pandemic* is when a particular outbreak extends beyond national borders, across a continent or even around the globe. It causes a deadly illness that is transmitted easily from one person to another, which spreads across a wide geographic area, affecting and even killing many people. In history, there have been a number of devastating pandemics. And the most impactful pandemic which till today has wrapped the globe is the coronavirus pandemic of 2020. On March 11, 2020, coronavirus was officially declared a pandemic by the World Health Organization.
- *An endemic* is the persistence of an infectious disease in a particular geographic region which affects a large part of the population. They remain at a stable state, but do not vanish from the population. For example, chickenpox is categorized as an endemic.
- *The term Outbreak* is used when the number of cases of a disease exceeds the normal expectation for the region or season.

The emerging infections may offer a real challenge for the entire globe. A strategic response must be approached towards the problem at the core level. A well-planned international strategy on fighting infectious diseases addressing the issues of emerging and re-emerging diseases should be taken up by both governmental and non-governmental agencies.

1.11 Benefits of Microbial Activity in Food and Industry

Nature utilizes microorganisms for fermentation, and since time immemorial mankind has used yeasts, moulds and bacteria to make food products such as bread, beer, wine, vinegar, yoghurt and cheese, as well as fermented fish, meat and vegetables without the prior knowledge of microbes being involved in the process. Fermentation is a traditional method for food transformation and preservation. This method allows to retain the nutritional and organoleptic qualities (relating to the senses: taste, sight, smell, touch) of food. The first discovery that microorganisms were involved in food production processes was made in the early years of 1800; yeast was found to have a role in alcoholic fermentation in the formation of beer and vinegar by French chemist and biologist Louis Pasteur (Pai 2003). However, it was during the World War I that the technological microbiology gained popularity. This started with the demand for glycerol for the manufacture of explosives during World War I (Wang et al. 2001) and the large-scale production of penicillin, discovered by Fleming, in the 1940s (Neushul 1993). The end of World War II witnessed the expansion of the American economy known as the Golden Age of Capitalism (Marglin and Schor 2000), and the growing interest in the knowledge of microbial genetics (Susman 1970; Bagdasarian 1982). This triggered the appearance of microorganism-based industrial processes, making way for modern technological microbiology. However, technological microbiology found legal validation in the 1980s, following a decision made by the US Supreme Court to patent *Pseudomonas putida* variant, by Ananda Chakrabarty, that it is efficient in naturally digesting compounds found in crude oil spills (Robinson and Medlock 2005). This patent was a revolution in biotechnology that resulted in the thousands of other patents that made way for establishing hundreds of bioengineering and food plants (Holloway 2015). Later with the progression of knowledge on genetic manipulation, the modification of *Escherichia coli* by genetic engineering led to the production of artificial insulin, which was the first product obtained and a breakthrough from recombinant DNA technology (Walsh 2012), approved by the US Food and Drug Administration in 1982 (Johnson 1983). Eventually applied microbiology became a science essentially applied to several branches of production, including food, chemical, agricultural and pharmacological. Here in this section, we present some application of microbiological fermentation in food industry.

1.11.1 Application

- *Production of organic acids*
  - Citric acid is produced by fermentation with *Aspergillus niger* in large corrosion fermenter using stirrer. The aid of yeast such as *Candida albicans* has also been useful in the process (Roehr 1996).
  - Lactic acid another important organic acid is produced by *Lactobacillus delbrueckii* (Rabbani Khorasgani and Shafiei 2017), *Lactobacillus casei* (Panesar et al. 2010) and *Lactobacillus acidophilus* (Bull et al. 2013).

  - *Gluconic acid*: It is produced by fermentation of *Aspergillus niger* (Shindia et al. 2006).
- *Production of industrial enzymes*: Enzymes have been used in food industry for various reasons such as making of bread, fermentation of juice and malt and clotting of milk to make cheese. Rennet a complex set of enzymes occupies 25% of worlds' market. It is produced in any mammalian (calf) stomach and is often used in the production of cheese. The active enzyme present in rennet is called chymosin or rennin. Glocoamylase (20%) and then glucose isomerase (5%) are high in demand for enzyme. Also genetically modified microorganisms such as *Eschericia coli* containing calf rennet were developed. *Bacillus spp*. such as *Bacillus acidopullulyticus* produces pullulanase; alpha-amylase is produced by *Bacillus amyloliquefaciens*, glucose isomerase by *Bacillus coagulans*, etc. (Pai 2003).
- *Production of amino acids*: Amino acids are an important component in food industry. Amino acids such as α-glutamate are used as flavour enhancer, glycine as sweetener, lysine and methionine as food and feed additive and phenylalanine and aspartame as a low calorie sweetener. All these are prepared either chemically or enzymatically. Microorganisms such as *Corynebacterium glutamicum* used for preparation of glutamate, lysine, threonine, etc. are used to produce these amino acids. Some genetically modified organisms such as *Escherichia coli*, *Serratia* spp., *Bacillus* spp., *Hansenula* spp., *Candida* spp., and Saccharomyces spp. are used in amino acid production (Pai 2003).
- *Effects of lactic acid fermentation on the nutritional aspects of food*: Lactic acid bacteria (*Lactobacillus*, *Leuconostoc*, etc.) have been used to enhance flavour, texture, preservation and nutritive value of dairy products and other foods. Some fermented dairy products are also used as probiotics (Rezac et al. 2018). The quality of a food is dependent on nutrient content as well as the digestibility, which may be improved by the process of fermentation (Nkhata et al. 2018). Fermentation also has great impact on nutritional diseases (Hill et al. 2017). Fermentation of food contributes with the bioavailability of vitamins such as niacin, thiamine, folic acid or riboflavin (Melini et al. 2019). Iron absorption of food is enhanced by breaking it into inorganic iron with vitamin C (Nkhata et al. 2018). Food fermentation also helps in uptake of the mineral and trace elements by reducing the non-digestible content in plants such as glucuronic and polygalacturonic acids, cellulose and hemicelluloses (Gupta et al. 2015). It also reduces blood cholesterol by inhibiting cholesterol synthesis in the liver and also inhibits dietary cholesterol absorption in the intestine (Jesch and Carr 2017). Lactic acid fermentation product also provides relief to infections such as diarrhoea and salmonellosis (Minh 2014).
- *Alcohol production using improved cultures*: Yeast is used for beer brewing. Strains such as *Saccharomyces cerevisiae* and *Saccharomyces uvarum* have been commonly used (Pai 2003).
- *Miscellaneous application*: *Candida utilis* has been used in industry to produce single-cell protein to be used as food and fodder, waste treatment and production of flavour enhancer (Pai 2003).

1.12 Conclusion

- The introduction on how microbes impact our lives and are involved both in good health and sickness through their benefit and harm is elaborated. This chapter gave a clear insight into isolation, mass cultivation, growth kinetics and preservation strategies for the industrial application of microbes. The types of microbes based on their ability to grow in certain conditions were explained. Also, strain improvement strategies were discussed with their industrial implications along with an overview on how microbes can be useful for the generation of novel products that have extensive applications in the biotechnology industry. This interactive and engaging chapter will be useful in the fields across the biological, environmental and health sciences, and for the interested reader wanting to explore more about these ubiquitous microbes.

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