Microorganisms in Fermentation

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What You Will Learn in This Chapter

Microorganisms [bacteria, fungi (yeasts and mold)] have been adopted successfully in a wide range of industries, from food and beverage processing industries to pharmaceutical operations. Additionally, microorganisms offer tremendous unexploited potential for value-added products such as amino acids, nucleotides and nucleosides, vitamins, organic acids, alcohols, exopolysaccharides, antibiotics, antitumor agents, etc., through various fermentation processes and parameters. This chapter reviews the involvement of various groups of microorganisms in fermentation. The measurement of microbial biomass, growth and kinetics, and factors affecting fermentation processes are also explained. The roles of microorganisms (bacteria and yeasts) involved in fermentation processes [solid-state fermentation (SSF) and submerged fermentation (SmF)] mostly related in processing industries are discussed.

1.1 Introduction

The term "fermentation" is borrowed from the Latin word *fevere* which means "to boil." According to Louis Pasteur, fermentation was defined as "La vie sans l'air", i.e., life without air, and the science of fermentation is also known as zymology or zymurgy. However, fermentation is one of the oldest food storage/preservation methods throughout the world dating back to the Neolithic period (10,000 years BC) [39, 41]. In biochemical sense, it is the metabolic measure in which complex organic compounds (particularly carbohydrates) are broken down into simpler compounds without the involvement of oxygen (exogenous oxidizing agent) along with the generation of energy (ATP molecules).

The end products of fermentation differ depending on the microorganism. The scientific motivation behind fermentation started with the naming and identification of microorganisms (in 1665) by Leeuwenhoek and Hooke [15]. Thereafter, Pasteur dismantled the "spontaneous generation theory" in 1859 by ideally designed experimentation. However, the performance of an exclusive bacterium *Lactococcus lactis* in fermented milk was searched out by Sir Joseph Lister in the year 1877 [44]. Generally, microbes in the form of distinct cell or group of cells, usually bacteria, sometimes fungi, algae, or cells of animal or plant origin, are involved in the process of fermentation. Several parameters are associated with the fermentation process, such as concentration of microbial cells and their constituents (enzymes), and conditions like pH, temperature, and fermented medium (aerobic/anaerobic). In general, the microbial fermentation is divided into four categories, viz., (i) generation of biomass (viable cellular material); (ii) production of metabolites; (iii) synthesis of enzymes, vitamins, and proteins; and (iv) transformation/conversion of substrate into value-added products [56]. The current chapter provides an overview of the microorganisms involved in fermentation, growth and kinetics, factors affecting fermentation, bio-products (primary and secondary metabolites) derived from fermentation processes, and uses of probiotics in food fermentation.

1.2 Microbial Diversity: an Overview

There is no universal accord on how to categorize/classify microorganism and such classification is rather arbitrary. However, there are two basic/primary cell types: *prokaryotic* and *eukaryotic*. The fundamental difference/characteristic between them is the presence or absence of membrane around the cell's genetic information.

Prokaryotes are unicellular organism and have simple structure with a single chromosome. *Prokaryotic* cell lacks membrane-bound organelles (i.e., mitochondria and endoplasmic reticulum) and also lacks nuclear membrane.

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Eukaryotes have a more complex internal structure, with more than one chromosome (DNA molecule) in the nucleus. *Eukaryotic* cells have a true nuclear membrane and hold membrane-bound organelles (i.e., golgi apparatus, mitochondria, endoplasmic reticulum) and a variety of functional/specialized organelles. A detailed contrast/comparison of *prokaryotes* and *eukaryotes* is presented in Table 1.1. Evidence/data recommends

Table 1.1 Comparison between *Prokaryotes* and *Eukaryotes* [48]

Characteristics	Prokaryotes	Eukaryotes
Genome		
Number of DNA molecules	One	More than one
Genetic (DNA) organization	DNA is circular, without protein	DNA is linear and associated with proteins to form chromatin
	Chromosome found freely in a cytoplasomic region called the *nucleiod*	Chromosome found in a membrane bound nucleus
	Naked i.e., not bound with protein and therefore doesn't form chromatin	Bound with histone proteins
DNA in organelles	No	Yes; (chloroplast and mitochondrial genome)
Plasmid	Contain extra-chromosomal plasmids	No (but organelle like mitochondria may contain their own chromosome)
Nuclear membrane	No	Yes
Cell division	Binary fission/budding	Mitosis or meiosis
Organelles		
Mitochondria	No	Yes
Endoplasmic reticulum	No	Yes
Golgi apparatus	No	Yes
Photosynthetic apparatus	Chlorosomes	Chloroplasts (plants)
Ribosome	70S	80S
Flagella	Single protein, simple structure	Complex structure, with microtubules
Spores		
Types	Endospores	Endo- and exospores
Heat resistance	High	Low
Example		
	Bacteria and archaea	Fungi, protozoa, algae and helminths

Table 1.2 Primary subdivision of cellular organisms

Group	Cell structure	Properties	Constituent groups
Eukaryotes	Eukaryotic	Multicellular	Plants (i.e., seed plants, ferns, mosses)
Eubacteria	Prokaryotic	Similar to *eukaryotes*	Most of bacteria
Archaebacteria	Prokaryotic	Distinctive properties	Methanogens, thermophiles,halophiles

that a universal/common ancestor (family history) gave rise to three distinctive/inherent branches of life: *Eukaryotes*, *Eubacteria* (or "true bacteria"), and Archaebacteria. Table 1.2 complies some of the inherent aspects/features of these groups.

1.2.1 Prokaryotes

The sizes of maximum or most prokaryotes differ from 0.5 to 3 micrometers (μm) in length (or equivalent radius). Different species have different shapes such as spiral or spirillum (e.g., *Rhodospirillum*), spherical or coccus (e.g., staphylococci), or cylindrical or bacillus (e.g., *Escherichia coli*). Prokaryotic cells grow rapidly, with usual doubling times of one-half hour to several hours. Moreover, prokaryotes can use/take advantage of a variety of nutrients as carbon source, including hydrocarbons, carbohydrates, proteins, and carbon dioxide [48].

Eubacteria

Eubacteria (genus of gram-positive bacteria in the family *Eubacteriaceae*) are microscopic single-celled organisms, characterized by rigid cell wall, and grow in diverse environments. The *Eubacteria* can be divided into several groups. One differentiation is established on the gram stain—the name comes from Danish bacteriologist Hans Christian Gram who developed the technique in 1884. The staining process first requires fixing/mending the cells (by heating), and then crystal violet (basic dye) is added; all bacteria will stain purple. Next, iodine is added (binds crystal violet), followed by the rapid decolorization/addition of ethanol (or acetone). ***Gram-positive*** cells stay purple, while ***gram-negative*** cells become colorless. Finally, counterstaining with safranin or fuchsine leaves *gram-positive* cells purple, while *gram-negative* cells are red. This ability to react with the gram stain differentiates bacteria by the chemical and physical properties of their cell wall (or structure of the cell envelope) [48].

A typical/ideal *gram-negative* cell is *E. coli* (Fig. 1.1). It has an outer membrane supported by a thin peptidoglycan (also known as *murein*) layer. Peptidoglycan is a polymer of sugars (polysaccharide) and amino acids that forms a structure similar to a mesh/chain-link fence/layer. Peptidoglycan provides structural strength as well as resists (opposing action) the osmotic pressure of the cytoplasm. A second membrane (the inner or *cytoplasmic membrane*) prevails and is detached from the outer membrane by the *periplasmic space*. The *cytoplasmic membrane* (bacterial *plasma membrane*) is composed of phospholipid bilayer

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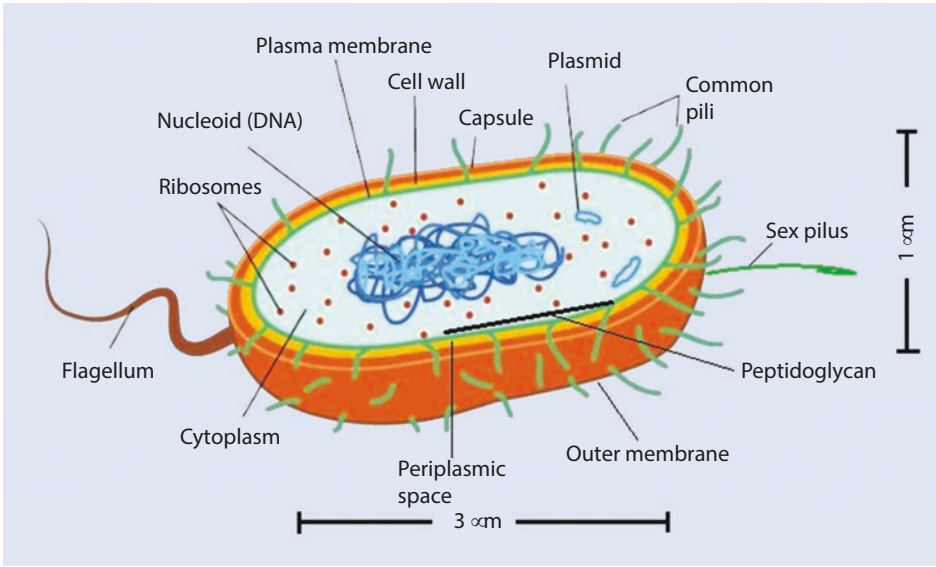

Fig. 1.1 A typical/ideal *gram-negative* cell (*E. coli*)

(50% protein, 30% lipids, and 20% carbohydrates). The cell envelope (capsule of polysaccharide) serves to protect, reserve/retain important cellular compounds, and in turn exclude/prevent undesirable compounds in to the cell environment. It provides structural/membrane integrity and loss of membrane integrity that leads to *cell lysis* and cell death. The cell envelope is essential to the transport of preferred material in and out of the cell [30].

A typical *gram-positive* cell is *Bacillus subtilis*. *Gram-positive* cells do not have an outer membrane. Rather they have a very thick, rigid cell wall with multiple layers of peptidoglycan and are made up of glycan strands that are cross-linked by peptide side chains. *Gram-positive* cells also contain *teichoic acids* covalently bonded to the peptidoglycan (wall *teichoic acids*) or are attached to the lipid membrane (lipo *teichoic acids*). Because *gram-positive* bacteria have a single cytoplasmic membrane, they are often much better suited/appropriate to extraction of proteins. Such extraction can be technologically advantageous when the protein is a desired product. Spore-forming *gram-positive* bacteria (*B. subtilis*) produce morphologically specific daughter cells by asymmetric cell division. The cell wall of spores varies from that of mother cells and has specific sets of proteins [36]. Unlike eukaryotic DNA, which is neatly packed into a cellular compartment called the *nucleus* (within the *nucleus*), bacterial DNA floats freely in the cytoplasm, in a twisted thread-like mass called the *nucleoid* [48].

Actinomycetes are bacteria (generally *gram-positive*, anaerobic), but, morphologically, they resemble molds with their long filaments and highly branched *hyphae*. However, the lack of a nuclear membrane and the composition of the cell wall require/appropriate classification as bacteria. *Actinomyces*, *Thermomonospora*, *Streptomyces*, *Microbacterium*, *Bifidobacterium*, *Brevibacterium*, *Corynebacterium*, *Micrococcus*, and *Brachybacterium* are examples of the genera convenient to this group [62]. *Actinomycetes* are essential sources of antibiotics. Over 500 species of *Streptomyces* bacteria have been recognized as the builders of abundant bioactive metabolites, such as antibacterials, antivirals, antifungals, and

enzyme inhibitors. Certain *actinomycetes* possess amylolytic [23, 26], pectinolytic [25], and cellulolytic [6] enzymes and are useful in enzymatic hydrolysis of starch, pectin, and cellulose, respectively.

Other/alternative characteristics within the *Eubacteria* can be made based on cellular nutrition and energy metabolism. One important example is photosynthesis. The ***cyanobacteria*** (*cyanophyta* or blue green algae) obtain their energy through photosynthesis and have chlorophyll, fix CO_2 into sugars, and are able to produce oxygen. Some of the *cyanobacteria* are nitrogen-fixing (fix atmospheric nitrogen in anaerobic condition) by means of specialized cells called *heterocysts*.

Prokaryotes may have other visible structures/organizations when observed/viewed under the microscope, such as *ribosomes*, *storage granules*, *spores*, and *volutins*. *Ribosomes* are the site of biological protein synthesis (translation). Prokaryotes have 70S *ribosomes*, are made of 50S and 30S subunits, and are highly complex cellular structures [48].

Storage granules (which are not present in every bacterium) act as reservoir—nutrients can be stored in the cytoplasm. *Storage granules* are source of basic/key metabolites in the form of polysaccharides (glycogen), polyphosphates, lipids, nitrogen, and sulfur granules. *Volutins* (polyphosphate bodies) are another granular intracytoplasmic/intracellular energy-rich compound, composed of inorganic polymetaphosphates, present in some species (e.g., A*grobacterium tumefaciens* and *Rhodospirillum rubrum*) [47]. It acts as a reserve store of energy and of phosphate, and, in some cases, nucleic acid, protein, and lipids are associated with these granules. Some photosynthetic bacteria (e.g., *Rhodospirillum rubrum*) have *chromatophores* which are large inclusion bodies (pigment-containing and light-reflecting cells) utilized in photosynthesis for the absorption of light [30].

Some bacteria make intracellular spores or *endospores*. Bacterial *endospores* (seed like) are dormant, tough, and nonreproductive structures and are produced as a resistance to adverse conditions (i.e., high temperature, radiation, and toxic chemicals). Spores can germinate under favorable growth conditions to yield actively growing bacteria. Certain bacteria (e.g., *Streptococcus, Bacillus antracis*) have a coating or outside cell wall called *capsule*, which is usually a polysaccharide (lipopolysaccharides) or polypeptide (lipoproteins). Many bacterial cells secrete some extracellular material in the form of *slime layer* which protects the bacterial cells from environmental changes (e.g., antibiotics and desiccation). Unlike bacterial *capsule*, which is attached tightly to the bacterium and has definite boundaries, the *slime layer* is loosely associated and can be easily washed off [48].
Table 1.3 summarizes the architecture of most bacteria.

Archaebacteria

Archaebacteria are similar to *eukaryotes*, but these cells differ greatly at the molecular level. Archaebacteria and *Eubacteria* or "true" bacteria both are considered to be part of same kingdom of "single-celled" organisms. However, sophisticated genetic and molecular studies allowed the major biochemical differences between Archaebacteria and *Eubacteria*. The differences between Archaebacteria and *Eubacteria* are as follows:

1. Archaebacteria are called ancient bacteria, whereas the *Eubacteria* are called "true" bacteria.
2. *Eubacteria* are usually found in soil and water and living in and on of large organism, whereas Archaebacteria are found in extreme environments and possess unusual metabolism (e.g., salt brines, ocean depths, and hot springs).

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Table 1.3 Characterization of various components in bacteria [48]

Component	Size/diameter	Characteristics/composition
Extracellular materials		
Microcapsule	5–10 nm	Protein-polysaccharides-lipid complex responsible for the specific antigens of enteric bacteria and other species.
Capsule	0.5–2.0 μm	Mainly polysaccharides (e.g., *Streptococcus*); sometimes polypeptides (e.g., *Bacillus antracis*).
Slime layer	–	Mainly polysaccharides (e.g., *Leuconostoc*); sometimes polypeptides (e.g., *Bacillus subtilis*).
Cell wall		
Gram-positive species	10–20 nm	Confer shape and rigidity to the cell; consists mainly mixed polymers of N-acetyl muramic peptide, teichoic acids, and polysaccharides.
Gram-negative species	10–20 nm	Consists mostly of a protein-polysaccharides-lipid complex with small amount of muramic polymer.
Cell membrane		
	5–10 nm	Semipermeable barrier to nutrients; consisting of 50% proteins, 28% lipid and 15–20% carbohydrate in double layered membrane.
Flagellum		
	10–20 nm by 4–12 μm	Long and helical filament located either ends. Protein of the myosin-keratin-fibrinogen; arises from cell membrane and is responsible for motility.
Pilus		
Fimbria	5–10 nm by 0.5–2.0 μm	Rigid protein projections from the cell. Especially long ones are formed by *E. coli*.
Inclusions		
Spore	1.0–1.5 μm by 1.6–2.0 μm	One spore is formed per cell intracellularly. Spores show great resistance to dryness, heat and antibacterial agents.
Storage granule	0.5–2.0 μm	Glycogen like, sulfur, or lipid granules may be found in some species.
Chromato-phore	50–100 nm	Organelles in photosynthetic species (e.g., *Rhodospirillum rubrum*).
Ribosome (70S)	10–30 nm (approx. 20 nm or 200 A°)	Organelles for synthesis of proteins; about 10,000 ribosomes per cell; contain 63% rRNA and 37% ribosomal protein.
Volutin (polyphos-phate bodies)	0.5–1.0 μm	Inorganic metaphosphates
Nuclear material		
		Composed of DNA that functions genetically as if the genes and arranged linearly on a single endless chromosome.

S Svedberg unit/rate of sedimentation, *rRNA* ribosomal ribonucleic acid, *DNA* deoxy-ribonucleic acid

3. *Eubacteria* are divided into two groups known as *gram-positive* and *gram-negative*; whereas three types of Archaebacteria are found: methanogens (methane-producing bacteria), halophiles (grow at very strong salt solutions), and thermophiles (grow at high temperatures and low pH values).
4. Archaebacteria have no peptidoglycan.
5. The nucleotide sequences in the rRNA are similar within the Archaebacteria and distinctly different from *Eubacteria*.
6. The lipid composition of the cytoplasmic membrane is very different for the two groups [48].

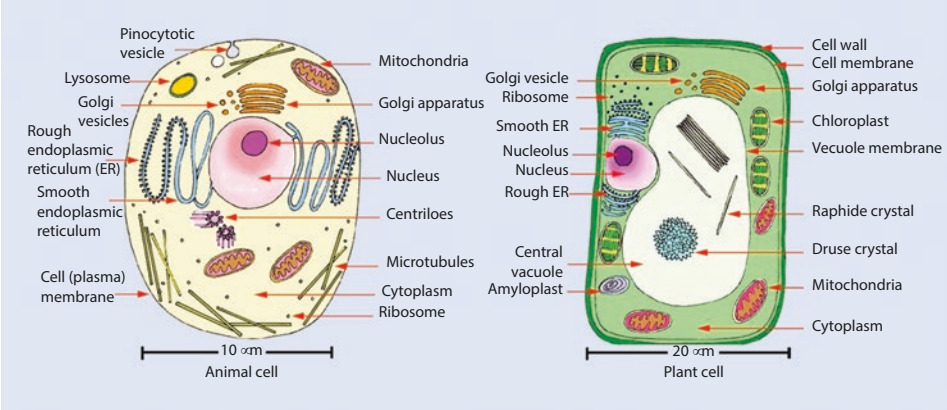
Proteobacteria

Proteobacteria is a major phylum of gram-negative bacteria. Under this phylum, *Acetobacter* sp. and *Gluconacetobacter* sp. are the main bacterial groups consisted with nine and eight species, respectively. They are chiefly employed in the making of vinegar but also of significance in the fermentation of coffee and cocoa [46].

1.2.2 Eukaryotes

Fungi (yeasts and molds), algae, protozoa, and animal and plant cells comprise the eukaryotes. Eukaryotes are five to ten times larger than prokaryotes in diameter (e.g., yeast about 5 μm, animal cell about 10 μm, and plant cell about 20 μm). Eukaryotes have a true nucleus (enclosed within membrane) and a number of cellular organelles (e.g., mitochondria, golgi apparatus etc.) inside the cytoplasm [48]. ◘ Figure 1.2 is a schematic representation of two typical eukaryotic cells.

In ***cell wall*** and ***cell membrane*** structure, eukaryotes are similar to prokaryotes. The plasma membrane is made of proteins and phospholipids that form a bilayer structure (lipid bilayer with embedded protein). Major proteins for the membrane are hydrophobic and are embedded in the phospholipids (50% of all lipids) matrix. Sterols are essential in all eukaryotic cell/cytoplasmic membranes. One major difference is in the presence of sterols (third major class of membrane lipid after phospholipids and glycolipids) in the plasma/cytoplasmic (plasmalemma) membrane of the eukaryotes. For instance,



◘ **Fig. 1.2** Schematic representation of two typical eukaryotic cells

cholesterol is the major sterol in animal plasma membrane. Sterols increase rigidity and strengthen the membrane structure (reduce membrane fluidity and permeability) and make the membrane less flexible [48].

The ***cell wall*** of eukaryotic cells (e.g., algae, plant, fungi) shows considerable variations. Some eukaryotes (e.g., algae) have polysaccharides and cellulose in their cell wall, while others have chitin and other polysaccharides (e.g., fungi). The true fungi do not have cellulose in their cell wall. The plant cell wall is composed of cellulose fibers and is linked via hemicellulose adhere/edge to form the network which is embedded in pectin aggregates. The cellulose-hemicellulose-pectin network imparts strength to the cell wall. Animal cells do not have a cell wall but only a cell/plasma/cytoplasmic membrane. For this logic, animal cells are very shear-sensitive and fragile (delicate) [48]. This aspect significantly complicates/upsets the design of large-scale bioreactor for animal cells [30].

The ***nucleus*** of the eukaryotic cells (except mammalian red blood cells) contains chromosomes as nuclear material (DNA-associated proteins), surrounded by a membrane. The nuclear membrane/envelope, a double membrane, consists of concentric and porous (nuclear pores) membranes. The nuclear envelope completely encloses the nucleus and separates the cell's genetic material from the surrounding cytoplasm. The nuclear pores are required to regulate nuclear transport of the molecules across the envelope. The nucleolus is an area in the nucleus that stains differently and is the site of ribosome and rRNA synthesis. It is not surrounded by a membrane. Nevertheless, many chromosomes have small amounts of RNA and basic proteins called histones adhered to the DNA. Each chromosome contains a single linear DNA molecule on which the histones are attached [48].

The ***mitochondria*** are double membrane-bound organelle and are the powerhouses (generate most of the cell's supply of adenosine triphosphate) of a eukaryotic cell, where respiration and oxidative phosphorylation occur. The mitochondria have a nearly cylindrical (vary considerably in size and structure) shape 1 μm in diameter and 2–3 μm in length. The regular structure of mitochondria is shown in Fig. 1.3. The external membrane is made of a phospholipid bilayer with proteins embedded in the lipid matrix. It has a protein-to-phospholipids ratio very much alike to that of eukaryotic plasma membrane (approx. 1:1). The mitochondria contain complex system of inner membranes (compartmentalized) called *cristae*. A gel-like matrix containing large amounts of protein (mixtures of enzymes) fills the space inside the *cristae*. Some enzymes of oxidative

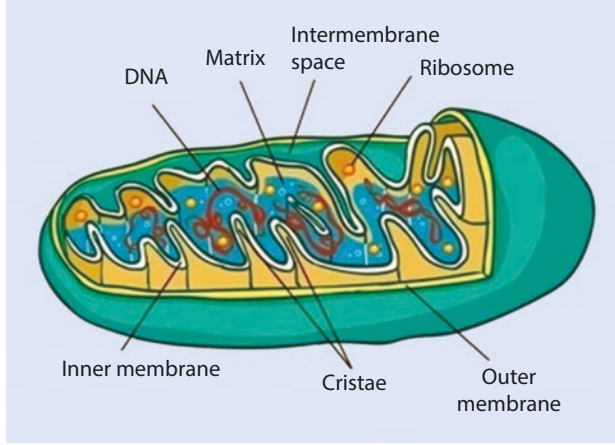


Fig. 1.3 Regular structure of mitochondria

respiration (oxidation of pyruvate, fatty acids, and citric acid cycle) are bound to the *cristae*. Mitochondria have its own DNA which is organized as several copies of single, usually circular chromosome and have protein-synthesizing system and reproduces independently [48].

The ***endoplasmic reticulum*** (ER) is a complex, convoluted/tangled membrane system of interconnected network of flattened, sac- or tubelike structure known as *cisternae*. There are two types of ER, rough *endoplasmic reticulum* (RER) and smooth *endoplasmic reticulum* (SER). The RER (granular) contains ribosomes on the inner surfaces (giving a rough appearance) and is the site of protein synthesis and modifications/corrections of protein structure after synthesis. The SER (agranular) is more engaged with lipid, phospholipids, and steroids synthesis.

Lysosomes are very small membrane-bound organelle that contain and release digestive/hydrolytic enzymes. *Lysosomes* add to the digestion of nutrients and invading substances/biomolecules (e.g., peptides, nucleic acids, carbohydrates, and lipids).

Peroxisomes (also known as *microbody*) are similar to lysosomes in their structure, but not in function. *Peroxisomes* contain oxidative enzymes (carry out oxidative reactions) that produce hydrogen peroxide.

Glyoxysomes (specialized *peroxisomes* found in plants) are also very small membrane-bound particles that contain the enzymes of glyoxylate cycle.

Golgi bodies (*golgi apparatus* or *golgi complex*) are endomembrane system in the cytoplasm and are composed of membrane aggregates/combinations engaged for the secretion of certain proteins. *Golgi bodies* are sites/centers where proteins (*glycosylation* enzymes) are modified by the addition of various sugars monomers in a process called *glycosylation*. Such modification/conversion is essential to protein function (responsibility) in the body.

Vacuoles are membrane-bound organelles (filled with cell sap) of low density (have no basic shape or size) and are enclosed by a membrane called *tonoplast*. *Vacuoles* are responsible for food digestion, osmotic regulation (hydrostatic pressure or turgor within the cell), and waste product storage. *Vacuoles* may occupy a large fraction of cell volume (up to 90% in plant cells) and maintain an acidic internal pH.

Chloroplasts (known as *plastid*) are comparatively large, chlorophyll-containing organelles that are responsible for photosynthesis in photosynthetic eukaryotes (algae and plant cells). Every *chloroplast* is characterized by its two membranes, an outer membrane and a large number of inner membranes called *thylakoids*. Chlorophyll molecules are combined with *thylakoids*, which contain a regular membrane structure with lipid bilayers. Chloroplasts are autonomous units consisting of their own DNA (ctDNA) and protein-synthesizing machinery.

Certain prokaryotes and eukaryotic organisms contain flagella—long, filamentous structures that are connected to one end of the cell and are responsible for the motion of the cell. Eukaryotic ***flagella*** contain two central fibers surrounded by 18 (nine fused pairs of microtubule) peripheral fibers, which exist in doublets (so-called "9 + 2" arrangement). Fibers are in a tube structure called *microtubule* and are composed of proteins called *tubulin*. The whole fiber assembly is installed/embedded in an organic matrix and is surrounded by a membrane. ***Cilia*** are flagella-like structures but are numerous and shorter. Only one group of protozoa, called *ciliates* (e.g., *Paramecium*), contains cilia. Eukaryotic cilia are structurally identical to eukaryotic flagella, although differentiations are made according to length and/or functions. Ciliated organisms move much faster than flagellated ones. The ***cytoskeleton*** (in eukaryotic cells) refers to filaments that provide an internal framework to organize the cell's internal activities and control its shape. These filaments are essential

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in cell movement and separation of chromosomes into the two daughter cells during cell division and transduction of mechanical forces into biological response. The types of fibers present are *actin filaments*, *intermediate filaments*, and *microtubules*.

Microscopic Eukaryotes

Fungi are heterotrophs (obtain their food by absorbing dissolved molecules) that are widespread in distribution and grow in a wide range of habitats. Fungal cells (fungal *mycelia*) are larger than bacterial cells, and their typical internal structures, such as nucleus and vacuoles. The fungal *mycelia* can become visible to naked eye and can also be seen easily with a light microscope. Two major/dominant groups of fungi are yeasts and molds.

Yeasts are eukaryotic and single-celled microorganism of 5–10 μm size. Yeast cells are usually cylindrical, spherical, or oval. Yeasts can reproduce by asexual or sexual modes. Asexual reproduction (vegetative growth) is by either *budding* or *fission*. In *budding*, a small bud (known as "bleb" or daughter cell) forms on the parent/mother cell; this progressively enlarges/swells and detaches from the mother cell. Asexual reproduction by *fission* is identical to that of bacteria. Only a few species of yeast (e.g., *Saccharomyces pombe*) can reproduce by *fission*. In *fission*, the cell grows to a definite size and divides into two equal cells. Sexual reproduction of yeasts concerns with the formation of a zygote (a diploid cell) from fusion of two haploid cells, each having a single set of chromosomes. The nucleus of the diploid cells divides several times to form *ascospores*. Each *ascopspore* finally becomes a new haploid cell and may reproduce by *budding* or *fission*. The life cycle/life process of typical yeast cell is pictured in Fig. 1.4. The classification (analysis) of *yeast* is based on reproductive means (e.g., *budding* or *fission*) and the nutritional requirement/demand of cells. The most widely

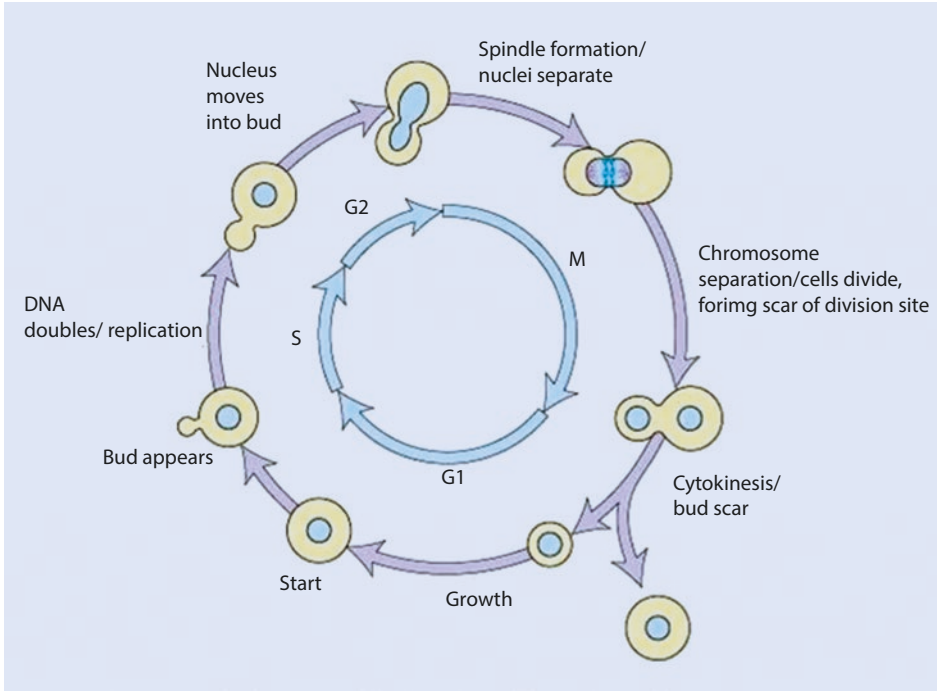

Fig. 1.4 Life cycle/life process of typical yeast cell

used yeast *Saccharomyces cerevisiae* is one of the main microorganisms of industrial interest such as baking, distilling, and wine making. *S. cerevisiae* is used in alcohol fermentation/formation under anaerobic or low-oxygen conditions (e.g. in beer, wine, and whisky making) and also for baker's yeast (leavening agent) production under aerobic conditions, where it converts fermentable sugar (present in dough) into carbon dioxide [48].

Molds are filamentous fungi and have a *mycelial* structure (i.e., network of tubular branching), the highly branched system (*mycelium*) of tubes that contains cytoplasm with many nuclei. Long and thin multicellular filaments on the mycelium are called *hyphae*. Certain branches of mycelium may grow in the air, and the asexual spores (or differentiation at the ends of *hyphae*) formed on these aerial branches are called *conidia*. *Conidia* are nearly spherical in structure and are often colored/pigmented. Some molds reproduce by sexual modes and form sexual spores. These spores provide resistance against heat, drying, freezing, and some chemical agents. Both sexual and asexual spores of molds can germinate and form *hyphae*. Figure 1.5 describes the structure and asexual reproduction of molds. ***Molds*** usually form long, highly branched cells, and easily grow on moist, solid nutrient surface. The growth of molds in the form of pellets can be an alternative in industrial fermentation processes. The growth of mold in submerged culture often forms cell aggregates and pellets. The pellets reach a diameter of 1.0–2.0 mm at the end of fermentation. Pellet formation can cause some nutrient transfer (mainly oxygen) problems inside the pellet. However, pellet formation reduces broth viscosity, which can improve bulk oxygen transfer. ***Molds*** are used for the production of citric acid (*Aspergillus niger*), and the pellet growth is being widely used in the production of citric acid. Several groups of antibiotics such as penicillin are produced by mold (*Penicillium notatum-chrysogenum* group) [48].

Algae are usually unicellular (eukaryotic) organisms (e.g., chlorella, diatoms). The size of a typical unicellular alga is 10–30 μm. However, multicellular algae (e.g., giant kelp, spirogyra, and stonewort) sometimes form a branched or unbranched filamentous structure. Some algae with multicellular structure are present in marine water (e.g., seaweeds).

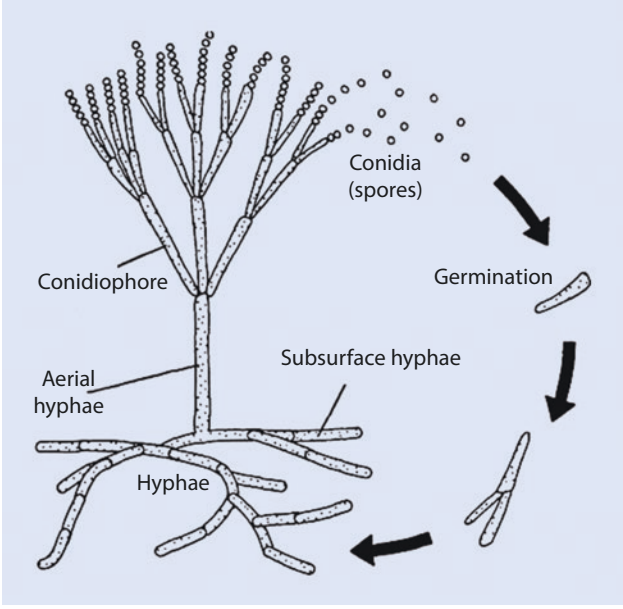

Fig. 1.5 Structure and asexual reproduction of molds

All algae are photosynthetic and contain chloroplasts (similar in structure to cyanobacteria), which commonly impart/transmit a green color to the organisms. The chloroplasts are the sites/centers of chlorophyll pigments and are important for photosynthesis. Some algae contain calcium carbonate or silica in their cell wall. Diatoms containing silica in their cell wall are used as filter aids/supports in industry. Some algae, such as *Chlorella*, *Spirulina*, *Scenedesmus*, and *Dunaliella*, are used for wastewater treatment with simultaneous single-cell protein production. Certain gelling agents (e.g., *alginic acid* or *alginate*) are obtained from marine/brown algae and seaweeds. Some algae are brown or red due to the occupancy of other pigments [30, 48].

Protozoa are unicellular, motile, either free-living or parasitic, and comparably large (1–50 mm) eukaryotic cells that lack cell walls. *Protozoa* are normally heterotrophic and obtain food by ingesting other small organisms or organic particles. *Protozoa* are usually uninucleate and reproduce by sexual or asexual modes. They are classified on the basis of their motion. The *amoeba* move by amoeboid motion, whereby the cytoplasm of the cell flows forward to form a pseudopodium (false foot), and the rest of the cell flows toward this lobe. The *flagellates* move using their flagella. *Trypanosomes* move by flagella and cause a number of diseases in humans. The *ciliates* move by motion of a large number of small appendages on the cell surface called cilia. These *protozoa* do not engulf/imbibe food particles, but absorb dissolved food contents through their membranes. The *sporozoans* are nonmotile and contain members that are human and animal parasites. *Protozoa* cause some diseases (human pathogens), such as malaria, giardiasis, and amoebic dysentery. Some *protozoa* are helpful in removing/eliminating bacteria from wastewater in biological wastewater treatment methods and obtaining clean effluent [48].

1.3 Methods of Fermentation

Fermentation has been classified into liquid fermentation (LF) or submerged fermentation (SmF) and solid-state fermentation (SSF) mainly based on the level of water used during the fermentation (Table 1.4).

1.3.1 Submerged Fermentation (SmF) or Liquid Fermentation (LF)

SmF exploits/utilizes free-flowing liquid substrates, broths, and molasses. The bioactive compounds are secreted into the fermentation broth. The substrates are utilized quite rapidly and hence need to be constantly replaced/supplemented with nutrients. This fermentation method is suitable for microorganisms such as bacteria that need high moisture content (Table 1.5). An additional choice of this technique/method is that purification/refining of products is easier. SmF is mainly used in the extraction of secondary metabolites that necessitate to be used in liquid form.

1.3.2 Solid-State Fermentation

SSF utilizes solid substrates, like bran, bagasse, and paper pulp. The main interest/advantage of using these substrates is that nutrient-rich waste materials can be easily/efficiently recycled as substrates. In this fermentation method/technique, the same substrate

Table 1.4 Comparison between liquid and solid substrate fermentations

Factor	Liquid substrate fermentation	Solid substrate fermentation
Substrates	Soluble Substrates (sugars)	Polymer Insoluble Substrates: Starch Cellulose, Pectines, Lignin
Aseptic conditions	Heat sterilization and aseptic control	Vapor treatment, non sterile conditions
Water	High volumes of water consumed and effluents discarded	Limited Consumption of Water; low A_w. No effluent
Metabolic heating	Easy control of temperature	Low heat transfer capacity
Aeration	Limitation of soluble oxygen. High level of air required	Easy aeration and high surface exchange air/substrate
pH control	Easy pH control	Buffered solid substrates
Mechanical agitation	Good homogenization	Static conditions preferred
Scale up	Industrial equipments available	Need for Engineering & new design Equipment
Inoculation	Easy inoculation, continuous process	Spore inoculation, batch
Contamination	Risks of contamination for single strain bacteria	Risk of contamination for low rate growth fungi
Energetic consideration	High energy consuming	Low energy consuming
Volume of equipment	High volumes and high cost technology	Low volumes and low costs of equipments
Effluent and pollution	High volumes of polluting effluents	No effluents, less pollution
Concentration of Substrate/Products	30–80 g/L	100/300 g/L

Source: Mienda et al. (2011) [32]

can be used for long fermentation periods and can be utilized very slowly and steadily. Henceforth, this technique supports controlled release of nutrients. SSF is best suited/adapted for fermentation techniques including fungi and microorganisms that depend on limited moisture content [6]. Nevertheless, it cannot be used in fermentation processes involving organisms that require high a_w (water activity), such as bacteria [4].

Bacteria and yeasts are equally involved in SmF and SSF, whereas fungi are mostly concerned with SSF processes. The roles of bacteria and yeasts in SmF are mostly related to food and beverage processing industries and will be discussed in detail in the subsequent chapters. Filamentous fungi are best suited for SSF owing to their physiological, biochemical, and enzymological properties and dominate in oriental foods, ensiling and composting processes [24].

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Table 1.5 Examples of main groups of microorganisms involved in SSF and SmF processes

Microorganisms	SSF	SmF
Bacteria		
Bacillus sp.	Composting, Natto, α-amylase	Enzymes (α-amylase, polygalcturonase, phytase, etc)
Clostridium sp.	Ensiling, food	Pesticide degradation
Lactic acid bacteria	Ensiling, food	Fermented foods (yogurt, lacto-pickle, sausage etc)
Pseudomonas sp.	Composting	Xenobiotic degradation
Serratia sp.	Composting	
Fungi		
Altemaria sp.	Composting	
Penicilium notatum, roquefortii	Cheese	Penicillin
Lentinus edodes	Shiitake mushroom	–
Pleurotus oestreatus, sajor-caju	Mushroom	–
Aspergillus niger, A. oryzae	Food, enzymes (glucoamylase	Food, enzymes (glucoamylase, amylopullulanase)
Amylomyces rouxii	Cassava tape	
Beauveria sp., *Metharizium* sp.	Bioinsecticide	Bioinsecticide
Phanerochaete chrysosporium	Composting, lignin degradation	–
Rhizopus spp.	Composting, food, enzymes, organic acid	Food, enzymes, organic acid
Trichoderma sp.	Composting Biological control, Bioinsecticide	Cellulase
Yeasts		
Endomicopsis burtonii, Schwanniomyces castelli	Cassava tape	–
Saccharomyces cerevisiae	Alcoholic beverages, ethanol	Alcoholic beverages, ethanol

The hyphal means/modes of fungal growth and their good tolerance to low a_w and high osmotic pressure conditions make fungi more efficient for bioconversion of solid substrates. *Koji* (in Japan) and *Tempeh* (in Indonesia) are the two most successful applications of SSF using filamentous fungi. In *Koji* production, the fungus *Aspergillus oryzae* is grown on soybean and wheat bran, which is the first step of soy sauce and citric acid production. Koji is an unpurified form of several hydrolytic enzymes required in further steps of the fermentation process. Similarly, *Tempeh* is a fermented food produced by the growth of *Rhizopus oligosporus* on soybeans. In *Tempeh* fermentation, the hydrolytic

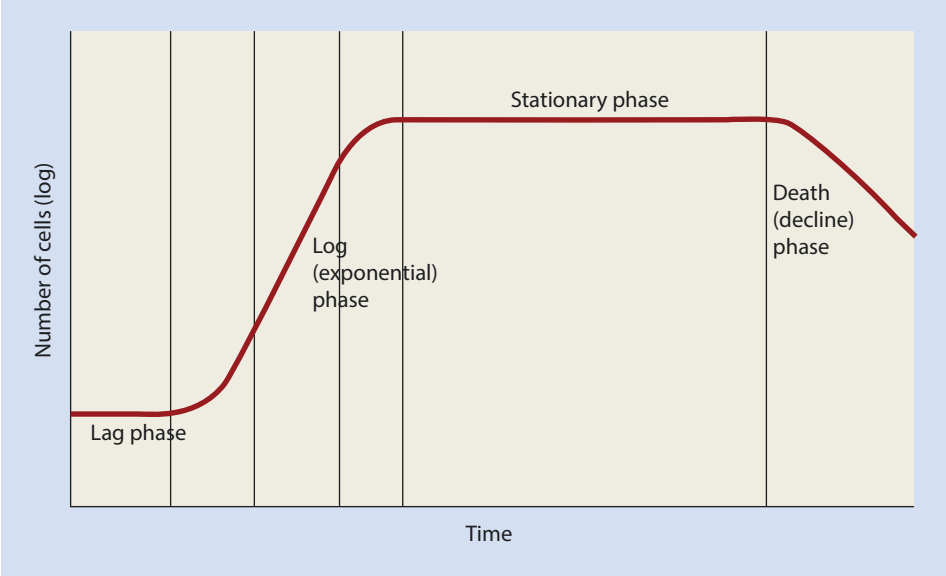
enzymes released by *R. oligosporus* degrade the anti-nutrients such as trypsin inhibitors and hemagglutinins (lectins) in crude soybean and improve taste, flavor, and nutritive quality. Further, the hyphal mode of growth has an added advantage to filamentous fungi over unicellular microorganisms in colonization of solid substrate and better utilization of nutrients [5]. The basic mode of fungal growth is a combination of apical extension of hyphal tips and the mycelium ensures a firm and solid structure. The hydrolytic enzymes are excreted at the hyphal tip, without large dilution like in the case of SmF.

1.4 Growth Measurement During Fermentation Process

In order to grow successfully, microorganisms must have a supply of water as well as numerous other substances including mineral elements, growth factors, and gas, such as oxygen. Virtually all chemical substances in microorganisms contain *carbon* in some form, whether they are proteins, fats, carbohydrates, or lipids.

1.4.1 Growth in SmF

Microbial growth is defined as the increase in all chemical components in the presence of suitable medium and the culture environment. Growth of the cell mass or cell number can be described quantitatively as a doubling of cell number per unit time for bacteria and yeast, or a doubling of biomass per unit time for filamentous organisms such as fungi. After the inoculation of a sterile nutrient solution with microorganisms and cultivation under physiological conditions, four typical phases of growth are observed as indicated in the ◘ Fig. 1.6. Growth is the result of consumption of nutrients.



◘ **Fig. 1.6** Four typical phases of microbial growth

The cells consume a part of the substrate/reactant and essential nutrients from the medium of fermentation. The cells initially multiply and grow. Depending on the type of cells, whether they are unicellular or molds, the growth pattern varies. For example, the unicellular organisms, which divide when they grow, will increase the number of cells or increase the biomass. As they increase in number, they consume more and more of substrate. The other types of cell (e.g., mold) will not increase in number, but they increase in size which also results in increase in viscosity of the broth.

Numerous procedures in biology require cells to be counted. By counting the cells in a known volume of a culture, the concentration can be assessed. For example, the concentration of microorganisms/cell number (e.g., bacteria, virus, and other pathogens) in blood or body fluids can reveal about the progress of an infectious disease and/or about a person's immune system. Knowing the cell concentration is important in molecular biology experiments in order to adjust the amount of reagents and chemicals applied to the experiment.

The various methods of measuring microbial growth are discussed below.

Plate Count

A viable cell count (number of colony-forming bacteria) allows one to identify the number of actively dividing/growing cells in a liquid sample. In this method, fixed amount of inoculum (generally 1 ml) from a broth/sample is placed in the center of sterile Petri dish (containing nutrient medium) using a sterile pipette (Fig. 1.7). The bacteria grow as a colony (i.e., cluster of cells or clones which arise from single bacterium by asexual reproduction) on a nutrient medium. When sample is plated, each colony that grows represents a single cell or spore in the original sample. The colony becomes visible to the naked eye and the number of colonies (colony count multiplied by the dilution factor) on a plate can be counted.

Serial Dilution

A serial dilution is the series of sequential dilution of a substance in solution. Each dilution will reduce the concentration of sample by a specific amount to a more suitable (usable) concentration. The first step in making a serial dilution is to take a known volume (usually 1 ml) of stock and place it into a known volume of distilled water (usually 9 ml) to make a ten-fold (0.1 M) serial dilution (Fig. 1.8). The progressive tenfold serial dilution could be 0.01 M, 0.001 M, 0.0001 M, etc. Usually the dilution factor at each step is constant, resulting in a geometric progression of the concentration in a logarithmic fashion.

A tenfold dilution reduces the concentration of a solution or a suspension by a factor of 10, that is, one-tenth the original concentration. A series of tenfold dilutions is described as tenfold serial dilutions. They are carried out in small sterile test tubes and are usually made of glass, and it is preferable if they have fitted lids to minimize the risk of contamination during the dilution.

The dilution factor is the number which multiplies the final concentration to get actual concentration (cell/ml) and thus decreases the final concentration of stock solution (Fig. 1.9).

Most Probable Number Method

The MPN method is a method used to estimate the concentration of viable microorganism in a sample by means of replicating liquid broth growth in tenfold or twofold (10× or 2×) dilutions. MPN analysis is a statistical method based on random dispersion

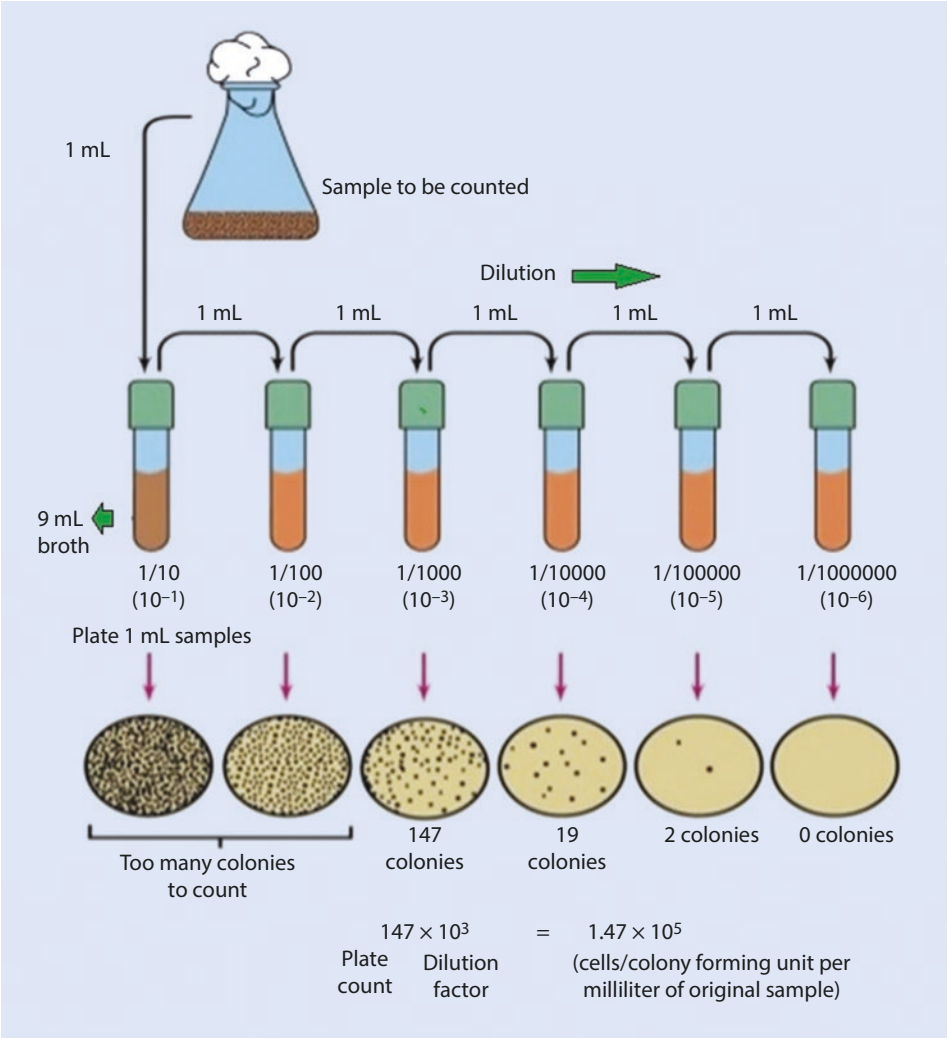


Fig. 1.7 Plate count of viable colony-forming bacteria

of microorganisms per volume in the given sample (Fig. 1.10). In this method, measured volumes of water are added to a series of tube containing a liquid indicator growth medium. The media receiving one or more indicator bacteria show growth and characteristic color change. The color change is absent in those receiving an inoculums of water without indicator bacteria. It is commonly used in estimating microbial populations in soils, waters, agricultural products and is particularly useful with samples that contain particulate material that interferes with plate count enumeration methods.

Optical Density

The optical density/spectrophotometer is usually taken to measure the concentration of growing bacteria. This method is completely based on Lambert–Beer's law. Optical density is directly proportional to the biomass in the cell suspension in a given range that is

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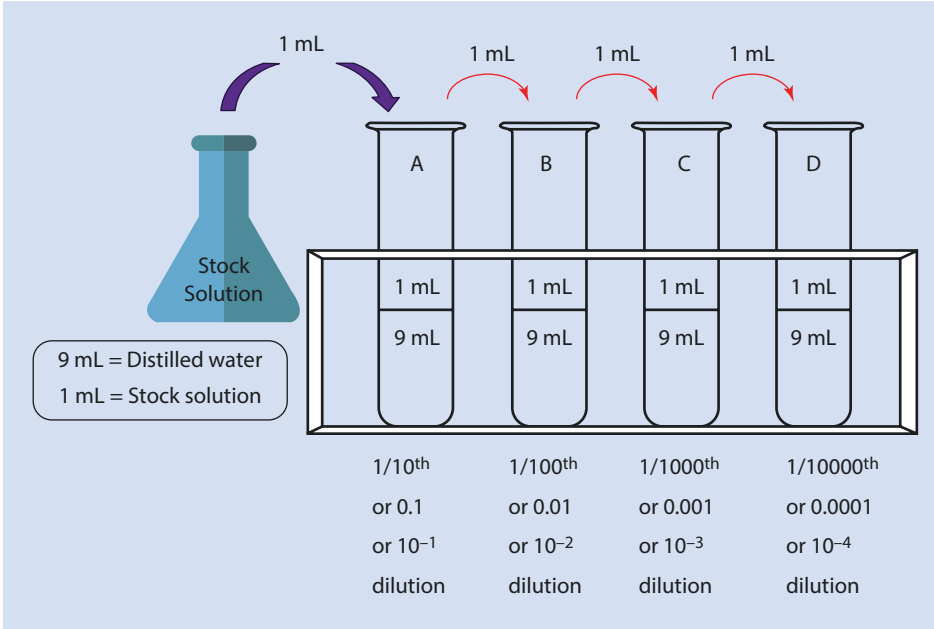
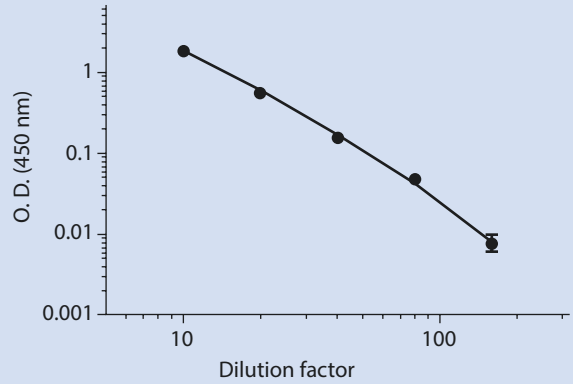


Fig. 1.8 Serial dilution/series of sequential dilution of stock solution

Fig. 1.9 Graph representing optical density (O.D.) with dilution factor



specific to the cell type. Cell suspensions/cell concentrations are turbid and absorb and scatter the intensity of light. The higher the cell concentration, the higher is the turbidity. The cell culture is placed in a transparent cuvette and the absorbance is measured relative to medium alone.

The Lambert–Beer Law

When a ray of monochromatic light of initial intensity (I_o) passes through a solution in a transparent vessel, some of the light is absorbed so that intensity of transmitted light "I" is less than "I_o". There is some loss of light intensity from scattering by particles in the solution and refection at the interfaces.

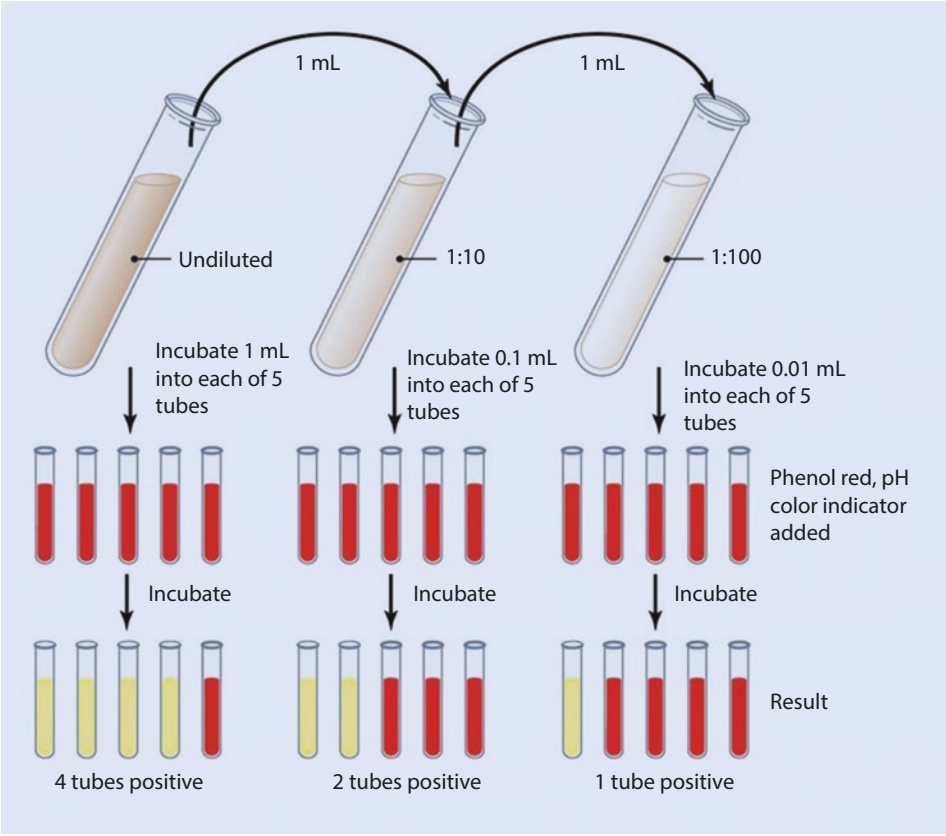


Fig. 1.10 Most probable number (MPN) method of microorganisms per volume of sample

The absorbance of an electronic transition depends on two external factors:

1. The absorbance is directly proportional to the concentration (C) of the solution of the sample used in the experiment.

$$A \propto C$$

2. The absorbance is directly proportional to the length of light path (l), which is equal to the width of the cuvette.

$$A \propto l$$

Combing the two relationships,

$$A = \varepsilon C l$$

This proportionality can be converted into an equation by including a constant.

This formula is known as the Beer–Lambert law (Fig. 1.11), and the constant ε is called molar absorptivity or molar extinction coefficient and is a measure of the probability of the electronic transition. The larger the molar absorptivity, the more probable is the electronic transition. In UV spectroscopy, the concentration of the sample solution is

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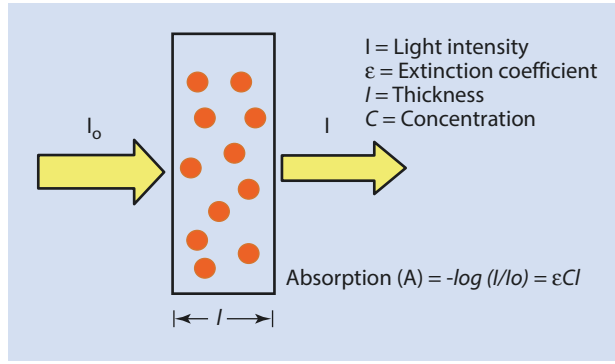

Fig. 1.11 The Beer-Lambert law represents $A = \varepsilon C l$; *A*, absorbance; constant ε is called molar absorptivity or molar extinction coefficient; *C*, concentration of the solution; *l*, light path (*l*), which is equal to the width of the cuvette

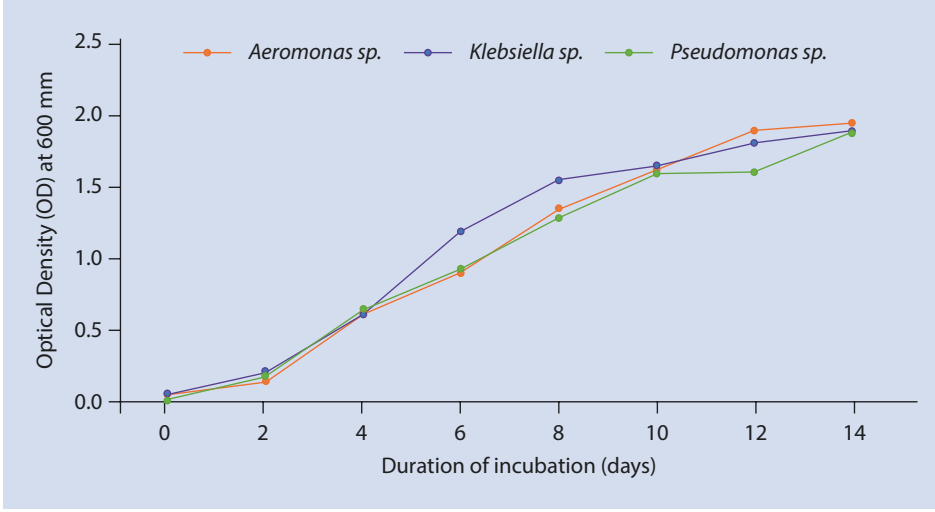

Fig. 1.12 Turbidimetry method (optical density with duration of incubation) of bacterial growth (*Aeromonas* sp., *Klebsiella* sp., *Pseudomonas* sp.,)

measured in mol/L and the light path in cm. Thus, given that absorbance is unit-less, the units of molar absorptivity are L/mol/cm.

Turbidimetry is an established method used to study bacterial growth (Fig. 1.12) since optical density measurements make it possible to follow bacterial population growth in real time. The electronic counting chambers or Coulter counter numbers are used to measure size distribution of cells. Turbidity measurement is done by estimations of large number of bacteria in clear liquid media and broth.

Kinetic Models for Cell Growth

It is generally believed that the growth rate of the cells at any time in the growth phase is proportional to the number of cells present at the time, which is popularly described by Malthus' law [63].

$$\frac{dx}{dt} = \mu x, \qquad x = x_o \quad at \quad t = o \tag{1.1}$$

where x is the mass of the cells per unit volume, μ is the proportionality constant known as specific growth rate in $(\text{hour})^{-1}$, and t is the time in hours.

On integration, we get

$$In\frac{x}{x_o} = \mu t \text{ or } x = x_o e^{\mu t} \tag{1.2}$$

$$In\, x = In\, x_o + \mu t \tag{1.3}$$

The time required to double the microbial mass is given by Eq. (1.2). The exponential growth is characterized by a straight line on a semi-logarithm plot of $In\ x$ versus time.

$$\tau_d = In\frac{2}{\mu} = \frac{0.693}{\mu} \tag{1.4}$$

Where τ_d is the doubling time of cell mass [40].

Monod Model

The growth phase passes through various phases, viz., high growth phase, low growth phase, and finally cessation. In other words, the specific growth rate varies with the residual concentration of the limiting substrate. The relation is well explained by an empirical equation proposed by Monod (1949) [34].

$$\mu = \frac{\mu_m S}{k_s + S} \tag{1.5}$$

where μ_m is the maximum growth rate achievable and k_s is the limiting substrate concentration when specific growth rate is equal to half the maximum specific growth rate, i.e., $\mu = \mu_m/2$.

This is evident by substituting $\mu_m/2$ for μ in Eq. (1.5), i.e.,

$$\frac{\mu_m}{2} = \frac{\mu_m S}{k_s + S} \tag{1.6}$$

By cancelling μ_m on both sides and on arrangement, $k_s = S$.

In Eq. (1.5), if $S >> k_s$, the denominator may be equated to simply S.

$$\mu = \frac{\mu_m S}{k_s + S} = \frac{\mu_m S}{S} = \mu_m \tag{1.7}$$

In other words, the above equation predicts the percentage in μ_m as compared to μ, when S is very large [40].

Example 1.1

A particular organism follows kinetics growth equation (Monod 1949). The kinetic parameter $\mu_m = 0.5\ h^{-1}$, $k_s = 2$ g/L and $S = 3.5$ g/L. Determine the value of μ.

Ans: $\mu = \frac{0.5 \times 3.5}{2 + 3.5} = \frac{1.75}{5.5} = 0.318\ h^{-1}$

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1.4.2 Growth in Solid-State Fermentation

Direct determination of biomass in SSF is very difficult due to the problem of separating the microbial biomass from the substrate. This is especially true for SSF processes involving fungi, because the fungal hyphae penetrate into and bind tightly to the substrate. On the other hand, for the calculation of growth rates and yields, it is the absolute amount of microbial biomass which is important. However, in the case of bacteria and yeasts, the microbial population (colony-forming units) can be calculated by serially diluting the fermented mash and pour plating on specific culture medium. Desgranges et al. [13] and Terebiznik and Pilosof [55] have outlined the following methods for biomass estimation of fungi (also applicable to bacteria) in SSF.

Metabolic Measurement of Biomass

Respiratory Metabolism

Oxygen (O_2) consumption and carbon dioxide (CO_2) evolution resulting from respiration are the metabolic processes by which aerobic microorganisms derive most of their energy for growth. Carbon compounds within the substrate are metabolized, which are converted into microbial biomass and carbon dioxide. Production of CO_2 causes the weight of fermenting substrate to decrease during growth, and the amount of weight lost can be correlated to the amount of growth that has occurred. The measurement of either CO_2 evolution or O_2 consumption is more powerful when coupled with the use of a correlation model. If both the monitoring and computational equipment are available then these correlation models provide a powerful means of biomass estimation since continuous on-line measurements can be made. Other advantages of monitoring effluent gas concentrations with paramagnetic and infrared analyzers include the ability to monitor the respiratory quotient to ensure optimal substrate oxidation, the ability to incorporate automated feedback control over the aeration rate, and the nondestructive nature of the measurement procedure.

Production of Primary Metabolites

Usually, a growing cell will use its primary metabolites for growth and not export them to the medium. However, in fermentation processes designed for production of primary metabolites, i.e., amino acids, enzymes, organic acids, or nucleotides, their production is often growth-related. It has been frequently observed that there is a good correlation between mycelial growth and organic acid production, which can be measured by the pH analysis or a posteriori correlated by HPLC analysis on extracts. In the case of *Rhizopus*, Sauer et al. [45] demonstrated a close correlation between fungal protein (biomass) and organic acids (citric, fumaric, lactic, or acetic). However, the ratio of product formed per unit cell mass is not necessarily constant and as a consequence correlation with cell mass is poor.

Biomass Components

Protein Content

The most readily measured biomass component is protein. The [*] *biuret method* is easy, with good reproducibility since it measures peptide bonds, but it is not very sensitive. [$] *Kjeldahl nitrogen* when multiplied by 6.25 to obtain crude protein is subject to error from

nonprotein nitrogen. The *Folin–Ciocalteu method* (**Lowry's method*) is more sensitive, but since it responds strongly to the aromatic amino acids, it can give an erroneous value unless samples and reference protein are similar in composition [20].

[$***Kjeldahl method or Kjeldahl digestion*** in analytical chemistry is a method for the quantitative determination of nitrogen contained in organic substances plus the nitrogen contained in the inorganic compounds ammonia and ammonium (NH_3/NH_4^+)].

[**Biuret method*: The chemical text used to assess the concentration of protein (i.e., the presence of peptide bonds). The intensity of color or absorbance measured at 540 nm is directly proportional to the protein concentration (according to the Lambert–Beer law).]

[***Lowry's method*: The biochemical assay for determining the total level of protein in a solution. The intensity of color or absorbance measured at 600 nm is directly proportional to the protein concentration.]

Nucleic Acid

Nucleic acid methods based on DNA or RNA determination are reliable only if there is little nucleic acid in the substrate and if no interfering chemicals are present.

Glucosamine

A useful method for the estimation of fungal biomass in SSF is the glucosamine method. This method takes advantage of the presence of chitin in the cell walls of many fungi. Chitin is a poly-N-acetylglucosamine. Interference with this method may occur with growth on complex agricultural substrates containing glucosamine in glycoproteins.

Ergosterol

Ergosterol is the predominant sterol in fungi. Glucosamine estimation was therefore compared with the estimation of ergosterol for determination of the growth of *Agaricus bisporus*.

1.5 Factors Affecting Microbial Growth in Fermentation

The growth of microbes is influenced by various internal and external factors, viz., pH, temperature, composition of the media, etc.

1.5.1 Temperature

Microorganisms need optimum temperature for growth. If grown at a temperature below the optimum, growth occurs slowly resulting in a reduced rate of cellular production (Fig. 1.13). The bacteria can be divided into following groups based on their optimum growth temperature.

Thermophiles are heat-loving bacteria (i.e., *Thermus aquaticus*, *Thermococcus litoralis, and Bacillus stearothermophilus*). The optimum growth temperature is between 45 and 70 °C and is commonly found in hot springs and in compost heaps. ***Hyperthermophiles*** are bacteria that grow at very high temperatures (i.e., genus *Sulfolobus*). Their optimum temperature is between 70 and110 °C. They are usually members of the *Archaea* and are found growing near hydrothermal vents of great depths in the ocean (Fig. 1.14b, d).

Mesophiles are bacteria that grow best at moderate temperatures (i.e., *Pesudomonas maltophilia, Thiobacillus novellus, Streptococcus pyrogenes, Streptococcus pneumoniae,*

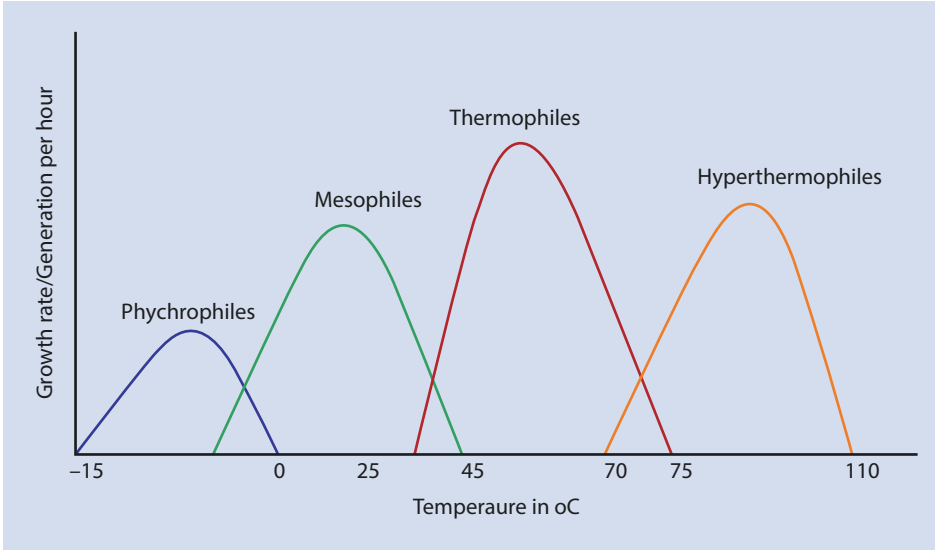

Fig. 1.13 Bacterial growth (growth rate vs. temperature in °C) based on temperature (thermophiles, hyperthermophiles, mesophiles, and psychrophile)

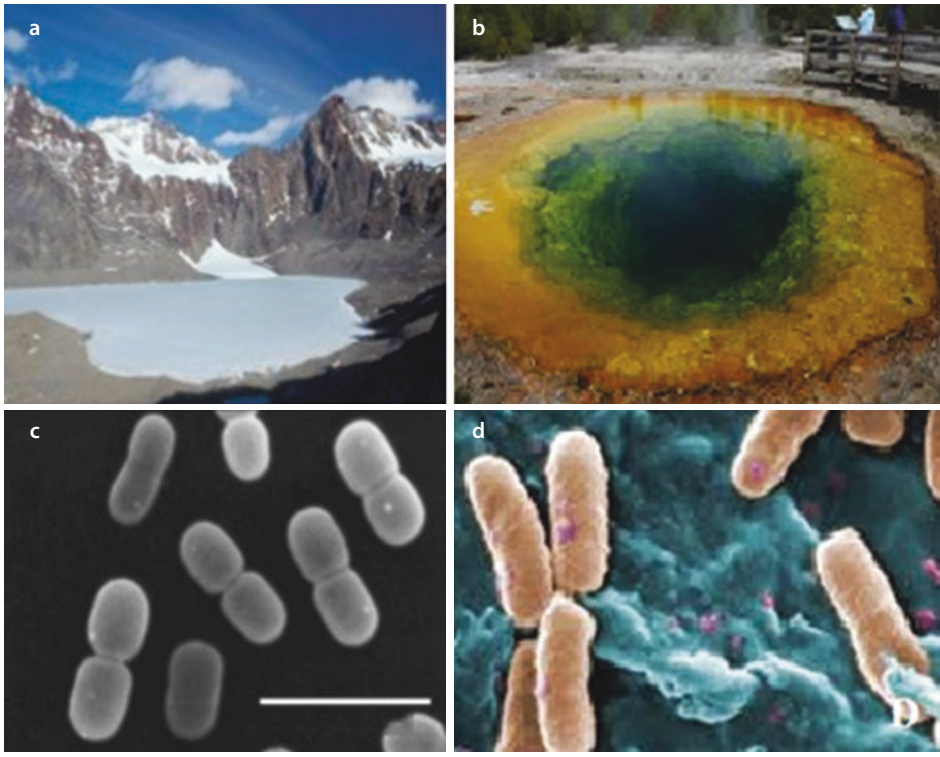

Fig. 1.14 Members of the *Archae* found near hydrothermal vents of great depths in the ocean (**b**, **d**) and Arctic and Antarctic regions (**a**, **c**) in the streams fed by glaciers

Clostridium kluyveri etc). Their optimum growth temperature is between 25 and 45 °C. Most bacteria are mesophilic and include common soil bacteria and bacteria that live in and on the body.

Phychrophiles are cold-loving bacteria (i.e., *Arthrobacter* sp., *Psychrobacter* sp.). Their optimum growth temperature is between −5 to 15 °C. They are usually found in the Arctic (Fig. 1.14a, c) and Antarctic regions and in the streams fed by glaciers. These bacteria are of little importance in fermentation.

1.5.2 pH

Most microorganisms grow optimally between pH 5 and 7 (fungi and yeast grow in acidic conditions) (Fig. 1.15). Microorganisms can be placed in one of the following groups based on their optimum pH requirements. ***Neutrophiles*** grow best at a pH range of 5 and 8. ***Acidophiles*** grow best at a pH below 5.5. ***Alkaliphiles*** grow best at a pH above 8.5.

1.5.3 Oxygen

Oxygen (aeration) is an important factor for aerobic organisms and is very strongly related to growth rate. Optimum growth of many microorganisms usually requires large amounts of dissolved oxygen. As oxygen is sparingly soluble in water (8.4 mg/L at 25 °C), it needs

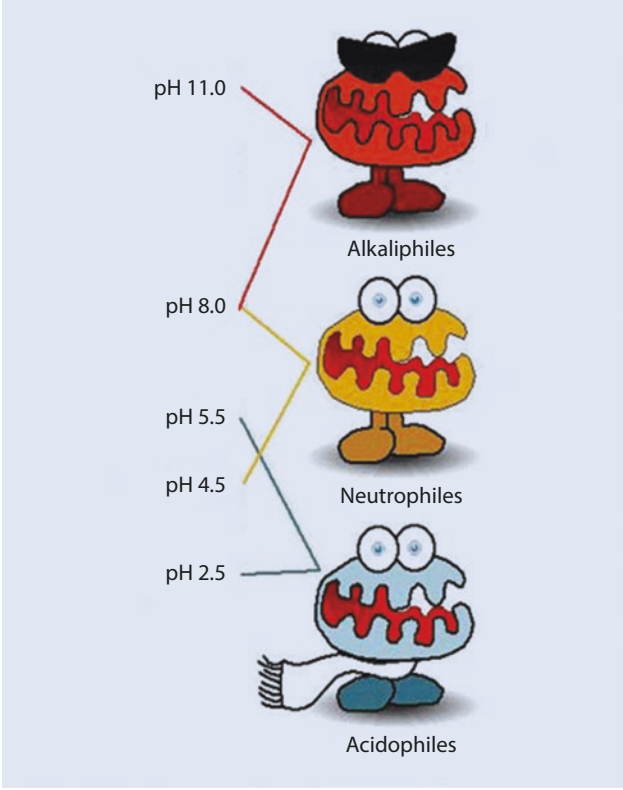

Fig. 1.15 Microorganisms growing in different pH range

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to be supplied continuously (generally in the form of sterilized air) to a growing culture. The air produces bubbles and the stirrer is used to break up the bubbles and mix content in fermentation. If airflow is inadequate or the air bubbles are too large, the rate of transfer of oxygen to the cells is low and is insufficient to meet oxygen demand.

1.5.4 Constituents of Growth Medium

Macro- and Microelements

All microorganisms require certain elements for growth and metabolism. Macro-elements are needed in concentrations larger than 10^{-4} M. Carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus, Mg^{2+}, and K^{+} are major elements. Carbon compounds in growth medium are major sources of cellular carbon and energy. However, micro-elements are needed in concentration less than 10^{-4} M. Trace elements such as Mo^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, Na^{+} and vitamins, growth hormones, and metabolic precursors are micro-elements.

C:N Ratio of Growth Medium

Carbon-to-nitrogen ratio (C/N ratio or C:N ratio) is a ratio of the mass of carbon to the mass of nitrogen in a substrate. The microorganisms decomposing organic matter with a higher C:N ratio are confronted with a surplus of C in relation to N and microorganisms confronted with a lower C:N ratio are facing a lack of C in relation to N [16]. The most common carbon sources in industrial fermentations are molasses (sucrose), starch (glucose, dextrin), corn syrup, and waste sulfite liquor (glucose). In laboratory fermentation, glucose, sucrose, and fructose are the most common carbon sources. Methanol, ethanol, and methane also constitute cheap carbon sources for the fermentation. Most industrially used microorganisms can utilize inorganic or organic sources of nitrogen. Inorganic nitrogen may be supplied as ammonia gas, ammonium salts, or nitrates. Ammonia has been used for pH control and as the major nitrogen source in a defined medium [52]. Ammonium salts such as ammonium sulfate usually produce acid conditions in the growth medium. However, ammonium nitrates normally cause an alkaline drift (provide basic environment) as they are metabolized.

Growth Factors

Some microorganisms cannot synthesize a full complement of cell components and therefore require preformed compounds called *growth factors*. Growth factors stimulate the growth and synthesis of some metabolites. Vitamins, hormones, and amino acids are the major growth factors. Some commonly used vitamins are thiamine (B_1), riboflavin (B_2), pyridoxine (B_6), biotin, cyanocobalamine (B_{12}), folic acid, lipoic acid, p-amino benzoic acid, and vitamin K. The growth factors most commonly required are vitamins, but there may also be a need for specific amino acids, fatty acids, or sterols. Depending on the organism, some or all of the amino acid may need to be supplied externally. Some fatty acids, such as oleic acids and sterols, are also needed in small quantities by some organisms. Many natural media formulations contain all or some of the required growth factors. For example, in processes used for the production of glutamic acid, limited concentrations of biotin must be present in the medium. Some production strains may also require thiamine (B_1).

1.6 Starter Culture(s)

1.6.1 Concept of Starter Cultures

A starter culture may be defined as a preparation containing large numbers of desired microorganisms, used for accelerating the fermentation process. The preparations may contain some unavoidable residues from the culture substrates and additives (such as antifreeze or antioxidant compounds), which support the vitality and technological functionality of the microorganisms. A typical starter after being adapted to the substrate facilitates improved control of a fermentation process and predictability of its products [18, 41]. Basically there are three categories of starter cultures: (1) single-strain cultures, contain only one strain of a species; (2) multi-strain cultures, contain more than one strain of a single species; and (3) multi-strain mixed cultures, contain different strains from different species [58].

1.6.2 History and Subsequent Development of Starter Culture

Microorganisms are naturally omnipresent and hence observed in raw substrates. This was the basis of the idea of spontaneous fermentation. Backslopping was the important technological phenomenon used in spontaneous fermentation by inoculating the raw material with a small quantity of a previously performed successful fermentation. Hence, the dominance of the best adapted strains resulted in backslopping. This technology is still used for production of foods and beverages where the ecology and concrete knowledge about microbial population and role are not clearly known. This is also an economical and reliable method of production of fermented foods.

Recombinant Starter Culture

Recombinant technology and genetic manipulation offer extensive possibilities to incorporate new traits into organisms and have positive applications in food and pharmaceutical industries. The recombinant *Lactococcus* is starter strains to produced peptidases from *Lactobacillus helveticus* 53/7 for potential use as an additional enzyme supply for accelerated proteolysis during cheese ripening [22]. It is possible to increase the production of expolysaccharides (EPS) by *Streptococcus thermophilus* strains through genetic engineering of galactose metabolism. In situ production of EPS by starter sutures (*S. thermophilus*) is an alternative to the addition of biothickeners of fat-free yogurts [42]. Bohmer et al. [8] reported both LABs (*Lactobacillus plantarum* NC8 and *Lactobacillus casei*) as potential expression hosts for recombinant enzyme [hyperthermophilic β-glycosidase (CelB)] production.

1.7 Facets of Fermentation

Fermentation process is used by human beings for various processes from industrial applications to food and agriculture, bioremediation of organic wastes to biovalorization of food and agricultural wastes, etc.

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1.7.1 Industrial Fermentation

Industrial fermentation is the intentional use of fermentation by microorganisms such as bacteria and fungi to make a product useful to humans.

Production of Biomass

Microbial cells or biomass is sometimes the intended product of fermentation, for example, single-cell protein (SCP), baker's yeast, lactobacillus, etc. SCP refers to edible unicellular microorganism. The biomass (protein extract) from pure or mixed cultures of algae, yeasts, fungi, or bacteria may be used as an ingredient or a substitute for protein-rich foods and is suitable for human consumption or as animal feeds. Baker's yeast (*Saccharomyces cerevisiae*) is commonly used as a leavening agent in baking bread and bakery products, where it converts the fermentable sugars present in the dough into carbon dioxide and ethanol.

Production of Extracellular Metabolites

Metabolites can be divided into two groups: primary metabolites (ethanol, citric acid, glutamic acid, lysine, vitamins, and polysaccharides) and secondary metabolites (penicillin, cyclosporin-A, gibberellins, and lovastatin) (*described earlier*).

Production of Intracellular Components

The intracellular components are several microbial enzymes, such as catalase, amylase, protease, pectinase, glucose isomerase, cellulase, hemicelluase, lipase, lactase, streptokinase, etc. Moreover, the industrially synthesized recombinant proteins, such as insulin, hepatitis B vaccine, interferon, and granulocyte colony-stimulating factor, play an important role in health care.

1.7.2 Food Fermentation

Fermentation of food is the ancient process of making bread, curds, idli, dosa, cheese, etc., from thousand years ago. However, similar process is employed specifically to the conversion of sugars into ethanol producing alcoholic drinks such as wine, beer, and cider and in the preservation of sour foods with the production of lactic acid such as in sauerkraut and yogurt. More localized foods prepared by fermentation may also be based on beans, grain, vegetables, fruit, honey, dairy products, fish, meat, or tea [7].

1.7.3 Biofuels

Fermentation is the main source of ethanol in the production of ethanol fuel (i.e., ethyl alcohol) and butanol (butyl alcohol). Common crops such as sugarcane, potato, cassava, and corn are fermented by yeast to produce ethanol and are further processed to produce motor fuel or biofuel additive to gasoline.

1.7.4 Sewage Treatment

Sewage treatment (also called "wastewater treatment") is the process of removing contaminants from municipal wastewater (containing mainly household sewage plus some

industrial wastewater). In the process of sewage treatment, sewage is digested by enzymes secreted by bacteria.

1.7.5 Animal Feeds

A wide variety of agricultural waste products can be fermented to use as food for animals, especially ruminants. Fungi have been employed to break down cellulosic wastes to increase protein content and improve in vitro digestibility.

1.8 Representative Metabolites Produced by Microorganisms

Microbial metabolites represent an incredibly diverse array of chemistry. Microbes can make molecules that synthetic chemists cannot access. While over 25,000 microbial metabolites have been reported in the scientific literature, fewer than 2% of these have ever been readily available to the wider research community. Most metabolites have only ever existed in small quantities in the research laboratory in which they were discovered, and their biological activity has never been fully investigated. Representative metabolites produced by various microbial species are shown in ◘ Table 1.6.

1.8.1 Primary Metabolites

Primary metabolites are vital molecules (e.g., amino acids, vitamins, nucleotides, solvents, or organic acids) of all living cells and are the end product of metabolic pathways or their intermediate products or are domicile of essential macromolecules or are converted into coenzymes.

Amino Acids

The amino acid market is over $6 billion and has been growing at 5–10% for each year [9]. For instance, monosodium glutamate, an effective flavor enhancer, is a leading amino acid in terms of tonnage. Various species of the genera *Corynebacterium* and *Brevibacterium* (e.g., *Corynebacterium glutamicum*, *Brevibacterium flavum*, and *Brevibacterium lactofermentum*) are claimed to be producers of glutamate.

Nucleotides and Nucleosides

The nucleotide/nucleoside fermentations are due to the activity of two purine ribonucleoside 5′-monophosphates, [i.e, guanylic acid (5′-GMP) and inosinic acid (5′-IMP)]. Three primary processes are involved: (i) hydrolysis and enzymatic deamination of yeast RNA to IMP, (ii) synthesis and phosphorylation of the nucleoside (inosine and guanosine) by *Bacillus subtilis* mutants, and (iii) direct fermentation of sugar to IMP (by *Corynebacterium glutamicum* mutants) and conversion of guanine to GMP (using intact cells of *Brevibacterium ammoniagenes*). The intracellular AMP and GMP are limited for effective accumulation of purine nucleotides and are mostly explained by control feeding of purine auxotrophs [35], for instance, adenine-needing mutants deficient of nucleotide degrading enzymes (e.g., adenylosuccinate synthetase), which caused accumulation of inosine or hypoxanthine, which resulted from breakdown of intracellular accumulated IMP.

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Table 1.6 Representative metabolites produced by different groups of microorganisms

Primarty Metabolites	Microorganism involved	Description and usage	References
Amino acids			
Monosodiumglutamate	*Corynebacterium glutamicum, Brevibacterium flavum* and*Brevibacterium lactofermentum*	Effective flavor enhancer	Burkoski and Kramer [9]
Nucleotides and Nucleosides			
Guanylic acid (5′-GMP) and Inosinic acid (5′-IMP)	*Bacillus subtilis, Corynebacterium glutamicum, Brevibacterium ammoniagenes*	ASS; auxotrophs	Nakayama et al. [35]
Inosine	*Bacillus subtilis*	IMP dehydrogenase; 35 g/L; auxotrophs	Miyagawa et al. [33]
Inosine monophosphate/guanosine	*Bacillus subtilis*	IMP gene; 7–20 g/L; auxotrophs	Miyagawa et al. [33]
Cytidine	*Bacillus subtilis*	HSD; 30 g/L; auxotrophs	Asahi et al. [3]
Vitamins			
Riboflavin (vitamin B2)	*Eremothecium ashbyii* and *Ashbyagossypii*	20 g/L; nutrition of animals and humans	Demain [10]
Vitamin B12 (Cyanocobalamin)	*Propionibacterium shermanii* and *Pseudomonas denitrificans*	206 mg/L; nutrition of animals and humans	Spalla et al. [49]
Biotin antimetabolites	*Serratiamarcescens*	600 mg/L; nutrition of animals and humans	Masuda et al. [31]
Vitamin C (L – Ascorbic acid)	*Erwinia herbicola* with *Corynebacterium* sp.	130 g/L; Potent antioxidant	Saito et al. [43]
Organic acids			
Oxalic and gluconic acids	*Aspergillusniger*	IDH; pH 3.0	Kubicek and Rohr [27]
gluconic acid	*Aspergillus niger*	150 g/L	Znad et al. [65]
Gluconic acid	*Aspergillusniger*	GO; pH 1.7–2.0	Kubicek and Rohr [27]
Citric acid	*Candida oleophila*	80% sugar; 100 g/L	Anastassiadis et al. [2]
Citric acid	*Aspergillusniger*	Batch or Fed-batch fermentations; 6–10 days; 150–180 g/L	Anastassiadis et al. [2]
Isocitric acid	*Candida* sp.	Aconitase; 225 g/L	Deppenmeier et al. [12]

Table 1.6 (continued)

Primarty Metabolites	Microorganism involved	Description and usage	References
Vinegar[a]	*Acetobacter polyoxogenes* withplasmid vector, *Acetobacter aceti* subsp. *xylinum*.	AldDH; 68–97 g/L	Fukaya et al. [14]
Lactic acid	*Lactobacilli*	100 g/L	Znad et al. [65]
Itaconic acid	*Candida* sp.	–	Wilke and Verlop [61]
Succinic acid	*Actinobacillus succinogenes*	110 g/L	Zeikus et al. [64]
Pyruvic acid	*Torulopsis glabrata*	69 g/L	Li et al. [29]
Alcohols			
Ethanol	*Escherichiacoli*	ADH II and PDC; 46 g/L	Ingram et al. [19]
Glycerol	*Candida glycerinogenes*	130 g/L	Taherzadeh et al. [54]
Mannitol	*Candida magnoliae*	213 g/L	Lee et al. [28]
Secondary Metabolites			
Antibiotics			
Penicillin	*Penicillium chrysogenum*	70 g/L	Jiang et al. [21]
Cephalosporin-C	*Acremonium chrysogenum*	30 g/L	Jiang et al. [21]
Antitumor agents			
Mitomycin-C, bleomycin, daunorubicin, doxorubicin, etoposide and calicheamicin	*Actinomycetes*	Effective agent against cancer	Strobel et al. [53]
Camptothecin (CPT)	Endophytic fungi	Used against cancer	Amna et al. [1]
Pharmacological agents			
Lovastatin	*Aspergillus terreus*	Cholesterol – lowering agents	Stabb et al. [50]
Cyclosporin-A	*Tolypocladium nivenum*	Immunosuppressants	Omura and Crump [37]

Abbreviation: *5′-IMP* Inosine 5′-monophosphates, *5′-GMP* guanosine 5′-monophosphates, *ASS* adenylosuccinate synthetase, *IMP* inosine monophosphate, *HSD* Homoserine dehydrogenase, *IDH* isocitrate dehydrogenase, *GO* glucose oxidase, *AldDH* aldehyde dehydrogenase, *ADH* alcohol dehydrogenase, *PDC* pyruvate decarboxylase

[a]5–20% acetic acid

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Vitamins

Several microbial strains are responsible for the production of vitamins or vitamin-like compounds (e.g., β-carotene, vitamin B12, riboflavin, vitamin C, linolenic acid, vitamin F, and ergosterol) [51]. Riboflavin (vitamin B2) has been produced commercially for many years by both fermentation and chemical synthesis; however, fermentation is the major route today. For instance, two yeastlike molds, *Eremothecium ashbyii* and *Ashbya gossypii*, were found to synthesize riboflavin in concentrations more than 20 g/L. Vitamin B12 (Cyanocobalamin) is formed industrially with *Propionibacterium shermanii* and *Pseudomonas denitrificans* [49]. The overproduction of vitamin B12 is absolutely dependent upon addition of betaine (the mechanism of control is unknown). The *P. freudenreicheii* can produce 206 mg/L of vitamin B12 and is not still a dominant industrial producing organism.

Organic Acids

Microbes have been widely used for the commercial production of organic acids (e.g., citric acid, acetic acid, lactic acid, gluconic acid, pyruvic acid, malic acid, tartaric acid, succinic acid, and itaconic acid). Among different types of organic acids, citric acid (CA) is easily palatable, assimilated, and has low toxicity. Therefore, it is extensively used (about15%) in the pharmaceutical and food industry (as an antioxidant, acidifying and flavor-enhancing agent). The CA is produced via the Embden–Meyerhof pathway (EMP) and the first step of the tricarboxylic acid (TCA) cycle. Harmsen et al. [17] reported that a high level of CA production is also linked with an increased level of fructose 2,6-biphosphate (an activator of glycolysis).

Vinegar production is best carried out with species of *Gluconacetobacter* sp. and *Acetobacter* sp. [61]. However, vinegar is consisting of about 5–20% acetic acid (AA). Fermentation has virtually waived chemical synthesis of lactic acid (LA). The LA is produced anaerobically with a 95% (w/w) yield based on carbohydrate, a titer of over 100 g/L, and a productivity of over 2 g/L. This is comparable to processes employing LA bacteria. *Lactobacilli* produce mixed isomers, whereas *Rhizopus* forms L -(+)- LA exclusively. *Rhizopus oryzae* is favored for formation since it makes only the stereochemically pure L -(+)-LA. Itaconic acid is used as a comonomer in resins and synthetic fibers and is produced from the selective fungal (i.e., *Candida*) sp. [61]. Although microbial processes exist for the other acids, they have not been exploited commercially on a large scale. Succinic acid can be made from the rumen organism *Actinobacillus succinogenes* at 110 g/L [64]. Pyruvic acid is formed at 69 g/L at 56 h with a yield of 0.62 g/g glucose using *Torulopsis glabrata* [29].

Alcohols

Ethyl alcohol (C_2H_5OH) is a primary metabolite that can be formed from fermentation of a carbohydrate/sugar or a polysaccharide that can be depolymerized to a fermentable sugar. Yeasts are preferred for these fermentations, but the species used depends on the substrate employed. *Saccharomyces cerevisiae* is used for the fermentation of hexose, whereas *Candida* sp. or *Kluyveromyces fragilis* may be employed if pentose or lactose, respectively, is the substrate. Ethanol is produced in Brazil from cane sugar at 12.5 billion liters/year and is used as a 25% fuel blend or as a pure fuel. With regard to beverage ethanol, some 60 million tons of beer and 30 million tons of wine are produced each year. Production of glycerol is usually done by chemical synthesis from petroleum feedstocks, but better fermentations processes are available [60]. Osmotolerant yeast strains (*Candida glycerinogenes*) can produce up to 130 g/L of glycerol. Six hundred thousand tons of glycerol

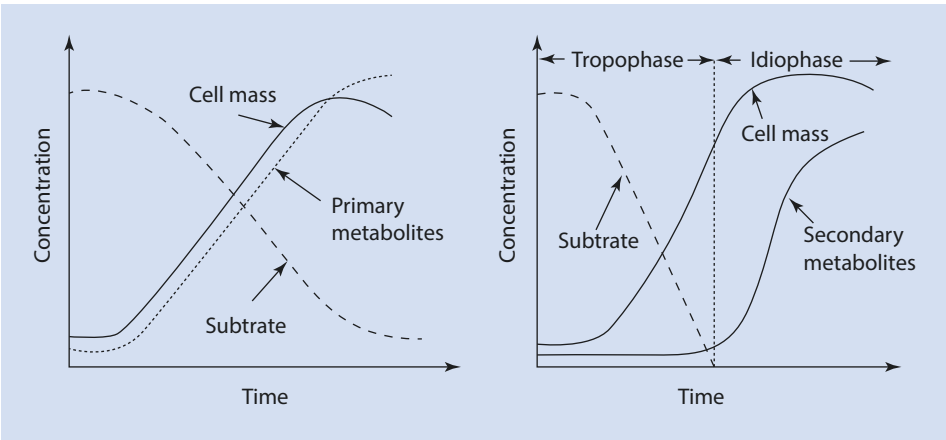
are produced annually by recovery as a by-product of the fat and oil industries, by synthesis from propylene, and, to a small extent, by glucose fermentation using *Saccharomyces cerevisiae* [54].

1.8.2 Secondary Metabolites

Microbial secondary metabolites are compounds produced by strains of certain microbial species (mainly by *actinomycetes* and *fungi*), usually late in the growth cycle (idiophase). The production of secondary metabolites starts when growth is limited with exhaustion of one key nutrient source (i.e., carbon, nitrogen, or phosphate). For example, penicillin biosynthesis by *Penicillium chryosgenum* starts when glucose is exhausted from the culture medium and the fungus starts consuming lactose, a less readily utilized glucose [57]. A characteristic of secondary metabolites is that they are usually not produced during the phase of rapid growth (trophophase) but are synthesized during a subsequent production stage (idiophase). The difference/comparison between primary and secondary metabolites is given in ◘ Fig. 1.16 and ◘ Table 1.7. The secondary metabolites have an enormous range of biological activities and are extremely important to our health and nutrition [10]. A group that includes antibiotics, toxins, pesticides, animal and plant growth factors, and other medicines has tremendous economic importance [11].

Antibiotics

The best known/recognized secondary metabolites are antibiotics (antimicrobial agent). The antibiotic revolutionized medicine in twentieth century and its market involves about 160 antibiotics and derivatives, (e.g., β-lactam peptide antibiotics, the macrolide polyketides and polyketides, tetracyclines, aminoglycosides, and others). The anti-infective market is framed of 62% antibacterials, 13% sera immunoglobulins and vaccines, 12% anti-HIV antivirals, 7% antifungals, and 6% non-HIV antivirals. Titers of *penicillin* with *Penicillium chrysogenum* have reached 70 g/L, whereas those of *Cephalosporin*-C by *Acremonium chrysogenum* are over 30 g/L. Published data on clavulanic acid production by *Streptomyces clavuligerus* indicate the titer to be above 3 g/L [21, 38].



◘ **Fig. 1.16** The difference/comparison between primary and secondary metabolites

Table 1.7 Differences between primary and secondary metabolites

Primary metabolites	Secondary metabolites
Produced during growth phase of cell	Produced during non-growth phase (near completion) of cell
Accumulated large quantities	Accumulated very small quantities
Growth phase where primary metabolites produced is called "Tropophase"	phase where secondary metabolites produced is called "Idiophase"

Antitumor Agents

Ever since the discovery/origination of the *Actinomycins* by Waksman and Woodruff [59] and the use of *Actinomycin*-D against the Wilms tumor in children, microbes have served as a prime source of anticancer agents. The essential microbial molecules are mitomycin-C, bleomycin, daunorubicin, doxorubicin, etoposide, and calicheamicin, all are produced by *Actinomycetes*. Taxol (paclitaxel) is a very productive compound against ovarian and breast cancer, produced by the endophytic fungi [1]. Another plant product is *camptothecin* (CPT), produced by certain angiosperms, which is a modified monoterpene indole alkaloid and is active against type I DNA topoisomerase. Its water soluble derivatives *irinotecan* and *topotecan* are used against cancer. It also can be made by endophytic fungi.

1.9 Concluding Remarks

A wide variety of organic chemicals like enzymes, amino acids, and antibiotics can be formed by fermentation. Fermentation is a metabolic process that converts organic substrates (mainly carbohydrates) into useful products (organic acids, gases, or alcohol) using diverse groups of microbes [bacteria, fungi (yeasts and mold)]. Most microorganisms used in commercial fermentation need disaccharides or six-carbon sugars as substrates, even though the microbial world contains organism that can break down essentially any organic compound. There has been a significant improvement in industrial products by strain selection/manipulation when related with the processes that were used in the first half of the twentieth century. However, further studies should aim to gather knowledge concerning several beneficial/microbial strains and processes involved in the formation of high-value fermented products.

Take-Home Messages

- Presence (in the case of eukaryotes) or absence (in the case of prokaryotes) of membrane around the cell's genetic materials is the fundamental difference between eukaryotes and prokaryotes.
- Gram staining differentiates bacteria into two groups, Gram +ve or Gram –ve, based on chemical and physical properties of their cell walls.
- Water level or water activity is the main difference between solid and submerged fermentation.

- Microorganisms have a supply of water as well as numerous other substances including mineral elements, growth factors, and gas such as oxygen to grow.
- Various methods of measuring microbial growth are plate count, serial dilution, most probable number (MPN), and optical density/spectrophotometer method.
- Monod equation can be used to measure microbial growth of microorganisms.
- Metabolic measurement of biomass includes respiratory metabolism, production of primary metabolites, biomass components of protein content, DNA or RNA components, and glucosamine method for the estimation of fungal biomass in solid- state fermentation.
- The growth of microbes is influenced by various internal and external factors, viz., pH, temperature, composition of the media, etc.
- In spontaneous fermentation, there is no control over the fermentation process and is mediated by natural microflora present on the surface of the substrate(s), whereas in controlled fermentation, the fermentation process is optimized for pH, temperature, incubation time and is mediated by specific microorganism or a group of microorganisms (starter culture).

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