1 Fundamentals and New Aspects

1.1 Biotechnological applications of animals, plants, and microbes

In transgenic biotechnology (also known as genetic engineering), a known gene is inserted into an animal, plant, or microbial cell in order to achieve a desired trait. Biotechnology involves the potential use of all living forms, but microorganisms have played a major role in the development of biotechnology. This is because of the following reasons: (i) mass growth of microorganisms is possible, (ii) cheap waste materials which act as the media for the growth of microorganisms can be rapidly grown, and (iii) there is massive diversity in the metabolic types, which in turn gives diverse potential products and results in the ease of genetic manipulation to improve strains for new products. However, mass culture of *animal cell lines* is also important to manufacture *viral vaccines* and other products of biotechnology. Currently, *recombinant DNA* (*rDNA*) *products* produced in *animal cell cultures* include *enzymes*, *synthetic hormones*, *immunobiologicals* (*monoclonal antibodies*, interleukins (ILs), *lymphokines*), and *anticancer agents*. Although many simpler proteins can be produced by recombinant bacterial cell cultures, more complex proteins that are glycosylated (carbohydrate-modified) currently must be made in animal cells. However, the cost of growing *mammalian cell cultures* is high, and thus research is underway to produce such complex proteins in *insect cells* or in *higher plants*. *Single embryonic cell* and *somatic embryos* are used as a source for direct gene transfer via *particle bombardment*, and analyze *transit gene expression*. *Mammarian cell-line products* (expressed by *CHO*, *BHK* (*baby hamster kidney*), *NSO*, *meyloma cells*, *C127*, *HEK293*) account for over 70% of the products in the biopharmaceutical markets including therapeutic monoclonal antibodies.

Biopharmaceuticals may be produced from microbial cells (e.g., recombinant *Escherichia coli* or yeast cultures), mammalian cell culture, plant cell/tissue culture, and moss plants in bioreactors of various configurations, including photo-bioreactors. The important issues of cell culture are cost of production (a low-volume, high-purity product is desirable) and microbial contamination by bacteria, viruses, mycoplasma, and

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so on. Alternative but potentially controversial platforms of production that are being tested include whole plants and animals. The production of these organisms represents a significant risk in terms of investment and the risk of nonacceptance by government bodies due to safety and ethical issues.

The important animal cell culture products are monoclonal antibodies; it is possible for these antibodies to fuse normal cells with an immortalized tumor cell line. In brief, lymphocytes isolated from the spleen (or possibly blood) of an immunized animal are fused with an immortal myeloma cell line (B cell lineage) to produce a hybridoma, which has the antibody specificity of the primary lymphoctye and the immortality of the myeloma. Selective growth medium (hyaluronic acid (HA) or hypoxanthine–aminopterin–thymidine (HAT)) is used to select against unfused myeloma cells; primary lymphoctyes die quickly during culture but only the fused cells survive. These are screened for production of the required antibody, generally in pools to start with and then after single cloning, the protein is purified. As mammals are also a good bioreactor to secrete the fully active proteins in milk, several species since 1985 have been cloned including cow, goat, pig, horse, cat, and most recently dog, but the most research has been on cloning of cattle. Genetically modified (GM) pigs, sheep, cattle, goats, rabbits, chickens, and fish have all been reported.

The main potential commercial applications of cloned and GM animals include production of food, pharmaceuticals ("pharming"), xenotransplantation, pets, sporting animals and endangered species. GM animals already on sale include cloned pet cats, GM ornamental fish, cloned horses, and at least one rodeo bull. Two pharmaceutical products from the milk of GM animals have completed (Phase III and Phase II) clinical trials, respectively, and may be on the market in the EU in the next few years. Cloned livestock (especially pigs and cattle) are widely expected to be used within the food chain somewhere in the world, though it would not be economical to use cloned animals directly for food or milk production, but clones would be used as parents of slaughter pigs, beef cattle, and possibly also milk-producing dairy cows. The first drug manufactured from the milk of a GM goat was ATryn (brand name of the anticoagulant antithrombin) by GTC Biotherapeutics in 2006. It is produced from the milk of goats that have been GM to produce human antithrombin. A goat that produces spider's web protein, which is stronger and more flexible than steel (BioSteel), was successfully produced by a Quebec-based Canadian company, Nixia.

Faster-growing GM salmon developed by a Canadian company is also awaiting regulatory approval, principally for direct sale to fish farming markets. Canada has also approved the GM pig (trade named "Enviropig") developed by University of Guelph and it is designed to reduce phosphorus pollution of water and farmers' feed costs. Enviropig excretes less phosphorous manure and is a more environmentally friendly pig. It will be years before meat from genetically engineered pigs could be available for human consumption. Molecular pharming can also produce a range of proteins produced from cloned cattle, goats, and chickens. An ornamental fish that glows in the dark is now available in the market. It was created by cloning the deoxyribonucleic acid (DNA) of jellyfish with that of a zebra fish. GM fish may escape and damage the current ecosystem by colonizing waters. Some tropical fish, like piranhas, could be engineered to survive in the cold and this could lead to major problems. These details will be covered in the section on Animal Biotechnology.

Recently, the production of foreign proteins in transgenic plants has become a viable alternative to conventional production systems such as microbial fermentation or mammalian cell culture. Transgenic plants are now used to produce pharmacologically active proteins, including mammalian antibodies, blood product substitutes, vaccines, hormones, cytokines, a variety of other therapeutic agents, and enzymes. Efficient biopharmaceutical

production in plants involves the proper selection of a host plant and gene expression system in a food crop or a nonfood crop. Genetically engineered plants, acting as bioreactors, can efficiently produce recombinant proteins in larger quantities than mammalian cell systems. Plants offer the potential for efficient, large-scale production of recombinant proteins with increased freedom from contaminating human pathogens. During the last two decades, approximately 95 biopharmaceutical products have been approved by one or more regulatory agencies for the treatment of various human diseases including diabetes mellitus, growth disorders, neurological and genetic maladies, inflammatory conditions, and blood dyscrasias. None of the commercially available products are currently produced using plants mainly because of the low yield and expensive purification costs; however, DNA-based vaccines are potential candidates for plant-based production in the future. After the cell is grown in tissue culture to develop a full plant, the transgenic plant will express the new trait, such as an added nutritional value or resistance to a pest. The transgenic process allows research to reach beyond closely related plants to find useful traits in all of life's vast resources. The details of transgenic plants will be covered in the section on Plant Biotechnology.

All the biopharmaceutical products are mostly manufactured commercially through various fermentation routes on using genetically engineered microorganisms like *E. coli*, yeast, and fungi. Some of the biopharmaceutical products produced commercially through fermentation routs are human insulin, streptokinase, erythropoietin, hepatitis B vaccine, human growth hormone, IL, granulocyte-colony stimulating factor (GCSF), granulocyte-macrophage colony stimulating factor (GMCSF), alfa-interferon, gamma interferon, and so on. All three domains – animals, plants and microbes – are not only involved in production of biopharmaceuticals but also find their application in manufacture of food products (Figure 1.1). Although there is a high level of public support for the development of new biotech, that is, for the production of new medicines (insulin, interferon, hormone, etc.), diagnostics (cancer detection kits), and food enzymes (recombinant

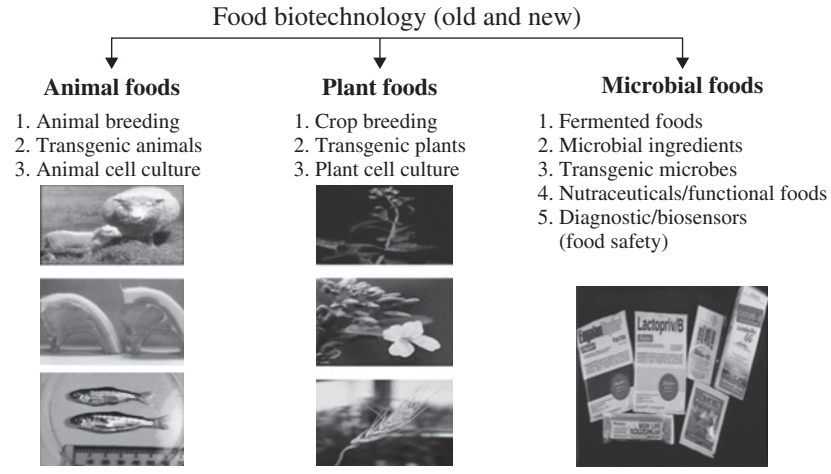

Figure 1.1 Concept of food biotechnology.

rennet, etc.), there is no support for the production of GM whole foods. This is because of the safety factor that is involved in the consumption of food. This is covered in detail in the section on Food Safety and New Biotechnology.

1.2 Cellular organization and membrane structure

Cellular organization comprises three levels of organization that exist within each cell. Cells are composed of organized organelles, which are unique structures that perform specific functions within cells. Organelles themselves are made up of organized molecules, and molecules are forms of organized atoms, which are the building blocks of all matter.

Most organisms share (i) a common chemical composition, their most distinctive chemical attribute being the presence of three classes of complex macromolecules: DNA, ribonucleic acid (RNA), and proteins (ribosomes, enzymes), and (ii) a common physical structure, being organized into microscopic subunits, termed cells. Cells from a wide variety of organisms share many common features in their structure and function. All cells are enclosed by a thin *cytoplasmic membrane*, which retains various molecules, necessary for the maintenance of biological function, and which regulates the passage of solutes between the cell and its environment. These generalizations apply to all living organisms, except for the virus because they cannot maintain life and reproduce by themselves.

Dissatisfaction with the existing classification of the biological kingdom led Haeckel (1866) to propose a third kingdom, the *Protists* (protozoa, algae, fungi, bacteria), besides the *plants* and *animals*. Observation with the electron microscope (developed in about 1950) revealed two radically different kinds of cells in the contemporary living world. Although the various groups of organisms are still linked by certain common features, we can distinguish two major groups of cellular organisms: the *Procaryotes* (or *Prokaryotes*) and the *Eucaryotes* (or *Eukaryotes*). As scientists learn more about organisms, classification systems change. Genetic sequencing has given researchers a whole new way of analyzing relationships between organisms. In recent years, the evolutionary relationships of prokaryotes are quite complex, in that the taxonomic scheme of life has been revised. The current system, the *Three Domain System*, groups organisms primarily based on differences in the structure of the ribosomal RNA, that is, a molecular building block for *ribosomes*. Under this system, organisms are classified into *three domains* and *six kingdoms*. The domains are Archaea, Bacteria, and Eukarya. The kingdoms are *Archaebacteria* (*ancient bacteria*), *Eubacteria* (*true bacteria*), *Protista*, *Fungi*, *Plantae*, and *Animalia*. The *Archaea* and *Bacteria* domains contain prokaryotic organisms. These are organisms that do not have a membrane-bound *nucleus*. Eubacteria are classified under the Bacteria domain and archaebacteria are classified as Archaeans. The Eukarya domain includes eukaryotes, or organisms that have a membrane-bound nucleus. This domain is further subdivided into the kingdoms Protista, Fungi, Plantae, and Animalia.

Figure 1.2 illustrates the relationship between the three domains. Archaea are sometimes referred to as *extremophiles*, inhabiting in extreme environments such as hot springs, hydrothermal vents, salt ponds, Arctic ice, deep oil wells, and acidic ponds that form near mines. In fact, many extremophiles cannot grow in ordinary human environment. Compared to eukaryotes, prokaryotes usually have much smaller genomes and an eukaryotic cell normally has 1000 times more DNA than a prokaryote. The DNA in prokaryotes is concentrated in the *nucleoid*. The prokaryotic chromosome is a double-stranded DNA molecule arranged as a single large ring. Prokaryotes often have smaller rings of extrachromosomal DNA termed *plasmids* in which most plasmids consist of only a few genes.

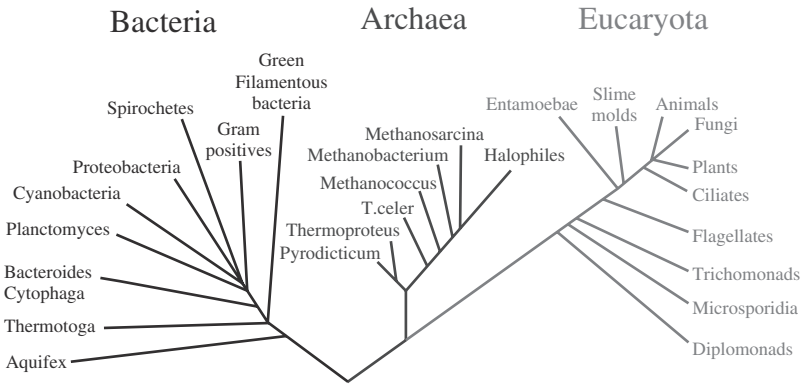

Figure 1.2 Concept of three life domains based on rRNA data, showing the separation of bacteria, archaea, and eukaryotes. *Source*: Wikipedia (June, 2007); http://en.wikipedia.org/wiki/Three-domain_system. (*See insert for color representation of this figure.*)

Plasmids are not required for survival in most environments because the prokaryotic chromosome programs all of the cell's essential functions. However, plasmids may contain genes that provide resistance to antibiotics, metabolism of unusual nutrients, and other special functions. Plasmids replicate independently of the main chromosome, and many can be readily transferred between prokaryotic cells. Prokaryotes replicate via binary fission, that is, simple cell division whereby two identical offsprings each receive a copy of the original, single, parental chromosome. Binary fission is a type of asexual reproduction that does not require the union of two reproductive cells, and that produces offspring genetically identical to the parent cell. A population of rapidly growing prokaryotes can synthesize their DNA almost continuously, which aids in their fast generation times. Even as a cell is physically separating, its DNA can be replicating for the next round of cell division.

Membranes are large structures that contain lipids and proteins as their major components, along with a small amount of carbohydrates. The ratio of lipid to protein can range from 4:1 in the myelin of nerve cells to 1:3 in bacterial cell membranes, though many have a similar lipid to protein ratio (1:1) as in human erythrocytes. The predominant lipids in cell membranes are *phospholipids*, *sterols*, and *glycolipids* (*sphingolipids*). The long-nonpolar hydrocarbon tails of lipids are attracted to each other and are sequestered away from water. Membrane proteins contain a high proportion of hydrophobic and acidic amino acids, but the study of membrane proteins are difficult, mainly due to loss of biological activity. However, it became apparent from earlier studies that protein was layered on both sides of a lipid bilayer which was confirmed by electron microscopy using OsO_4 (*Osmic acid*) staining. Several difficulties were encountered in explaining the properties of cell membranes in terms of this structure. Later several micellar models suggested that the nonpolar tails of the lipids formed a close association within the micelles with their polar carboxyl heads on the outside and surrounded by protein. However, the stability of this system was difficult to explain because highly nonpolar compounds must pass through the polar protein layer. Controversy continued about the exact location of the protein in the membranes. The cell membrane functions as a semipermeable barrier, allowing very few molecules across it, while fencing the majority of organically produced chemicals inside the cell. Electron microscopic examinations of cell membranes have led to the development of the lipid

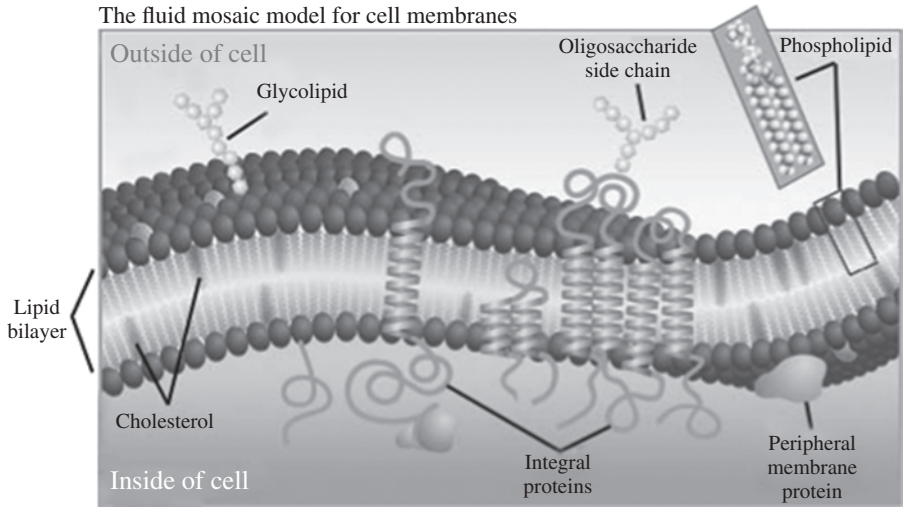

Figure 1.3 Fluid mosaic model of the structure of a membrane. *Source*: http://www.biology.arizona.edu/cell_bio/problem_sets/membranes/fluid_mosaic_model.html. (*See insert for color representation of this figure.*)

bilayer model (referred to as the *fluid mosaic model* proposed by Singer and Nicolson in 1972). This model suggested that the integrated proteins are located within the lipid bilayer in a number of ways. The hydrophobic amino acid residues of the protein are in close contact with the hydrophobic side chains of the phospholipids and the hydrophilic amino acid residues are on the surface in contact with water (Figure 1.3).

The oligosaccharide side chains of glycoproteins and glycolipids are always present on the outer membrane surface and never on the inside of the cell. The lipid bilayer is fluid at physiological temperatures, so that the phospholipid molecules are more mobile in the membrane plane to flow laterally and membranes are distinctly asymmetric. Membranes perform a variety of important functions, where their principal role is to control the flow of ions, metabolites, and other foreign compounds into and out of the cell and between the various cellular compartments. Membrane transport can occur by *diffusion* (nonmediated transport) or by means of a carrier (carrier-mediated transport). Transport can also be described as either passive or active. Further references on the structure and transport of membranes are listed. Figure 1.4 shows the differences of typical three cells.

Animal cells are typical of the eukaryotic cell, enclosed by a plasma membrane and containing a membrane-bound nucleus and organelles. Unlike the eukaryotic cells of plants and fungi, animal cells do not have a cell wall. This feature gave rise to the kingdom *Animalia*. Most cells, both animal and plant, range in size between 1 and 100 μm and are thus visible only with the aid of a microscope. The lack of a rigid cell wall allowed animals to develop a greater diversity of cell types, tissues and organs. The animal kingdom is unique among eukaryotic organisms because most animal tissues are bound in an *extracellular matrix* by a triple helix of protein known as *collagen*. Plant and fungal cells are bound in tissues or aggregations by other molecules, such as *pectin*. Animals are a large and incredibly diverse group of organisms. Making up about three-quarters of the species on Earth, they run the gamut from corals and jellyfish to ants, whales, elephants, and, of course, humans. Unlike plants, however, animals are unable to manufacture their own food, and therefore,

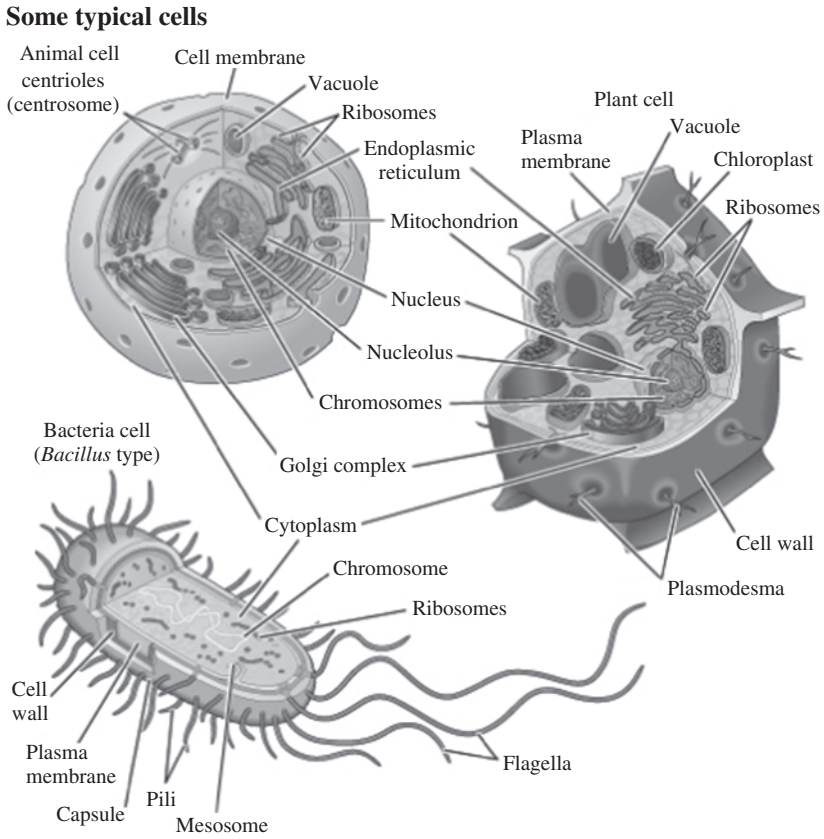

Figure 1.4 Anatomy differences of typical animal, plant, and bacterial cells. *Source*: Reprinted with permission from Encyclopædia Britannica, ©2010 by Encyclopædia Britannica, Inc. (*See insert for color representation of this figure.*)

are always directly or indirectly dependent on plant life. Most animal cells are *diploid*, meaning that their chromosomes exist in homologous pairs. Different chromosomal ploidies are also, however, known to occasionally occur. For the proliferation of animal cells in sexual reproduction, the cellular process of *meiosis* is first necessary so that haploid daughter cells, or *gametes*, can be produced. Two haploid cells then fuse to form a diploid *zygote*, which develops into a new organism as its cells divide and multiply.

Animal cells have a similar basic structure like bacteria in that there is a nucleus surrounded by cytoplasm contained in a cell membrane. As animals are multicellular organisms, there is a centrosome that splits in two when the cells divide during a process called mitosis. Lysosome has a similar job to chloroplasts in plant cells as they are responsible for absorbing and digesting.

Similarities and differences among cells are shown in Table 1.1 (www.k12.de.us /richardallen/science/comparing_cells/). The most striking difference among plant cells and other cells is the uniform shape. Each plant cell is roughly square or rectangular in shape, whereas an animal cell varies in shape. Around the nucleolus of the plant cell is a

Table 1.1 Comparision of features in bacterial, plant, and animal cells

Cell feature	Bacterial cells	Plant cells	Animal cells
1. Cell size (μm)	1–10	10–100	10–100
2. Cell wall	Yes (murein)	Yes (cellulose)	No
3. Cell nucleus	No	Yes (double nucleus membrane)	Yes (d.n.m)
4. Plasmids	Yes (double stranded, circular)	No	No
5. Chromosome (DNA)	Single circular without histone	Multiple linear with histone	Multiple linear with histone
6. Mitochondria	No	Yes (double membrane)	Yes (d.m)
7. Ribosomes	Small (70S)	Large (80S)	Large (80S)
8. Chloro plasts	No	Yes	No
9. Golgi	No	Yes	Yes
10. Vacuoles	No (except for blue–green bacteria)	Yes	No
11. Flagella	Yes	No	No
12. Capsule	Some	No	No
13. Cell division	Fission or budding	Mitosis	Mitosis
14. Cytoplasm (cytoskeleton)	No	Yes	Yes

d.n.m, double nucleus membrane; d.m, double membrane

layer of chromatin, which is a DNA protein complex nourishing and protecting the cells and is the most important element of the plant cell. Another vital element of a plant's cell structure is the chloroplasts, which are responsible for photosynthesis. Contained in the chloroplast are the granum, stroma, and thylakoid. The peroxisome is another unique plant cell element that removes hydrogen from the air and facilitates water absorption during photosynthesis. Plant cells also possess a cell wall and a membrane. The cell wall does roughly the same job as the membrane but its solid nature allows plant cells to maintain a ridged shape. Bacteria are single-celled organisms with a basic cellular structure that has a nucleolus, which is the brain of the cell; it is surrounded by cytoplasm, a jelly-like substance containing nutrients and a cell membrane. Although animals, plants and bacteria may seem vastly different, there are more similarities among the cell's structures than differences. All cells have a nucleus and most of the body space is taken up by the cytoplasm. Plants and animals then share more components than bacteria due to more complex structures. The vacuole is a sack filled with water within the cell. It is much larger in plants and sometimes comprises 90% of the total cell. It contains ions, sugars, and enzymes. The Golgi body contains proteins and carbohydrates and helps maintain the cell membrane. Mitochondrions produce energy for the cell by converting glucose into adenosine triphosphate (ATP). The rough and smooth endoplasmic reticulum (ER) can be seen as the intestines of the cell as they transport proteins though the cell. These are covered in ribosomes, which are small grains of cytoplasmic material responsible for protein synthesis.

Many transgenic (or genetically modified) microorganisms are particularly important in producing large amounts of pure human proteins for use in medicine. GM bacteria are now used to produce the protein, insulin, to treat diabetes. Similar bacteria have been used to

produce clotting factors to treat hemophilia and human growth hormone to treat various forms of dwarfism. These microbial recombinant proteins are safer than the products they replaced because the products obtained earlier were purified from cadavers and could transmit diseases. In fact, the human-derived proteins caused many cases of AIDS and hepatitis C in hemophilliacs and the Creutzfeldt–Jakob disease from human growth hormone. Recombinant proteins derived from microrganisms will be discussed in the section of microbial products.

1.3 Bacterial growth and fermentation tools

Growth and applications of animal cells and plant cells will be separately covered in the chapters on Animal Biotechnology and Plant Biotechnology.

Microbes are the *tools of fermentation* because they produce enzymes, amino acids, vitamins, biogums, other valuable recombinant proteins, and organic acids. This discussion will thus mainly focus on the growth of unicellular bacteria as they are ideal objects for study of the growth process, current scale-up process for the manufacture of industrial products, and many aspects of food biotechnology. Negative aspects of microrganisms are also the most common causes of food-borne illness and food spoilage and thus the detection of pathogens, and so on, using biosensors and nanobiotechnology will also be covered in a different section.

Fermentation technology is becoming increasingly important in the production of various bulk chemicals, fine chemicals, and pharmaceuticals. Compared to the chemical manufacturing processes of various compounds, the fermentative production process is a very promising technology to produce enantiomer pure chemicals with low environmental burden. High conversion efficiencies are often achieved in fermentative production processes. For this reason, chemical industries are now investigating the field of biotechnology as a more economic alternative for the chemical synthesis of compounds. Moreover, by means of fermentation, it is possible to convert abundant renewable raw materials or waste materials to produce high-value products.

1.3.1 Classification and reproduction of biotechnologically important bacterial system

In contrast to the taxonomy of plants and animals, which show a diversity of cell types, a bacterial system is very simple and is classified based on artificial criteria such as structure, shape, motility, nutrition, propagation and immunological reactions. Tables 1.2 and 1.3 summarize the most important bacterial species that are involved in biotechnology processes on the basis of the classification in *Bergey's Manual of Systematic Bacteriology*. This familiar reference work differentiates the bacteria into the 19 parts listed in Table 1.2, each of which is subdivided into orders, families, genera and species. These classifications show differences in many characteristics of energy and nutritional requirements, growth and product release rates, method of reproduction, motility, and habitats. All these factors are of great practical importance in applications of biotechnology. Other differences in the morphology or the physical form and structure are also important in the calculation of the rate of nutrient mass transfer and the fluid mechanics of a suspension containing microbes. Table 1.3 lists some bacteria of technological importance by group, family, genus and process. The detailed fermentation processes and tools related to the important food fermentations are described in Part II.

Table 1.2 The important bacterial family in biotechnological processes

Part	Type
1	Photosynthetic bacteria
2	Gliding bacteria
3	Sheathed bacteria
4	Budding and/or appendaged bacteria
5	The spirochetes
6	Spiral and curved bacteria
7	Gram-negative aerobic rods/cocci
8	Gram-negative facultative anaerobic rods
9	Gram-negative anaerobic rods
10	Gram-negative cocci and coccobacilli
11	Gram-negative anaerobic cocci
12	Gram-negative chemolithotrophic bacteria
13	Methane-producing bacteria
14	Gram-positive cocci
15	Endospore-forming rods and cocci
16	Gram-positive asporogeneous rod-shaped bacteria
17	Actinomycete and related organisms
18	The richettsias
19	The mycoplasmas

Source: Adapted from *Bergey's Manual of Systematic Bacteriology*, Vol. 3, J. T. Staley, Ed. Baltimore: Williams & Wilkins, 1989.

The basic unit is the species, which is characterized by a high degree of similarity in physical and biochemical properties, and significant differences from the properties of related organisms. The Gram-positive bacteria are those that retain the purple stain of crystal violet/iodine after it is washed with ethanol, while Gram-negative species are those that decolorize. The Gram stain developed by Christian Gram in 1884 reflects an important chemical property of the cell wall and has proved to be a valuable taxonomic criterion.

Most prokaryotes reproduce by asexual means in the haploid state. The asexual process involves simple fission, in which DNA replication is followed by the formation of a septum, which divides the cell into two genetically identical clones (i.e., descendants of a single bacterial cell). Sexual reproduction involves the fusion of two reproductive cells (i.e., gametes), each of which contains a complete set of genetic material, producing more individuals. Therefore, only incomplete sets of genetic material can be transferred between bacteria. Sexual reproduction, which is characteristic of many eukaryotes (persistent diploidy), rarely occurs in prokaryotes. Genetic transfer among prokaryotes always occurs by means of a unidirectional passage of DNA from a donor cell to a recipient. This can be mediated either by conjugation, which involves direct cell-to-cell contact, or by transformation and transduction. However, genetic exchange of prokaryotes is rather an occasional process, but it occurs quite frequently in eukaryotes.

1.3.2 Bacterial growth

This discussion focuses mainly on the growth of unicellular bacteria, which are ideal objects for study of the growth process. In an adequate medium to which microorganisms have

Table 1.3 Some bacteria of biotechnological importance among the 19 bacterial groups

Group and family	Genus	Process
7. Gram-negative aerobic rods/cocci		
Pseudomonadaceae	*Pseudomonas*	Single cell protein (SCP) from methanol, oxidation of steroids/hydrocarbon, polysaccharides (alginate); oxidation of alcohols
Methylomonadaceae	*Methylomonas*	SCP from methanol
	Methylococcus	Oxidation of methane
Azotobacteriaceae	*Azotobacter*	Nonsymbiotic binding of nitrogen
8. Gram-negative facultative anaerobic rods		
Enterobacteriaceae	*Escherichia*	Many different processes, productions of amino acid (lysine)
	Aerobacter	Nucleotides, 2-ketoglutaric acid, pullulanase, 6-aminopenicillanic acid, recombinant rennet
12. Gram-negative chemolithotrophic bacteria		
	Thiobacillus	Leaching of copper, zinc, iron, manganese, other sulfides
13. Methane-producing bacteria		
Methanenobacteriaceae	*Methanobacterium*	Methane from sewage
	Methanococcus	
Nocardiceae	*Norcardia*	Oxidation of hydrocarbon, steroids
14. Gram-positive cocci		
Micrococcaceae	*Micrococcus*	Oxidation of hydrocarbon, meat starter culture
Streptococcaceae	*Streptococcus* (*Lactococcus*)	Production of lactic acid, diacetyl; cheese and fermented dairy product starter
Pediococcaceae	*Pediococcus*	Meat starter
	Leuconostoc	Dextran production; cheese starter, wine starter (heterofermentation)
15. Endospore-forming rods/cocci		
Bacillaceae	*Bacillus*	Antibiotics, many enzymes, amino acids, and vitamins (B_2, B_{12})
	Clostridium	Butanol, acetone, butyric acid, botulins
16. Gram-positive asporogeneous rod-shaped bacteria		
Lactobacillaceae	*Lactobacillus*	Lactic acid, fermented milk products, fermented sausage and vegetables; silage, spoilage of foods
	Bifidobacterium	Bifidoyogurt, bifidotablets
17. Actinomycete-related organisms		
Coryneform group	*Corynebacterium*	Oxidation of hydrocarbon, amino acids
	Arthrobacter	Transformation of steroids
	Cellulomonas	Cellulose fermentation
Propionibacteriaceae	*Propionibacteria*	Vitamin B_{12}, propionic acid, cheese fermentation
Mycobacteriaceae	*Mycobacterium*	Oxidation of hydrocarbons and steroids

become fully adapted, cells are in a state of balanced growth. Cultures undergoing balanced growth maintain a constant chemical composition with an increase of the biomass. In higher organisms, growth is defined as an increase either in size or in organic matter. In unicellular microbes, however, increases in number (population) or mass of cells normally are used as indicators of growth.

The rate of increase in bacteria at any given time is proportional to the number or mass of cells present, which is similar in many aspects to first-order chemical reaction kinetics. The velocity of a chemical reaction is determined by the concentration of the reactants, but the growth rate of bacteria remains constant until the limiting nutrient of the medium is almost exhausted. This can be explained by the action of carrier proteins known as permeases, which are capable of maintaining saturating intracellular concentrations of nutrients over a wide range of external concentrations.

In *batch culture*, a pure culture is grown in a suitable medium containing the substrate, and incubation is continued until transformation of the substrate ceases. In this process, the biocatalyst is used only once and then discarded. The procedure is useful for screening purposes. If the concentration of one essential medium constituent is varied, while the other medium components are kept constant, the growth curves to nutrient concentration are typically hyperbolic and fit the *Monod equation*:

$$\mu = \frac{V_{\max}[S]}{K_s + [S]}$$

where μ is the specific growth rate at limiting nutrient concentration, $\mu_{\max}$ is the maximum growth rate achievable when $[S] > K_s$ and all other nutrient concentrations are unchanged, and K_s is the value analogous to the Michaelis–Menten constant of enzyme kinetics, being equal to the concentration supporting a growth rate to $\mu_{\max}/2$. The K_s values for glucose and tryptophan utilization by *E. coli* are 1×10^{-6} and 2×10^{-7} M/mL, respectively. These very low values can be attributed to the high affinities characteristic of bacterial permeases. In the following equation, the constant of proportionality K is an index of the growth rate, which often is called the growth rate constant, and t_d is the mean generation or doubling time:

$$K = \frac{\ln 2}{t_d} = \frac{0.693}{t_d}$$

For example, the mean doubling time t_d of the culture may be $0.693/2.303 = 0.3$ h ($\approx$18 min), which is a relatively high growth rate for a bacterium. In a typical batch growth, the cell numbers vary with time, as shown in Figure 2.7. The lag period of adjustment, where no increase in cell numbers is evident, is extremely variable in duration depending on the period of the preceding stationary phase. After this lag phase, a straight-line relationship is obtained between the log of cell number and time, with a slope equal to $K/2.303$ and an ordinate intercept of a log N_0. This stage of batch growth is called the *exponential* (or *logarithmic*) *phase*.

Bacterial growth in a closed vessel is normally limited either by the exhaustion of available nutrients or by the accumulation of toxic by-products. As a consequence, the growth rate declines and growth eventually stops; at this point, however, the population has achieved its maximum size. This stage is called the stationary phase. The transition between the exponential phase and the stationary phase involves a period of unbalanced growth during which the various cellular components are not synthesized at equal rates. Eventually, bacterial cells held in a nongrowing state die; this is the death phase. Death results from a number of factors, such as depletion of the cellular reserve of energy.

The death rate of bacteria is highly variable, depending on the environment as well as the particular species, and the age and size of the transferred inoculum.

Each phase is of potential importance in a biotechnological process. The general objective of a good fermentation design is to minimize the length of the lag phase and to maximize the rate and length of the exponential phase for achieving the largest possible cell density at the end of the process. When cells switch rapidly to a new environment, an adaptive period is required for the synthesis of the new enzymes and cofactors needed for assimilation; thus a lag will appear. Multiple lag phases can sometimes be observed when the medium contains multiple carbon sources. This phenomenon, called diauxic growth, is carried out by a shift in metabolic patterns in the middle of growth. For example, during the growth of *E. coli* in the presence of glucose and lactose, glucose is consumed during the first phase of exponential growth and lactose in the second. The enzymes for glucose utilization are constitutive, which means that the enzymes are always present, while those for lactose utilization are inducible in that they are produced only in the presence of lactose.

The net amount of bacterial growth is the difference between cell mass or number used as an inoculum and cell mass obtained at the end of culture. When growth is limited by a particular nutrient, a linear relationship between nutrient and the net growth results. The cell mass produced per unit of limiting nutrient is a constant called the growth yield (Y), and the value of Y can be calculated by the following equation.

$$Y = \frac{X - X_0}{[N]} \quad \text{or} \quad Y = \frac{X - X_0}{[S]}$$

where X is the dry weight per milliliter of culture at the beginning of stationary growth, X_0 is the initial cell mass immediately after inoculation, and the concentration of limiting nutrient (organic substrate) is $[N]([S])$.

In the case of chemoheterotrophic bacteria, which use the organic substrate as the sole source of carbon and energy, the growth yield can be measured in terms of the organic substrate and biomass resulting. Many microorganisms utilizing sugars as the sole source of carbon reveal that the ratio of the sugars to cellular carbon varies between 20% and 50%. The microbes usually use about half the carbon source to make cells and metabolize the other half to CO_2 or other by-products. The differences in conversion of efficiency probably reflect differences in the efficiency of generating ATP through catabolism of the substrate.

In batch cultures discussed so far, nutrients are not renewed and growth remains exponential for only a few generations. Thus, the physiological state of cells in batch cultures varies continuously throughout the growth cycle. In continuous cultures, however, cells can be maintained in a steady physiological state for long periods of time by adding fresh medium continuously and removing equal amounts of spent medium. Although exponentially growing cells in batch cultures may suffice for some studies, many studies on microbial physiology require a cell that is not constantly changing. A batch fermentation can be extended by feeding, either intermittently or continuously, nutrients containing a substrate that limits cell growth. This so-called *fed-batch* operation can forestall the inevitable accumulation of too much cell mass; but since there is no built-in provision for product removal, at some point the cell mass will become unsustainable. Growth may be prolonged, but depletion of selected nutrients and accumulation of metabolic by-products change the environment.

In the absence of genetic selection, continuous culture offers the means of obtaining a cell population that grows indefinitely in an unchanged environment. This is accomplished by feeding a complete medium to a fermentation and removing whole broth to maintain a fixed volume. The turbidostat and the chemostat are the two most widely used devices for

promoting growth at the maximal rate. The cell density is controlled by washing the cells out of the vessel to maintain a certain turbidity, as ascertained by optical density measurements of the medium.

Chemostatic operation involves maintenance of the microbial culture density by exhaustion of either a limiting substance or the nutrient. The flow rate is set at a particular value and the growth rate of the culture adjusts to this flow rate. Thus cell growth is limited by a selected nutrient, and the rate at which the medium is supplied dictates the growth rate of the organism. Continuous culture systems offer a few valuable features:

1. They provide a constant source of cells in an exponential growth phase.
2. They allow cultures to be grown continuously at extremely low concentrations of substrate, which is valuable in studies on the regulation of synthesis or catabolism of the limiting substrate, or in the selection of various classes of mutants.
3. They offer an increase (over batch or fed-batch systems) in productivity per unit of product manufactured and a reduction of scale-up and capital costs.

Nevertheless, continuous culture is not widely used as an industrial process, mainly because of the problems of chance contamination, and the danger of strain degeneration by spontaneous mutation, which produces a new strain of low product formation.

In the Monod chemostat model (Figure 2.8), the concentration of the limiting nutrient remains constant. Thus, the rate of addition of the nutrient must equal the rate at which it is utilized by the culture together with that lost through the overflow. The flow rate F is measured in culture volumes V per hour. The expression F/V is the dilution rate D. Thus

$$\mu = D$$

$$\mu_{\max} = \frac{[S]}{K_s + [S]} = D \quad \text{and} \quad C = \mu \frac{D}{\mu_{\max} - D}$$

solving for X (cell concentration)

$$X = Y(C_r - C)$$

where Y = yield factor.

In the relationship between cell concentration (X), limiting nutrient concentration (C), and the dilution rate (D), cell number and the concentration of limiting nutrient change little. As $D_{\max}$ approaches $\mu_{\max}$, it is near washout. It is equivalent to μ, and the concentration of the limiting nutrient approaches its concentration in the reservoir (C_r).

1.3.3 Environmental factors affecting bacterial growth

The growth of microorganisms is influenced by various factors, including nutrients, which have already been discussed, and the interactions between the microbial cell and its environment, which are shown in Figure 1.5.

1.3.3.1 Solutes Transport mechanisms play two essential roles in cellular function. First, they maintain the intracellular concentration of all metabolites at levels high enough to operate both catabolic and anabolic pathways at near-maximal rates, even when nutrient concentration of the external medium is low. This is known to be true because the

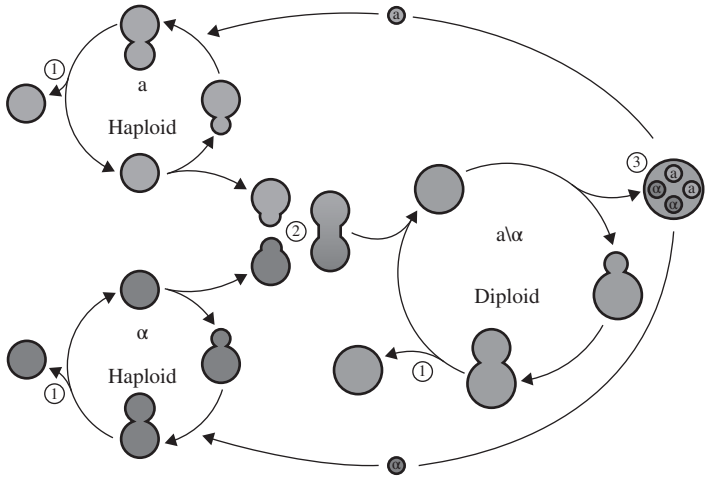

Figure 1.5 Sexual reproduction in the yeast life cycle. *Source*: http://en.wikipedia.org/wiki/File:Yeast_lifecycle.svg. (*See insert for color representation of this figure.*)

exponential growth rate of a microbial population remains constant until one essential nutrient in the medium falls to zero. Second, transport mechanisms function in osmoregulation, which maintain the solutes (principally small molecules and ions) at levels optimal for metabolic activity, even under a wide range of the osmolarity (i.e., the osmotic pressure exerted by any solution). If the internal osmotic pressure of the cell falls below the external osmotic pressure, water leaves the cell and the cytoplasmic volume decreases with accompanying damage to the membrane. Thus, the lysis of cells can be achieved by osmotic shock. In Gram-positive bacteria, this pressure causes plasmolysis: the pulling away of the cell membrane from the wall. Plasmolysis can be induced in a strong solution of sodium chloride.

Bacteria vary widely in their osmotic requirements. Microorganisms that can grow in solutions of high osmolarity are called osmophiles. Halophiles are microbes that grow in saline environments. Halophiles such as *Pediococcus halophilus* can tolerate high concentrations of salt in the medium but can also grow without salt. Other bacteria, such as marine bacteria and certain extreme halophiles, require NaCl for growth.

1.3.3.2 Temperature Temperature has a marked effect on microbial growth. Note given in Chapter 1 (Figure 1.18) in the Arrhenius plot that a plot of log velocity V of chemical reaction, as a function of temperature T, yields a straight line with a negative slope. This relationship can be expressed as follows:

$$\log_{10} V = \frac{-\Delta H}{2.303RT} + C$$

where V is the reaction velocity, ΔH the activation energy of the reaction, R a gas constant, and T the temperature in Kelvin's.

Unlike chemical reactions, however, the growth curve is linear only over the upper limit for survival of the microbes. Within certain limits, the rate of growth increases with a rise in temperature; but the cells die if the temperature is too high. Most microbes have preferred

Table 1.4 Temperature ranges of bacterial growth

Group	Temperature (°C)			Organism examples (temperature range, °C)
	Minimum	Optimum	Maximum	
Psychrophiles	−5 to 5	15–30	19–35	*Bacillus globispolus* (−10 to 25) *Micrococcus cryophilus* (−8 to 25) *Vibrio marinus* (−8 to 20) *Xanthomonas pharmicola* (0–40) *Pseudomonas rinicola* (3–40)
Mesophiles	10–15	30–45	35–47	Many species
Thermophiles	40–45	55–75	60–110	*Bacillus thermophilus* (45–60) *Thermus aquaticus* (65–100) *Pyrococcus* spp. (100–103)

temperature ranges, reflecting the chemical and physiological properties of their proteins and membranes. The range of temperature that is capable of supporting life lies roughly between −5 and 95 °C (up to 110 °C). Bacteria are often divided into three main broad groups: psychrophiles, which grow well at 0 °C, mesophiles, which grow well between 30 and 45 °C, and thermophiles, which grow at elevated temperatures above 55 °C.

Psychrophiles that grow at temperatures above 20 °C are often called facultative psychrophiles, while the ones that cannot grow above 20 °C are called obligate psychrophiles. The psychrophiles maintain the fluid nature of the membranes and are active at low temperatures. However, most bacteria stop growing at a temperature well above the freezing point of water. Some isolates from a cold environment can grow at temperatures as low as −10 °C, since high solute concentrations prevent the medium from freezing. Some bacteria isolated from hot springs such as *Pyrococcus* are capable of growth at temperatures as high as 110 °C, that are called extremophiles. Fluidity in psychrophiles is believed to be a function of the length and structure of the fatty acids in the phospholipid bilayer of the cell membrane. In *E. coli*, as the temperature decreases, the increase of unsaturated fatty acids (hexadecenoic and octadecenoic) is observed, and there is an increase in the amount of saturated fatty acids, such as palmitic, in membrane lipids. At low temperatures, all proteins also undergo slight conformational changes, attributable to the weakening of their hydrophobic bonds, which are important in determining the three-dimensional structure. Therefore, mutations that decrease the temperature at which growth can occur are likely to be present in genes encoded in these proteins.

Similarly, the adaptation of a thermophile to its thermal environment can be achieved through mutations affecting the structure of most proteins of the cell. Carbohydrates in glycoproteins and the rigidity of the protein structure in the presence of salts are considered to be the causes of increased thermoresistance. A large number of enzymes (e.g., α-amylase from thermophiles) depend on calcium for their high thermotolerance. Thermostability in thermophiles is controlled by plasmid DNA (Table 1.4).

1.3.3.3 Oxygen The oxygen requirements among bacteria are remarkably variable, and the fermentation conditions are decisively affected by whether the organism is aerobic or anaerobic. For aerobes, an adequate amount of dissolved oxygen (DO) must always be available in the medium. Facultative aerobes (or anaerobes) tolerate a wide range

of oxygen tensions. Anaerobes cannot utilize O_2, and there are two types: the *obligate anaerobes* (e.g., *Clostridium*), which will grow only in its absence and for which O_2 is toxic, and the aerotolerant anaerobes, which are not killed by exposure to O_2. Some obligate aerobes (e.g., lactic acid bacteria) show optimum growth at low oxygen levels (2–10% v/v); these organisms are called *microaerophiles*.

Some bacteria contain certain enzymes capable of eliminating O_2 toxicity. The oxidations of flavoproteins by O_2 produce a toxic compound H_2O_2, but most aerobes and aerotolerant anaerobes contain the enzyme catalase, which decomposes hydrogen peroxide to oxygen and water. In these organisms, a more toxic compound, superoxide, is decomposed by superoxide dismutase, which catalyzes its conversion to oxygen and hydrogen peroxide. Members of the other bacterial group that are able to grow in the presence of air, the microaerophiles, do not have catalase but contain peroxidases, which decompose H_2O_2. All strict anaerobes so far studied lack both superoxide dismutase and catalase. Thus, these three enzymes play roles in protecting the cell from the toxic consequences of oxygen.

Many enzymes of strict anaerobes are rapidly and irreversibly denatured by exposure to O_2, and thus their purification and study must be conducted under anaerobic conditions. A notable example is nitrogenase, responsible for nitrogen fixation (e.g., Azotobacter). In most filamentous nitrogen-fixing cyanobacteria, however, nitrogenase is protected from oxygen inactivation by specialized cells (heterocytes) lacking photosystem II. The primary metabolic function of O_2 in strict aerobes is to serve as a terminal electron acceptor; but it also serves as a cosubstrate for enzymes like oxygenases, which catalyze some steps in the dissimilation of aromatic compounds and alkanes. Many aerobic pseudomonas can grow anaerobically using nitrate in place of O_2 as a terminal electron acceptor.

1.3.3.4 pH Since protein structure and enzyme activity are pH dependent, we expect cellular transport mechanisms, reactions, and growth rates to depend on pH. Bacterial growth usually is maximum in the pH range of 6.5–7.5, as exemplified by the effects of medium pH on the growth rates of *E. coli* and *Methylococcus capsulatus*. Most microbes are able to tolerate a variation of about 1–2 pH units on either side of a definite optimum. There are exceptions, however, including acidophiles, which grow at pH 2.0, and *Thiobacillus thiooxidans*, which can grow below pH 1 for generation of sulfuric acid. At the other extreme, the urea splitters can tolerate pH values greater than 10.

Other factors – such as ultraviolet (UV) irradiation, which causes lethal mutations, and biotic factors, which require the production of secondary metabolites – can also affect microbial growth.

1.4 Fungal growth and fermentation tools

Among eucaryotic organisms, the most frequently known species in biotransformation work are the subgroups of *fungi*, namely the *yeasts* and *molds*. Most fungi are aerobic microbes that form long filamentous, nucleated cells known as *hyphae*. The cell sizes are larger than bacteria, being 4–20 μm wide and >100 μm long. Hyphae grow intertwined to form mycelia. Fungal classification is based more on morphological characteristics than on dye staining and biochemical reactions.

Based on the nature of their life cycle, fungi are classified into (i) *Zygomycetes* (or *Phycomycetes*), (ii) *Ascomycetes*, (iii) *Basidiomycetes*, and (iv) *Fungi imperfecti*. Two characteristics are common to all fungi: *heterotrophic* and *saprophytic*. *Heterotrophic* fungi require a source of organic carbon for growth. Many also require particular amino acids

and vitamins. *Saprophytic* fungi grow on dead organisms and are parasitic and others are mutualistic. The second feature of fungi is that they are true eucaryotes that possess nuclei, and many cytoplasmic organelles such as an ER, cytoskeletal components, and mitochondria. The cell wall is mostly composed of chitin and rarely cellulose, and thallus consisting of hyphae. They are aerobic, rarely facultative anaerobic. There are about 70,000 species.

Yeasts form one of the important subgroups of fungi which have lost the mycelial habit of growth. Although most of the fungi have a relatively complex morphology, yeasts are distinguished by their usual existence as unicellular, small cells (5–30 μm long × 1–5 μm wide). Yeasts are classified in all three classes of higher fungi; *Ascomycetes*, *Basidiomycetes*, and *F. imperfecti*. The well-known yeast, *Saccharomyces cerevisiae*, is an ascomycetous yeast; budding ceases at a certain stage of its growth and the vegetative cells become transformed into asci, each containing four ascospores. The various paths of reproduction of yeasts are *asexsual* (*budding* and *fission*) and *sexual* (Figure 2.6). In budding, a small offspring cell begins to grow on the side of the original cell, and physical separation of mature offspring from the parent and formation of clumps of yeast cells involving several generation are then achieved.

Although budding is the predominant mode of multiplication in yeasts, there are a few that multiply by *binary fission*, much like bacteria. Fission occurs by division of the cell into two new cells. Sexual reproduction occurs by conjugation of two *haploid* cells (each having a single set of chromosomes) with dissolution of the adjoining wall to form a diploid (two sets of chromosomes/cell) zygote. The nucleus in the *diploid zygote* may undergo one or several divisions and form *ascospores*, and each of these eventually becomes a new haploid vegetative individual which may then undergo subsequent reproduction by budding, fission, or sexual fusion again. Besides playing an important role in the manufacture of wine and beer and in the leavening of bread, yeast supplies flavoring ingredients, nucleic acids, protein supplements, and other useful chemicals is described (Chapter 2).

A typical representative of the haploid yeasts is the fission yeast, *Schizosaccharomyces pombe*, in which the diploid phase is restricted to the zygote. Other example is the alkane yeasts, *Saccharomycopsis lipolytica* for the production of *single cell protein* (*SCP*) from paraffins and *Saccharomyces fibrigera* for the SCP from starch.

Molds are higher fungi with a vegetative structure called a *mycelium*, which is a highly branched system of tubes. Within these tubes is a mobile mass of cytoplasm containing many nuclei. The long, thin filaments of cells within the mycelium are called *hyphae*. Molds do not contain chlorophyll, are nonmotile and reproduction, which may be sexual or asexual, is accomplished by means of spores. The mycelium (which is very dense), coupled with the mold's oxygen-supply requirements for normal function, can cause complexities in their cultivation, as the mycelium offers a substantial mass-transfer resistance. The most important classes of molds industrially are *Aspergillus* and *Penicillium*. Major useful products of these organisms are antibiotics, organic acids (oxalic acid, citric acid), and biological catalysts (enzymes). The fungi of biotechnologically importance are summarized in Table 1.5. Filamentous fungi are also large-scale producers of pigments and colorants for the food industry and some fermentative food grade pigments from filamentous fungi exist in the market are: *Monascus* pigments, Arpink red™ from *Penicillium* oxalicum, riboflavin from *Ashbya gossypii*, lycopene and *b*-carotene from *Blakeslea trispora*. The production yield in the case of *b*-carotene could be as high as 17 g/L of the *B. trispora* culture medium. For more detailed information about this group of organisms, the reader should consult specialized books dealing with this subject.

Table 1.5 Some of recognized biotechnologically important fungi

Genus	Products
Mucor	Organic acids, enzymes
Rhizopus	Organic acids, enzymes
Blackeslea	β-Carotene
Choanephora	β-Carotene
Ashbya	Riboflavin
Cryptococcus	Riboflavin
Candida	Citric acid
Rhodotorula	Lipids
Saccharomyces	Ethanol, wine, beer
Saccharomycopsis	Proteins
Torulopsis	Citric acid
Aspergillus	Enzymes, antibiotics, organic acids
Cephalosporium	Antibiotic
Penicillium	Antibiotic, organic acid, enzymes
Fusarium	Protein, fat
Gibberella	Hormone like substances, Gibberellins

Fungal growth is often carried out in *solid-state fermentation* (*SSF*), in which microbial growth and product formation occur on solid, normally organic materials such as cereal grains, wheat bran, legumes, and lignocellulosics, and so on. Traditionally SSF has been used in Oriental foods to produce *Koji* using rice in order to manufacture alcoholic beverages such as *Sake* or *Koji* using soybean to produce soy sauce or temph, sufu, and so on. In western countries, the traditional manufacturing process of many foods requires SSF for fermented bakery products such as bread or for the maturing of mold-ripened cheese and sausage. SSF is also widely used to prepare raw materials necessary for some of our daily food or cacao bean and coffee bean fermentation.

One important application of SSF is the manufacture of industrial enzymes; that is, SSF is particularly well suited for the production of various enzymatic complexes composed of many different enzymes. SSF enzymes are widely used in fruit and vegetable transformation (pectinases), baking (hemicellulases), animal feeding and bioethanol (hemicellulases, cellulases), brewing and distilling (hemicellulases), and in the production of digestives (crude form of mixed enzymes). SSF has several advantages in lower cost and simple technology, and higher and reproducible product yields, but have problems with heat buildup, slower microbial growth, and high power requirement in continuous agitation as well as the risk of bacterial contamination.

Liquid-, submerged-, and SSF have been used for the preservation and manufacturing of foods. However, *liquid-state fermentation* has been developed on an industrial scale to manufacture vital metabolites such as antibiotics. Economic changes and the growing awareness for environmental criteria generate new perspectives for SSF. The renewal of SSF has now become possible with a new generation of industrial equipments dedicated to that technology. The Fujiwara company in Japan can transform substrate volumes up to 400 m^2 for the production of soy sauce or sake. SSF companies for the production of enzymatic complexes can be found at LYVEN (France) for the manufacturing pectinases and hemicellulases on beet pulp and wheat bran. LYVEN has been taken over by SOUFFLET group in 2003.

1.5 Classical strain improvement and tools

After an organism is chosen for a particular fermentation, the next step is to increase its yield. The aim of strain improvement is to block the regulatory mechanisms of an organism so that maximum metabolic energy is devoted to a single product. The major aim is to achieve economic viability because the metabolite concentrations produced by wild strains are usually too low for use in economical processes.

1.5.1 Natural selection and mutation

The oldest method, *screening*, does not require complicated biochemical and genetic information on the strain. The screening process is often the most direct and least expensive means of improving most industrial microorganisms. Improvement in the quality of agricultural animals and plants has also for many years relied on the selection of desirable characters from natural variants or the hybridization of related species. Natural variants are often the products of chance mutations. Another selection technique, the so-called *enrichment* procedure, frequently uses special environmental conditions that are toxic to a majority of cell types but less or nontoxic to a desired minority of cells, to enrich a cell population for the desired mutants. Many enrichment procedures take advantages of the evolutionarily conserved natural regulatory mechanisms that control primary metabolism of microorganisms. By applying *analogs* of amino acids and vitamins, which regulate their own synthesis, mutants that lack feedback regulation can be selected to overproduce these metabolites. In the wild type cell, the analog prevents synthesis of an adequate amount of the primary metabolite. The primary metabolite is required for growth and maintenance. Thus, most normal cells die and the remaining population is enriched for *deregulated mutants*. Selection of deregulated mutants has been applied extensively to the microbial production of amino acids, vitamins, and nucleic acid precursors (Table 1.6).

The enrichment procedures of *auxotrophic mutants* can also be valuable tools for strain improvement. Blockage of a known metabolic pathway represents a simple and direct method of shunting metabolism in predictable patterns. Many economically important primary metabolites such as amino acids and nucleotides have been formed through branching biosynthetic pathways (Table 1.7). As auxotrophs lack one or more enzymatic step in one or several biosynthetic pathways, one or several end products of the pathway are not synthesized and the missing metabolites must be fed to grow a mutant. When the unsynthesized metabolite is a repressor, such auxotrophic mutants (lacking repressor synthesis) can be made to overproduce an intermediate metabolite.

By keeping the concentration of repressor low in the medium, feedback inhibition and repression of pathway enzymes are minimized. The intermediate, normally substrate for that enzyme which is absent from the mutant, will then achieve much higher concentrations than in the native organism. As compared with an auxotroph, which requires the nutrient, a strain that synthesizes its own nutrient is said to be *prototroph* (parent strain).

The role of auxotroph in commercial *L-lysine* production using *Corynebacterium glutamicum* is illustrated in Figure 1.6. A mutant of *C. glutamicum* lacks *homoserine dehydrogenase*, so that the inhibition of end product, threonine on lysine synthesis by *asparto* (*aspartate*) *kinase* does not occur. As the auxotrophic mutant does not synthesize threonine or methionine, these amino acids must be added to the growth medium. In *E. coli* having similar biosynthetic pathways, the aspartokinase system differs from *C. glutamicum* in that lysine inhibits its own production through the reaction leading to dihydropicolinate.

Table 1.6 Examples of analog-resistant deregulated mutants which overproduce primary and secondary metabolites

Product	Analog resistance	Microbe
L-Tryptophane	5 or 6-Methyltryptophane	*Bacillus subtilis*
L-Threonine	α-Amino-β-hydroxyvalerate	*Brevibacterium flavum*
L-Arginine	D-Arginine, arginine hydroxamate	*Corynebacterium glutamicum*
L-Isoleucine	α-Amino-β-hydroxyvalerate α-Methylthreonine	*Brevibacterium flavum*
L-Histidine	Triazolealanine	*Corynebacterium glutamicum*
L-Glutamate	Anthracycline	*Corynebacterium* sp.
Inosine	8-Azoguanine	*Bacillus subtilis*
Guanosine	8-Azoxanthine	*Bacillus subtilis*
Biotin	5-Valeric acid, actithiazate	*Bacillus* sp.
Thiamine	Pyrithiamine	*Propionibacterium* sp.
Cellulase	2-Deoxyglucose	*Trichoderma* sp.
Amylase	Tunicamycin	*Bacillus subtilis*

Source: Author's compiled data.

Table 1.7 Examples of auxotrophic mutants that overproduce primary metabolites

Product	Organism	Auxotrophic requirement	Substrate	Yield (g/L)
L-Lysine	*Corynebacterium glutamicum*	Threonine, methionine	Glucose (double mutant)	50
	Brevibacterium flavum	Homoserine, threonine	Acetate	75
L-Phenylalanine	*Arthrobacter paraffineus*	Tyrosine	*n*-Alkanes	15
	Brevibacterium lactofermentum	Methionine	Glucose	25
L-Tryptophane	*Brevibacterium flavum*	Tyrosine	Glucose	19
L-Ornithine	*Corynebacterium glutamicum*	Arginine	Glucose	26
L-Leucine	*Brevibaterium lactofermentumis*	leucine, methionine	Glucose	28
L-Threonine	*Escherichia coli*	Lysine, methionine, isoleucine	Glucose	20
L-Tyrosine	*Corynebacterium glutamicum*	Phenylalanine	Glucose	18
L-Valine	*Corynebacterium glutamicum*	Isoleucine	Glucose	11
Inosine	*Brevibacterium ammoniagenes*	Adenine, guanine	Glucose	30
Guanosine	*Bacillus subtilis*	Adenine, histidine	Glucose	16

Source: Author's compiled data.

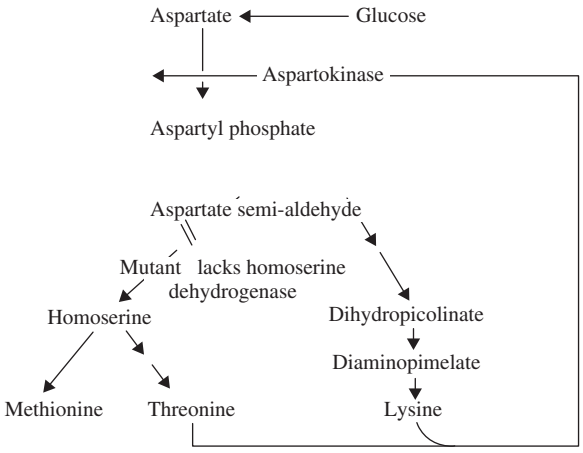

Figure 1.6 Pathway and control of lysine production in auxotrophic mutant of *Corynebacterium glutamicum*.

Similar strategies using an auxotrophic mutant of *Brevibacterium ammoniagenes* have been successfully used to overproduce the flavor enhancing *ribonucleotides* such as *inosine monophosphate* (*IMP*) and *guanine monophosphate*.

Almost half of increased crop yields and dramatic improvements in the efficiency of livestock production have come about through conventional genetic improvements, such as bulk breeding and selection techniques. These techniques have been extended to microorganisms and have contributed to the development of strong fermentation technologies. The biotechnological process based on classical microbial fermentation has been augmented by single genetic manipulation using chemical or physical mutagens to improve microorganisms for food fermentation and to enhance the production of bioingredients.

Mutants of *C. glutamicum* and *Brevibacterium flavum* can accumulate in the medium even in the presence of high concentrations of *biotin* (up to 100 g/L). Similarly, *α-amylase* yields from *Bacillus subtilis* have been improved a 1000-fold by mutation and screening. Pathways for the synthesis of antibiotics are often more complex than others and the effects of mutation are less predictable. Nevertheless the progressive selection of *high-yielding mutants* has been the basis for the development of today's highly efficient commercial strains. The yields of penicillin have been significantly increased from 0.15 to 7 g per liter by both *spontaneous* and *induced mutations*.

As a result of normal chromosomal replication or exposure to certain chemicals or physical agents, called *mutagenic agents* (*mutagens*), the nucleotide sequence of a gene occasionally changes. Any such change is called a *mutation*. If the mutant protein differs functionally from the wild type (the unmutated form), then it may result in a corresponding change in an observable character and produce a mutant organism. The difference could be one of enzyme activity or stability or one which affects the role of protein as a carrier or as a structural component of the cell. If the mutation affects the DNA of the reproductive cells, it is expressed as a heritable variation and is subject to natural selection. Such mutations provide the process for evolutionary change. Mutations in non-reproductive cells are called *somatic mutations* that produce local, non-heritable changes such as the pigmented cells in human cancer and possibly in aging.

Mutations may be due to different types of change in the DNA sequence and these are described by a number of specialized terms. *Point mutations* result from a single base

change in the DNA sequence and show a characteristic tendency to back mutation, that is, to revert to the wild type. This is because a further mutation at the same site has a one in three chance of restoring the original base sequence of wild type. Four types of single base change, transition, transversion, insertion, and deletion are known. *Transitions* are changes from a pyrimidine to a pyrimidine bases (T → C or C → T) or from a purine to a purine base (A → G or G → A). *Transversions* refer to the substitution of a pyrimidine with a purine or vice versa (T or C → A or G; A or G → T or C). A transition or transversion may change the codon for an amino acid residue to another codon for the same amino acid residue (same-sense mutation), to the codon for a different amino acid residue (missense mutation), or to a termination codon (nonsense mutation).

Frameshift mutations, another category of change, results when one nucleotide or more is inserted or deleted, thus altering the reading frame in the following transcription and translation processes. Although genome mutations are important in plant genetics, mutations used in microbial strain improvement usually are point mutations. Chromosome mutations also occur (e.g., deletions, duplications) but are of minor significance. Multisite mutations that affect more than one base do not back-mutate; that is, they are stable. This stability can be an advantage in industrial microbial strains which must retain the same characteristics over long periods. However, an extensive deletion will completely alter the resultant protein, and even a short deletion will have a severe effect if it alters the reading frame.

Spontaneous and induced mutations To some extent, mutation is a spontaneous process that is constantly occurring but the rate of spontaneous mutation is rather low. The naturally occurring error rate is as low as about one error in 10^{10} bases. The spontaneous mutation rate means that one in 10^7 cells will contain a point mutation, depending on the growth conditions of the organism. The causes of spontaneous mutations are likely due to the existence of *tautomeric* forms of all four bases in DNA, the integration and excision of *transposons*, along with errors in the functioning of several enzyme DNA polymerases, recombinant enzymes, DNA repair enzymes. *Tautomer* is a chemical that exists as a mixture of two interconvertable forms (keto or enol; amino or imino). Tautomerization can lead to mispairing during replication. *Transposon* is a DNA sequence (several kilobases in length) that can insert copies of itself into any DNA molecule in the same cell and hence disrupt the transcription and translation of any gene in which they insert. The movement of transposons constitutes transposition. Because of such low frequency of spontaneous mutations, it is not a cost-effective approach to isolate such mutants for the industrial strain improvement. The mutation frequency of cells can be significantly increased by using the chemical mutagens such as *nitrosoguanidine*, which interferes with DNA function. The chemical or physical agents work in many different ways but basically all interfere with DNA replication or repair. The agents may increase to 10^{-5} to 10^{-3} for the isolation of improved secondary metabolite producers or even up to 10^{-2} to 10^{-1} for the isolation of auxotrophic mutants. Commonly used chemical mutagents and their roles are shown in Table 1.8.

Except for UV radiation, the alkylating agents are the most potent mutagenes for practical application. The alkylating agents add methyl or ethyl groups to the heterocyclic nitrogen atoms of the bases and are known to cause transition, transversion, and −1 (but not +1) frameshift mutations. Because of their structural similarity, base analogs such as 5-bromouracil or 2-aminopurine are incorporated into replicating DNA in place of the corresponding bases, thymine, and adenine. The analogs tautomerize more frequently than the natural bases. Base analogs are of less importance for practical applications because of the costs required to set up the optimal conditions and of the complications in strain development.

Table 1.8 Examples of chemical mutagens and their mode of action

Chemical agent	Action
Mutagens that affect nonreplicating DNA	
Alkylating agents	
Nitrosoguanidine (*N*-methyl-*N*-nitro-*N*-nitrosoguanidine, NTG)	Transition, transversion, deletion, and frameshifts
Ethyl or methylmethanosulfonate	
Other DNA modifiers	
Hydroxylamine	Hydroxylates six amino groups of cytosine, causing G–C to A–T transitions
Nitrous acid (HNO_2)	Deamination of purine and pyrimidine bases of DNA
Mutagens that associate with or become incorporated into replicating DNA	
Base analogs	
5-Bromouracil	Incorporates into DNA
2-Aminopurine	Causing transition mutations
Intercalating agents	
Acridine dyes (acridine orange)	Frameshift mutations
Proflavine/acriflavine	

Intercalating agents (*frameshift mutagens*) such as acridine dyes, proflavine, and acriflavine are planar molecules that insert between the stacked pairs of bases. Such insertion distorts the backbone of the double helix, causing errors that result in the formation of faulty protein or no protein. Although acridines are useful for research, they are not very practical for a routine isolation of mutants. They are strong mutagens for bacteriophages (T_2 and T_4) but they have little mutagenic effect in bacteria. Various types of radiations are also powerful mutagens. UV light at a short-wavelength (200–300 nm) is strongly absorbed by DNA and the energy released causes dimerization between adjacent thymine residues, so that replication of the DNA cannot occur. UV light rapidly kills most cells, but the surviving cells exhibit a higher rate of mutation. However, there is one enzymatic repair mechanism that is specific for thymine dimers and can cleave them back to the monomer. Unlike the general repair mechanisms, this is totally error free and is light dependent. To maximize the yield of mutants, *in vitro* UV-treated cells should be kept in the dark for a few hours. In the presence of light, photoreactivating enzyme splits thymine dimers into monomer pyrimidines. Up to 80% of the thymine dimers in the genome (1000 pyrimidine dimers) can be repaired.

A different repair mechanism after UV damage is *excision repair*, which is possible with the complementary strand. Repair of DNA-containing pyrimidine dimers is achieved by nucleotide excision using specific endonucleases, DNA polymerase I and polynucleotide ligase. Excision repair can be partially prevented by inhibitors such as caffeine, acriflavine, and 8-methoxypsoralen. Ionizing radiations such as X-ray, γ-ray, and β-ray act by causing ionization of the medium and breaking single and double strands. Ninety percent of the single-strand breaks are repaired by nucleotide excision. Double-strand breaks result in major structural changes such as translocation, inversion, or similar chromosome mutations. Thus, in industrial strain development, UV radiation or chemical agents are normally preferable for mutations.

For most antibiotics from the discovery, the titer of the producing strain has increased by the order of magnitude by classical mutations. Although medium development and process engineering have been successful, strain improvement by radiation and chemical agents has been the key to improve the final antibiotic titer in fermentation. A typical strain improvement involved first generating genotype variants in the cells by physically or chemically induced mutations or by recombination among strains, that was followed by selection or screening of those with improved phenotype properties. The most popular agent of these, because of its very high mutant to survivor ratio and multiplicity of mutations, has been nitrosoguanidine. Many possible mechanisms to contribute toward enhanced antibiotic production may be due to (i) the increased flux of a precursor primary metabolite, (ii) the increased resistance of the strain to the antibiotics, and (iii) the enhanced gene expression and the resulting concentration of biosynthetic pathway enzymes. Besides random screening for mutants with the desired phenotype properties, selection of auxotrophe mutants resistant to analogs involved in *de novo* synthesis.

Phenotypic expression of mutations Many mutations that result in increased formation of metabolites are recessive. When a recessive mutation takes place in a uninuclear, haploid cell (e.g., bacteria, actinomyces spores, asexual conidia of fungi), the mutant phenotype can be expressed only after further growth and reproduction have taken place. In diploid or eucaryotes, recessive mutations are allowed to undergo phenotypic expression after meiosis or mitotic recombination. Delays in expression are not directly the result of genetic effects. A regime can be selected in which the lethal mutation is not expressed (or the wild type phenotype is expressed) and the organism survives, the so-called permissive condition. The mutations cause death only under certain conditions, termed the restrictive condition, in which the mutant phenotype is expressed.

Temperature sensitive mutations (t^s) are an example of conditional lethals that can be understood in biochemical terms. t^s mutations cause the gene to become nonfunctional at either high (heat sensitive) or low (cold sensitive) temperatures. The biochemical basis of t^s mutation is probably changes in the amino acid sequence of a protein which affects thermostability rather than activity because they affect the overall secondary or tertiary structure of the protein rather than an active site. Osmotically, remedial mutations cause the gene product to be particularly sensitive to the osmotic strength of the growth medium. The protein is usually stable only in the presence of higher concentrations of solutes. Streptomycin-remedial mutants express a wild-type phenotype in a medium that contains low levels of aminoglycoside antibiotic (Streptomycin, Neomycin, or Kanamycin). Restrictive condition denotes growth in a medium that lacks the antibiotic by altering the translation mechanism rather than the gene products.

A gene activity that has become lost through mutation can be restored at least partially through a second mutation, called a *suppressor mutation*. Suppressor mutations act in several different ways. Suppressor mutation like streptomycin changes the translation mechanism, thereby producing some gene products that are functionally active, but often only as a somewhat abnormal pseudo wild-type. In contrast, error-free repair restores the DNA sequence to give a normal wild-type.

1.5.2 Recombination

The genetic information from two genotypes can be brought together into a new genotype through genetic recombination. This is another effective way of increasing the genetic variability of a cell. The advantages of genetic recombination are (i) different alleles of the

parent strains with increased metabolic production can be brought together in one strain, so that the cumulative effect of these mutation is greater than the effect of the single mutation. However, a significant yield increase by recombining two high-yielding mutants has only been successful in a few instances and in most cases, the productivity of the recombinants is intermediate between the values of the parent strains; (ii) there is frequently a decline in the increase in yield and in the development of inapparent mutations, which prevent a further increase in the metabolite production by pleiotrophic influences. With genetic recombination, these unfavorable mutant alleles can be replaced with alleles of one of the parents in the cross; and (iii) high-yielding mutants can actually increase the cost of the fermentation because of the changed physiologies such as greater forming, nutrient requirements and others. By crossing back to the wild type strains, high-yielding strains with improved fermentation properties may be obtained.

Sexual and parasexual recombination in eucaryotes When a sexual cycle is known, *nuclear fusion* (*karyogamy*) results after fusion of hyphae, leading to a recombination of nuclei in the heterokaryotic mycelium. After diploid formation in some fungi (*Aspergillus*, *Sacharomyces*, etc.), recombination takes place during the subsequent meiosis process. A new genotype results either from the combination of parent chromosomes or from crossover between two paired homologous chromosomes both of which are *replicons* (called general recombination). This type of recombination is known to be catalyzed by the *recA* gene. The main role of the product of the *recA* gene is in the process of recombination, which creates crossover exchange between DNA molecules by binding single-strand DNA in order to unwind double-stranded DNA. The so-called *tetrad analysis* is often applied to eucaryotes, especially for the yeast, *S. cerevisiae*. This method consists in the possibility of isolating the four products (four spores) and cultivating them separately. The attainment of recombinants through the sexual process has been confined to commercial mushrooms and yeasts. Some of the most economically important fungi, such as *Penicillium chrysogenum* and *Cephalosporium acremonium*, which are producers of penicillin and cepharosporin antibiotics do not have a sexual cycle.

In parasexual reproduction, the fusion of two hyphae of equal or different polarity results in a mycelium with nuclei of both parent strains. This heterokaryon is normally stable with the nuclei mingling but not interacting. In rare cases, nuclear fusion occurs and a diploid nucleus is formed. In such mitotic crossover between homologous chromosomes, genetic recombination can be obtained. To obtain a recombinant, haploid cells or spores must be formed, but spontaneous haploid formation is relatively rare. It can be induced with *p*-fluorophenylalanine.

Recombination in bacteria Although the parasexual mechanisms such as conjugation, transformation, and transduction in bacteria are established, only a fragment of the genome of the donor cell is transferred into a recipient cell, becoming a partial diploid. After homologous pairing, recombination occurs, but the rate of recombination is far lower than when the perfect sexual cycle is used.

Conjugation generally involves the participation of plasmid and single-stranded DNA is transferred from the donor cell to the recipient cell after the two cells have come into contact. In transformation, short pieces of DNA are taken up by the competent recipient cells. In *generalized transduction*, *temperate phage* particles, which have lost a piece of their own genomes, transfer a chromosome DNA fragment of the host bacteria at the rate of 10^5

per phage. In specialized transduction, recombination occurs within homologous segments shared by the phage (phage) and the chromosome at the attachment site of the phage. The insertion of the prophage into the chromosome results in further incorporation of the attached piece of DNA into the genome of the host cell. This is an example of site-specific recombination, which does not require the participation of the *recA* protein.

Transposable genetic elements, termed *insertion sequences*, and *transposons* have the ability to transpose to various sites on the bacterial genome. Insertion sequences are small elements (~1 kb) that encode only their capacity for replicative recombination. Transposons are larger (up to 10 kb in length) composite elements terminated by insertion sequences. The discovery of transposons that undergo replicative recombination at relatively high frequency (Tn_5) provided an explanation for the spread throughout a bacterial population of certain genes. Transposon Tn_5 contains the gene for resistance to an aminoglycoside antibiotic (Kanamycin), which can be expressed in a wide variety of procaryotes and eucaryotes. Several transposons have been integrated into plasmids (Tn_1 and Tn_3) and others in either plasmids or chromosomes (Tn_5). Thus, transposons can form multiple drug-resistant plasmids that can then be transferred to other strains. Thus, transposons are available for a wide variety of purposes in gene technology.

Protoplast fusion Recently developed methods such as protoplast fusion have extended the number of organisms in which two genotypes can be recombined. *Protoplasts* are wall-less cells, which retain full respiratory activity and can synthesize protein and nucleic acids. Under certain special conditions of cultivation, they can even be induced to regenerate cell walls and again assume a rod shape. The artificial production of protoplasts can be prepared by subjecting cells to the actions of cell wall-lysing enzymes (lysozyme for bacteria, chitinase, or cellulase for fungi) in isotonic sucrose solution. Protoplast fusion is normally rare because of the strong negative charge of the protoplast surface. However, in the presence of polyethylene glycol (PEG), the protoplasts fuse relatively easily accompanied by DNA exchange. Many yeast species like *Saccharomyces lipolytica*, it is not necessary to degrade the cell wall completely. Such cells in which cell wall residues still adhere to the plasmalemma are called *sphaeroplasts*.

Besides the use of PEG to bring about fusion, the method of electric-field-induced fusion (electrofusion) of protoplasts has also been developed. With electrofusion, two or more protoplasts can be fused under microscopic control of several cells into one giant cell. Protoplasts can also be induced to fuse artificial phospholipid vesicles, called liposomes. The fusion rate is about 60% using PEG and 80–90 % with electrofusion. Protoplast fusion can be used for intraspecific recombination of strains, which lack sexual or parasexual systems or whose frequency of recombination is too low, and interspecific hybridization to obtain completely new organisms capable of synthesis of new or modified metabolites. Protoplast fusion has been achieved with the filamentous fungi, yeasts, *Bacillus* sp., *Brevibacterium* sp., *Streptomyces*, *Aspergillus*, *Pennicillium*, mucor, lactic acid bacteria, and many more strains.

In vitro rDNA technology made possible for the development of methods for transformation of protoplasts with plasmid, chromosomal, or viral DNA. Protoplasts are usually treated with DNA in the presence of PEG and Ca^{2+} and in this way, all of the numerous technologies of genetic engineering become available for use with the industrial strains. Even microorganisms for which conventional host-vector systems are not available can be transformed. For example, protoplast transformation is inefficient in dairy lactic acid

bacteria such as *Lactococcus* and *Lactobacillus* strains. Most recently, however, electroporation has developed into a very efficient method for physically introducing DNA into the Gram-positive microorganisms. Electroporation uses short electric pulses of a certain field strength, which alters the permeability of membranes such that DNA molecules can enter the cell. Using virus vectors, transfection (or transformation) systems have also been described for *Streptomyces*, *Thermomonospora*, *Mycobacterium*, and *Brevibacterium lactofermentum*.

Summary

Biotechnology involves the potential use of all living organisms including cells of animals, plants, and microorganisms. However, microorganisms have played a major role in this field for millennia, since the discovery of fermentation, and will continue to do so more extensively for the foreseeable future. There are many reasons for the choice of microbes: their rapid growth rate, ease of mass cultivation in many cheap substrates, diverse metabolic types (which give rise to many different products), and the tremendous possibility of genetic improvement for new products. Of the many thousands of microbial species known, relatively few are currently exploited. Microbes are responsible for the photosynthesis of energy from the sun, and they participate in the cycles of nitrogen, oxygen, sulfur, and other elements essential for life. They are also responsible for many diseases of humans, animals, and plants.

Among the vast contents in the science of microbiology, we have concentrated primarily on the cellular organization, reproduction and classification, genetics of useful microorganisms for foods and commodity products. Except for the viruses, most organisms share a common chemical composition, the presence of three complex macromolecules (DNA, RNA, proteins), and common physical structural and functional properties.

Two major groups of cellular organisms, the prokaryotes and the eukaryotes, were compared. The prokaryotes are the Eubacteria and Archaebacteria. The rapid growth and the biochemical versatility of prokaryotes make them important tools for biological and genetic research, and for biochemical scale-up processing. Eukaryotes are the unit of structure in plants, animals, and protists (algae, fungi, protozoa). The eukaryotic cells possess several unique organelles: the nuclear envelope, the endoplasmic reticulum, and the Golgi apparatus. A basic knowledge of eukaryotic cells and the function of organelles is assumed.

The microbial classifications are based on many different factors, which are of great practical importance in applications of biotechnology. With the exception of the Actinomycetes, most biotechnology-related bacteria belong to the Eubacteria.

The fungi are nonphotosynthetic, aerobic, filamentous, and nucleated cells known as hyphae. Fungal classification is based more on morphological and habitat characteristics than on staining and biochemical reactions. The fungi can be classified into zygomycetes, ascomycetes, basidomycetes, and fungi imperfecti. Of these, the most frequently known species in biotransformation work are the yeasts and the molds.

The principal macromolecules of all cells are proteins and nucleic acids, and the biochemical reactions leading to their formation are similar among prokaryotes and eukaryotes. However, a greater divisity is formed in the synthesis of other classes of cell constituents such as polysaccharides and lipids, which are the group-specific substances. The reactions of biosynthesis and polymerization common to all organisms were discussed. Also classical strain improvement and tools were described.

1.6 Systems/synthetic biology and metabolic engineering

Classical strain improvement is too labor intensive for the improvements. In classical strain improvement, cells are treated with a chemical or physical mutagen that kills off most of the cells, and screen the survivors for that rare clone which produces more of the interested product. This new strain becomes the starting point for the improvement, but it is not easy to know why the strain is improved. In the era of metabolic engineering (ME), more specifically targeted in the way that one can improve strain productivity more efficiently. That might have been true 20 years ago, but with the use of miniaturized cultivation along with automated colony picking and liquid handling, a large number of colonies can be screened regardless of whether their genetic variability was introduced by ME or chemical mutagenesis. Now biology companies are producing biofuels and renewable chemicals using classical strain improvement as a supplement to synthetic biology or other ME approaches. The term *synthetic biology* was used to describe concepts that would be classified today as *ME*, but in the last 10 years, terms such as "unnatural organic molecules," "unnatural chemical systems," "artificial, biology-inspired systems," and "functions that do not exist in nature" have been used to describe synthetic biology. Synthetic biology can thus be defined as "the design and construction of new biological components, such as enzymes, genetic circuits, and cells, or the redesign of existing biological systems" as well as "reconstruction of a complete bacterial genome." Synthetic biology has application to many fields, including cell-free synthesis, tissue and plant engineering and drug discovery, but in industrial biology, synthetic biology offers some tremendous opportunities to create cell factories that are tailor-made for efficient production of commodity chemicals and fuels from renewable resources. In most cases, the design and construction of cell factories for use in industry requires both synthetic biology and ME (Figure 1.7).

In Figure 1.7a, the first approach is a traditional biotech step where a naturally producing organism is selected as the cell factory for production of the desirable product. Typically, the flux toward the product is naturally low but through the use of classical strain improvement or the use of directed genetic modifications (ME), the increase of the sufficient flux toward the product is possible. Economically feasible processes are currently on the production of industrial enzymes, antibiotic (adipoyl-7-amino-3-deacetocephalosporannic acid: adipoyl-7-ADCA), a precursor for cephalexin using fungal and bacterial cell factories, and fuel production using *S. cerevisiae*. In Figure 1.7b, the platform cell factory does not naturally produce the product of interest, but through insertion of a synthetic pathway in the organism (illustrated by the red pathway), the cell factory can often produce small amounts of products initially, but through pathway optimization, the flux through this synthetic pathway can be increased to ensure a high flux toward the product. As all metabolites produced in nature are derived from a set of only 12 precursor metabolites that are intermediates of the central carbon metabolism, when a new synthetic pathway is inserted into a designated cell factory, a drain of one of these precursor metabolites will occur to the unadapted cells, resulting in a low yield initially. However, through engineering of the central carbon metabolism, the flux can be redirected toward the precursor metabolite for the desired product, thus enabling enhanced yield and productivity. Once engineering of the central carbon metabolism is successful, many different synthetic pathways can be inserted into the cell platform with the intermediate result of high-level synthesis of the target products. This approach clearly applies concepts from

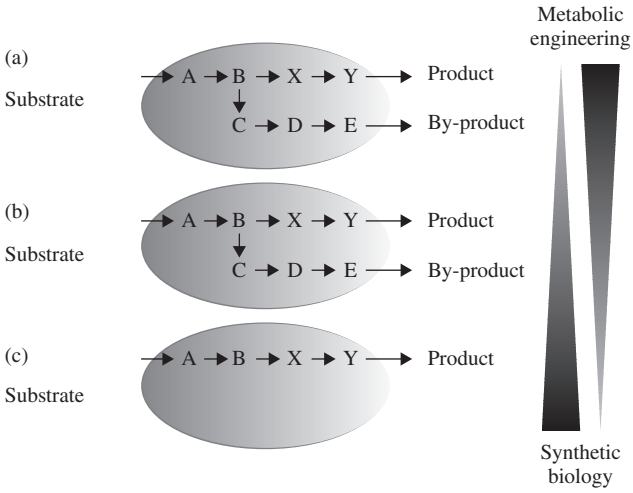

Figure 1.7 (a–c) Illustration of the overlap between metabolic engineering and synthetic biology by the use of three different approaches to produce a desirable product. *Source*: Nielsen and Keasling 2011. Reproduced with permission of Nature Publishing Group.

both ME and synthetic biology. If the cell factory that efficiently converts different sugars to acetyl-coA is developed, such a cell factory can be a good platform for producing many ranges of products including polyketides (antibiotics, anticancer drugs, immunosuppressors), lipids (dietary supplements, pharmaceuticals, biodiesels), isoprenoids (perfumes, antimalarial drugs, antibiotics, dietary supplements, food ingredients, vitamins), and so on. In Figure 1.7c, a complete synthetic cell is constructed to produce the desirable product. In this approach, the limited platform cell factories like *E. coli* and *S. cerevisiae* will function as the important model organisms for prokaryotic and eukaryotic cells. These two organisms already have the detailed mathematical models *in silico* representation, that are the most probable cells to study in terms of *systems biology* that seeks to integrate biological data as an attempt to understand how biological systems function. By studying the relationships and interactions between the various parts of a biological system (e.g., organelles, cells, physiological systems, organisms, etc.), it is hoped that an understandable model of the whole system can be developed. In this kind of *in silico* representation, mathematical modeling of the interaction between all the components in the system is carried out to obtain a predictive model for the system. Our knowledge is currently quite limited in the field of engineering of entire cells (that is, the ultimate goal of *synthetic biology*), but through advances in systems biology, the necessary knowledge and mathematical models can be obtained. In a short term, advance of systems biology will affect our ability to reengineer cells (that is, the goal of *ME*), which further speed up to develop more efficient cell factories. Therefore, there are synergies between systems biology, synthetic biology, and *ME* (Figure 1.8). Although the term "synthetic biology" has been around since the mid-1970s, the definition has been very vague. The activities related to synthetic biology are considered by some to be just extensions of already existing fields, like molecular biology, genetic engineering, and microbiology, but systems biology describes the overall cell function through quantitative description of the interaction between all the individual components in the cell, such as gene transcription, translation, protein–protein interaction,

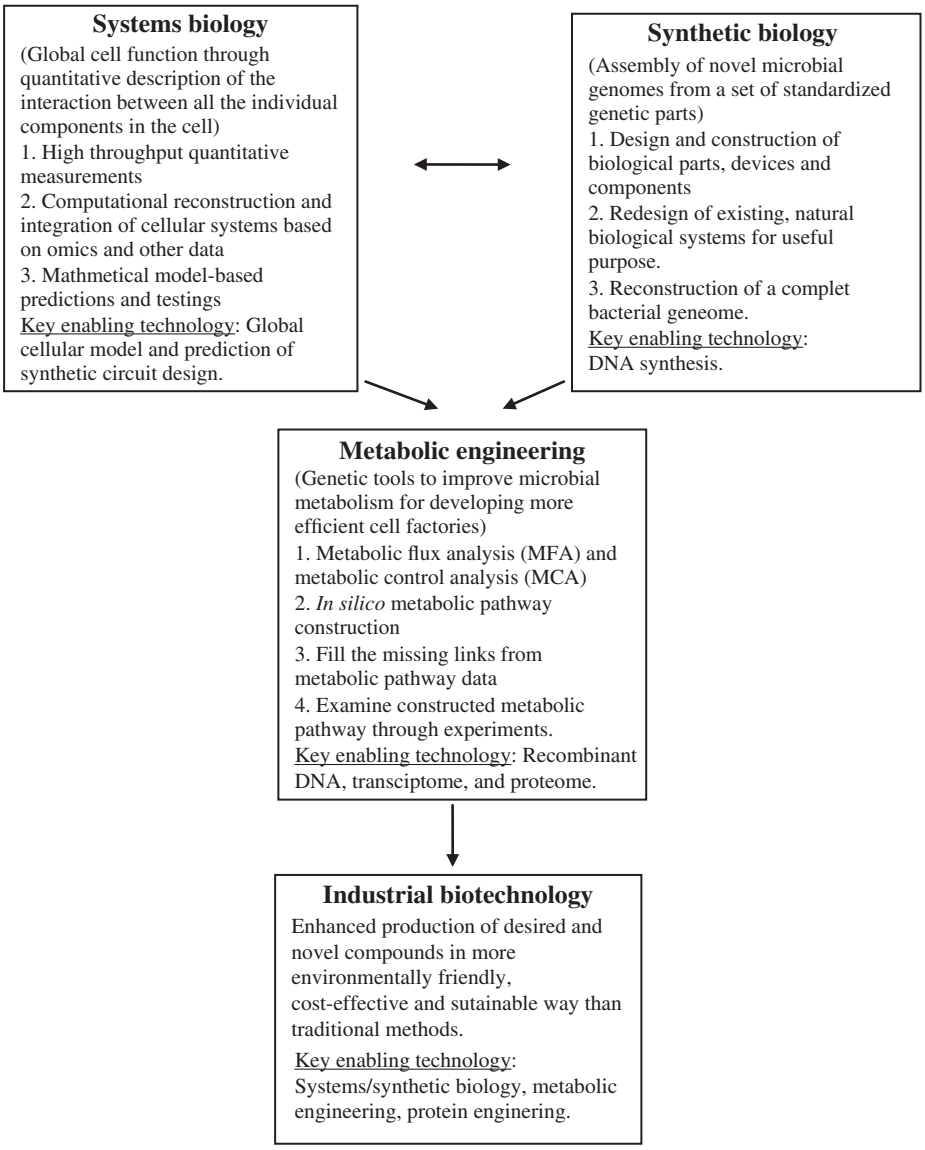

Figure 1.8 Contribution and overlap of systems/synthetic biology to metabolic engineering for industrial biotechnology.

enzyme catalysis of biochemical reactions, and receptor–metabolite interaction. That is the global-scale study of biological processes at the cell or organism level in terms of their molecular components and their interactions. Systems biology does not investigate individual cellular components at a time, but it is about the behavior and relationships of all of the elements in a particular biological system while it is functioning. In fact, the goal of synthetic biology is to make the engineering of biology faster and more predictable,

and to harness the power of biology for the common good. Systems biology focuses on the interaction of myriad components and how these give rise to the dynamic and complex behavior of biological systems. More precisely, synthetic biology is to gain an increasingly precise control over cellular processes, considering ground elements – that is, custom DNA sequences, custom proteins produced by genetic code expansion, standard biological parts, and basic synthetic circuits – but also higher-order elements, composite synthetic circuits, and engineered metabolic pathways. The degree and the tools with which synthetic biology intends and controls cellular processes are what distinguish it from any other field of the biological sciences. Even a simple biocatalyst like *E. coli* is a complex system of an estimated 4603 genes, 2077 reactions, and 1039 unique metabolites, and while the engineering steps are relatively straightforward, it is still difficult to quickly and reliably engineer a biocatalyst to perform desired behaviors. Systems biology, the standardization of biological systems, and metabolic evolution are all vital to compensate for this disconnect between the expected and actual biocatalyst behaviors. Applications of synthetic biology have already been successful in the (i) production of antimalarial drug precursors, *artemisinic acid* in *E. coli* and yeast, (ii) creation of synthetic oscillators and light sensors, (iii) transplantation of synthetic genomes and chromosomes, engineering of cells able to break down toxins, detection of explosives by plants, creation of bacteria that produce toxins in response to pathogens, and (iv) creation of cell factories tailor-made for efficient production of fuels and chemicals. One successful achievement by synthetic biology is the microbial production of the antimalarial drug *artemisinin*. Artemisinin, a sesquiter lactone endoperoxide extracted from *Artemisia annua* L (a sweet wormwood) is highly effective against the multi drug-resistant malaria parasite *Plasmodium falciparum*, but it is in short supply. Most people suffering from malaria are unable to get it due to its high costs. It was possible to modify *S. cerevisiae*, which can produce high titers (up to 100 mg/L) of artemisinic acid using an engineered mevalonate pathway, amorphadiene synthase, and a novel cytochrome P450 monooxygenase (CYP71AV1) from *A. annua* that performs a three-step oxidation of amorpha-4,11-diene to artemisinic acid (http://newscenter.berkeley.edu/2013/04/11/launch-of-antimalarial-drug-a-triumph-for-uc-berkeley-synthetic-biology/). The synthesized artemisinic acid is transported out and retained on the outside of the engineered yeast. Although the engineered yeast is already capable of producing artemisinic acid at a significantly higher specific productivity than *A. annua*, cost-effective optimization and industrial scale-up will be required to produce artemisinic acid production to significantly lower their current prices. However, challenges in synthetic biology are also many because biology is complex and synthesis capabilities far exceed design capabilities. Potential benefits are enormous in the fields of medicine, food, and energy, but the safety and ethical issues are being revisited. Similar to the genetically modified organism (GMO), potential risks include unintended harmful consequences for human health or the environment or deliberate misuse for hostile purposes. ME is basically the use of directed genetic modification to improve the properties of a given cell, for example, to improve the yield or productivity, expanded substrate range, and production of novel products. ME is to improve cellular activities by manipulations of enzymatic, transport, and regulatory functions of the cell with the use of rDNA technology. In fact, ME studies are also considered to be part of synthetic biology. Recent developments in synthetic biology promise to expand the ME toolbox further by creating novel biological components for pathway design. The holistic understanding of metabolic flux, promoter activity, preferential codons, and scaffold proteins are the basics for synthetic ME. These integrated approaches promise to do more than simply improve product yields by recombination DNA and other molecular biological tools; they can expand the array of products

that are tractable to produce biologically. That is why ME has recently been upgraded to systems level (so-called systems ME) by the integrated use of global technologies of systems biology, fine design capabilities of synthetic biology, and rational–random mutagenesis (RM) through evolutionary engineering.

By metabolic flux analysis (*MFA*), the intracellular metabolic fluxes can be quantified by the measurement of extracellular metabolite concentrations in combination with the stoichiometry of intracellular reactions. MFA can be applied to evaluate the intracellular metabolic conditions and to identify key metabolic pathways or metabolites. *Metabolic control analysis* (*MCA*) is a statistical modeling technique used to understand how metabolic fluxes are controlled by certain enzyme activities and metabolic concentrations. Those responses of small changes to metabolic flux distribution can be predicted by MCA. Tools of ME require: (i) molecular biological tools such as *genomic*, *proteomics*, *transcriptomics*, *interactomics*, *bioinformatics*, and so on; and (ii) metabolic pathway analysis tools such as identification of the metabolic network structure (or pathway topology), and quantification of the fluxes through the branches of the metabolic network, and identification of the control structures within the metabolic network. The metabolic flux is an essential concept in the practice of metabolic engineering. Because gene expression levels and concentrations of proteins and metabolites in the cells provide clues to the status of metabolic networks, they have inherent limitations in describing the cellular phenotype. In *in silico* construction of a strain's metabolic pathway, (i) one must obtain the complete sequence of the strain, search *open reading frames* (*ORFs*) from sequences (using ORF finder or ORF data from Genbank), (ii) identify the functions of ORFs based on literature information (e.g., *MetaCyc database*), (iii) search enzymatic reactions using metabolic pathways (*KEGG* or *Swiss-Prot*), (iv) construct metabolic pathway from enzymatic reaction list, (v) fill the missing links from reference metabolic pathways, and (vi) compare the constructed metabolic pathway through real experiments. Knowledge acquired from this research will benefit the society in a number of ways, including the ability to modify biological pathways to produce biological substitutes for less desirable chemical processes. This technology will allow greater agricultural production, permitting more efficient and safer energy production, and provide better understanding of the metabolic basis for some medical conditions that could assist in the development of new cures. The continued development of tools in synthetic biology is a way to reduce the cost and time required to engineer biological systems for the production of pharmaceutical ingredients, fine chemicals, biofuels, and so on. However, in the classical method of ME, identifying a rate-determining step in a pathway and alleviating the bottleneck by enzyme over-expression has enjoyed only limited practical success. Thus, an alternative method for cellular perturbations to identify targets, termed *inverse metabolic engineering* (*IME*), takes advantage of recent advances in high-throughput screening and genome sequencing. Although this IME approach is potentially very powerful, elucidating the genetic basis of a relevant phenotype can be difficult. This is especially true in the context of modern evolutionary techniques such as genome shuffling and directed enzyme evolution among others. In other words, the classical approach of ME requires detailed knowledge of the enzyme kinetics, the system network, and intermediate pools involved, and on such bases, a genetic manipulation is proposed for some presumed benefits.

In contrast, the concept of IME inverse metabolic is first to identify the desired phenotype, then to determine environmental or genetic conditions that confer this phenotype, and finally to alter the phenotype of the selected host by genetic manipulation. The process of IME consists of: (i) construction or identification of a certain phenotype, (ii) determination of the factors conferring that phenotype, and (iii) engineering the factors into another

strain or organism. However, the use of functional genomics on IME is still in its infancy, and thus the numbers of published data are still small.

Summary

Strain improvement is an essential part of process development for biotech products as a means of reducing costs by increasing productivity and yield by redirecting multiple-step metabolic pathways (catabolism and anabolism). ME aims to control this complexity in order to establish sustainable and economically viable production routes for valuable chemicals. Recent advances in systems-level data generation and modeling of cellular metabolism and regulation together with tremendous progress in synthetic biology has provided the tools to put biotechnologists on the fast track for implementing novel production processes.

The rapid advance of ME has been in part due to notable advances in fields adjacent to ME such as rapid DNA-sequencing techniques, extensive databases of gene expressions, metabolic reactions, and enzyme structures, new genetic tools enable more precise control over metabolic pathways, new analytical tools enable the metabolic engineer to track RNA, protein, and metabolites in a cell to identify pathway bottlenecks, and detailed models of biology aid in the design of enzymes and metabolic pathways (systems biology).

Yet even with these substantial developments, metabolic engineers must weigh many trade-offs in the development of microbial catalysts. In particular, there are many feedback loops in the control structure; that's why it is difficult to predict the overall effects of a specific genetic modification. It is thus necessary to go through the cycle of ME and inverse ME several times when intracellular fluxes needed to be redirected. Even with these many challenges, ME has been successful for many applications, and with continued developments, more applications will be possible. Recently, ME has been upgraded to systems level, called systems ME by the integrated use of global technologies of systems biology, fine design capabilities of synthetic biology, and rational–RM through evolutionary engineering.

1.7 Bioengineering and scale-up process

As the food science major does not have sufficient background on bioengineering, a detailed description has been added in this subject. *Biotechnological engineering or Bioengineering* is a branch of engineering that focuses on biotechnologies and biological science. It includes different disciplines such as biochemical engineering, biomedical engineering, bioprocess engineering, biosystem engineering, and so on. The definition of a *bioengineering* is in general an integrated approach of fundamental biological sciences and traditional engineering principles. Bioengineers are often involved to *scale up bioprocesses* from the *laboratory scale* to the *manufacturing scale*. Moreover, as with most engineers, they often deal with management, economic, and legal issues. Since *patents* and *safety regulations* are very important issues for biotech enterprises, bioengineers are often required to have knowledge related to these issues.

Processing of biological materials such as *cells*, *enzymes*, *metabolites*, or *antibodies* are the central domains of bioengineering. Success in bioengineering requires integrated knowledge of governing biological properties by process-oriented microbiologists, and the methodology and strategy of chemical engineers. In terms of annual world-wide

sales, biotechnology-derived products can be divided into the three categories. First, fine chemicals as low volume products and as bulk chemical products usually fall within the 100 kg per year to 100 tons per year range. This category broadly includes bioproduction of high-value molecules such as *vaccines*, *rDNA products*, *5′-nucleotides*, some of the *amino acids*, *enzymes* (for medical applications), *monoclonal antibodies*, and bioconversion of high-value starting materials such as antibiotics, and steroids, and so on. A significant fraction of the production costs of these products are involved in purification and testing of the product to meet the demand of quality and safety specifications. Intermediate volume chemicals are usually chemicals or ingredients that are produced microbiologically in the range of 100 and 20,000 tons per year. Such products have less vigorous quality and safety specifications than do fine chemicals. Some examples are *glutamic acid* (*monosodium glutamate* (*MSG*)), which is used as a flavor enhancer, *antibiotics* used for protecting agricultural crops, *food* and *industrial enzymes*, *organic acids* (*citric*, *lactic*, *gluconic acids*), *solvents* (*acetone*, *butanol*), many *fermented beverages*, and *food products*.

Finally, the bulk product sector consists of products that are usually produced in continuous reactors, their number exceed 20,000 tons per year. These products are marketed on the basis of commodities and overall product performance criteria rather than on the basis of rigid quality specifications. The microbial products that fall within this category are *SCP*, *gasohol* (*ethanol*), *biogas* (*methane*), and *biopolymers* for enhanced oil recovery.

The primary goal of bioengineering practice is then to create processing systems that economically transform raw materials into marketable products using *fermentation* and *downstream* (*or product recovery*) processing. However, the recovery process that involves extraction, purification, drying, and so on, of biological products differs from chemical recovery in that these materials are much more labile than chemicals. Many of the techniques used in the biological products are still similar to those of chemical processes (e.g., separation, distillation, heating, cooling, drying). There is, however, increasing use of non-denaturing methods such as various chromatography and electrophoresis techniques for biological materials. In the fermentation process, engineering is only an aid in the development and regulation of biological processes such as regulation of microbial metabolism by optimizing culture media, oxygen requirements under sterile conditions, genetic manipulation, and so on. The economic evaluation of industrial processes and process routes is also an essential activity of the engineers. The gross profit obtained as a result of operating a particular plant is the difference between the net income from the annual sales, after distribution, promotional and sale costs as well as the annual manufacturing costs have been deducted.

To improve its profitability by process integration and optimization, it is essential to understand the technological factors that affect the overall economics of manufacture significantly. In commercial processes, the *microbial factors* with the greatest impact on the process are (i) yield coefficients for the product(s), (ii) growth rates and/or product production rates, (iii) affinity of the culture for carbon energy substrates, (iv) stability and fastidiousness of the culture. The *process engineering factors* with the greatest impact on the process are (i) feedstock conversion efficients, (ii) productivity, and (iii) product concentration. Both the microbial and the process engineering factors are closely related in processes that are technologically and commercially successful. When evaluating any new production process, it is then essential to understand the technical factors that affect the overall economics of manufacture significantly. To improve profitability by process integration and optimization, it is essential to understand the technological factors that affect the overall economics of manufacture significantly. In commercial processes, the microbial factors having the greatest impact on process are yield coefficients for the product(s), growth rates and/or product production rates, affinity of the culture for carbon

energy substrates, and stability of the culture. The process engineering factors having the greatest impact on the process are feedstock conversion efficiency, productivity, and product concentration.

1.7.1 Microbial and process engineering factors affecting performance and economics

The general fermentation process is largely divided into a fermentation section and a product recovery section as shown Figure 1.9. The upstream section receives and stores raw materials for eventual preparation of media; the downstream part is the site of final product preparation, packaging, and shipping. A full-process design must include for

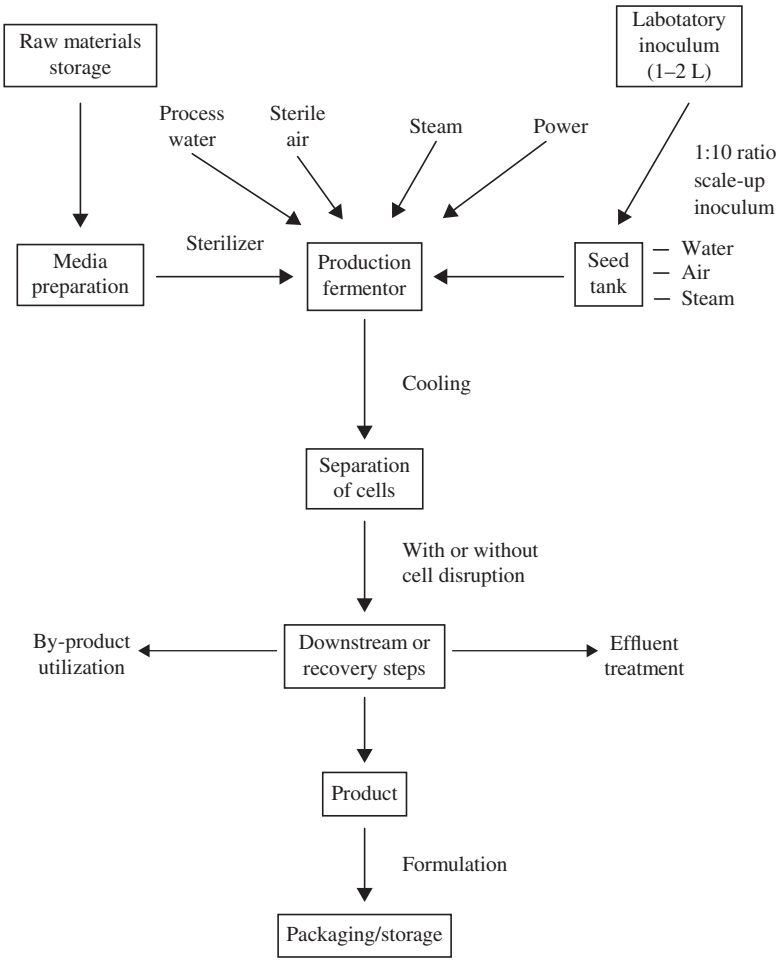

Figure 1.9 The general process scheme for upstream and downstream fermentation.

each section operations, utilities (water, gas, electricity, etc.), and effluent or by-product disposal. Since bulk chemicals from fermentation involve large-scale operations, and cell sludge or waste disposal is responsible for a major fraction of plant operating costs, a market for by-products must be found. Most modern fermentation cultures have been pure monocultures isolated from natural sources under aseptic operation. However, the cultures have been subjected to strain improvements by manipulating growth conditions, encouraging mutations, or using rDNA techniques. The concepts of microbial growth kinetics, yield coefficients, and batch or continuous cultures have been discussed. However, the overall suitability of a selected culture for a commercial process, which is a critical factor for process scale-up, has not been covered.

Heat and mass transfer in fermentors/bioreactors are often achieved by an intense mechanical agitation to disperse air and to promote adequate mixing. This shearing action of rotating impellers tends to damage the cells, particularly those of filamentous molds, actinomyces, and bacteria with appendages (*flagella* and *fimbriae*). Microbial strains whose morphology is significantly affected by such culture conditions are obviously not suitable for commercial process application. It is thus important to select more robust microbes that are better suited to the harsh conditions encountered in intense processes. This option is restricted for animal and plant cell cultures.

Microbial strains that exhibit broad ranges of pH, temperature, and DO optima are certainly more advantageous for process application than those of fastidious strains. Robust microbes that can adapt to high levels of performance under rapidly changing physical and chemical environments are essential for good bioprocess economics.

Any microbial process can be divided into the production of live microorganisms and the production of the desired end product or the decomposition of feedstock by chemical transformation due to, for example, the actions of enzymes present in the cells. On the industrial scale, biotransformation by growing microbial cells is the most commonly used technology. The immobilized systems using either cells or enzymes are developing rapidly for some industrial-scale transformations. However, problems still exist with respect to maintaining longer shelf life of enzymes, preventing microbial contamination, generating cofactor, and overcoming the intrinsic diffusional resistance. Productivity and conversion efficiency are the key factors that affect process economics, but each in turn is controlled by the physical phenomena occurring in the fermentor through its mass and heat transfer capacities.

For a project that eventually achieves commercialization, life cycle stages include the product idea, preliminary evaluation of economics and markets, development of data needed for final design, final economic analysis, detailed engineering design, procurement of site and equipment, construction of buildings and process, process start-up and trial runs, and regular production operation. It is beyond the scope of this text to describe the elements of total product cost and typical values for estimation.

1.7.2 Fermentor and bioreactor systems

Basically, there is not much difference between fermentors and bioreactors. Regardless of device type and size, the basic fermentor consists of a closed vessel, fitted with an air inlet and an agitator, in which microbial or biochemical reactions are carried out for commercial ends under a controlled environment. Vessels used to grow microbial cells are often referred to as *fermentors*, while those in which plant and mammalian cells can be cultured are called *bioreactors*. Essentially, however, all such equipment features heterogeneous systems consisting of two phases, usually gas and liquid. We will use the term *bioreactors* to denote cells of all types in this text.

Table 1.9 Historically developed types and applications of bioreactors

Reactors	Applications
1. Anaerobic vats	Alcohol and yeast production
2. Surface koji (solid state) culture	Acetic acid, citric acid, fungal commercial enzymes
3. Stirred tank–submerged culture (e.g., mixed flow, continuous flow (CSTR), backmixing)	Many examples of cell-free enzymes (e.g., glucoamylase) Free cells (e.g., steroid) Immobilized beads
4. Single-cell protein (SCP) culture (e.g., continuous, large volume, tower, high density)	Biomass
5. Packed bed (e.g., immobilized system)	Glucose isomerization Penicillin hydrolysis Selective separation of racemic amino acids
6. Bubble column (e.g., tower, air lift)	Beer production Biomass Vinegar Plant metabolites
7. Fluidized bed	Continuous beer and cider production
8. Trickle bed	Wastewater treatment Vinegar manufacture

Bioreactor classification is usually based on whether the reaction occurs in a single-phase or a multiphase environment. Reactions in the first case are termed *homogeneous reactions*, while those occurring in multiple phases are called *heterogeneous reactions*. For biological reactions involving microbes and for most enzymatic reactions, however, the concepts of homogeneity and heterogeneity cannot be applied consistently.

With a few exceptions, such as the continuous flow of aerobic wastewater and sewage treatment, the making of vinegar, and microbial biomass production, most bioindustries still prefer to use the batch mode of operation for transformation and the semicontinuous mode of operation for microbial biomass production, for reasons of reliability and versatility. Table 1.9 shows how bioreactors have been developed historically and compares reactor types. The discussion of reactors has been restricted in this text because there are so many different types and also because newer systems are continually developing, especially for systems that offer more savings in energy. Alternative nonstirred reactors such as immobilized cells and enzymes are also developing rapidly and may replace fermentation processes in the future.

Packed-bed bioreactors *Packed-bed reactors* are operated in most cases with a continuous gas atmosphere. The nutrient solution is evenly distributed over the packing through a feed device. In packed-bed (or fixed-bed) reactors, columns packed with immobilized biocatalyst particles are currently used for glucose isomerization, selective penicillin hydrolysis, and selective reactive separation of racemic mixtures of amino acids. Many immobilized cell systems in packed-bed reactors have been examined, and a useful model is the *plug-flow reactor*, which is also one of the simplest ones. The flow is from the bottom to the top, affording gradients of concentration of substrate and product in which the product of unreacted substrate flows out of the reactor continuously. It is very difficult to provide effective aeration at a significant scale as well as to regulate the temperature and pH.

Bubble-column bioreactors All reactors in which the compressed gas at the bottom of the vessel rises through the liquid because of its buoyancy belong to the bubble-column group. In several recent designs, the so-called *air-lift reactors*, an external loop is used to provide fluid circulation; this arrangement permits high efficiency of heat exchange and enhances flow and mixing in the vessel. It has been stated that the energy demand for a loop reactor is about one-fifth that of a conventional stirred vessel. As shown in Figure 1.10a, the air is introduced at a high velocity by static gas distributors (perforated or sintered plates). The use of a draft tube enables the liquid to circulate with considerable turbulence. For efficient oxygen transfer, it is necessary for the reactor to have a much higher tower (height-to-diameter ratio of 10:1), than a conventional stirred-tank reactor. As the bubble ascends the column, however, the partial pressure of the oxygen within it decreases, and this decreases the rate of oxygen transfer to the liquid.

The other reactor design that is able to overcome the foregoing disadvantages is the *deep-shaft reactor* (Figure 1.10b). The air is introduced at above midheight of the reactor and this forces the liquid up and down. Both the air-lift and deep-shaft reactors can be operated continuously. Reactors of the bubble-column type have been used for the chemical industry; in biological systems, they have been used mainly for the production of beer, vinegar, and single-cell protein (or animal feed).

Fluidized-bed reactors *Fluidized-bed reactors* are generally similar to those of bubble-column geometry but are considerably more complex than the systems discussed thus far. Heterogeneous biocatalyst particles such as flocculated organisms, pellets of immobilized enzymes, or cells are suspended by drag forces exerted by the rising liquid. By maintaining a careful balance between operating conditions and cell characteristics, the biocatalyst is

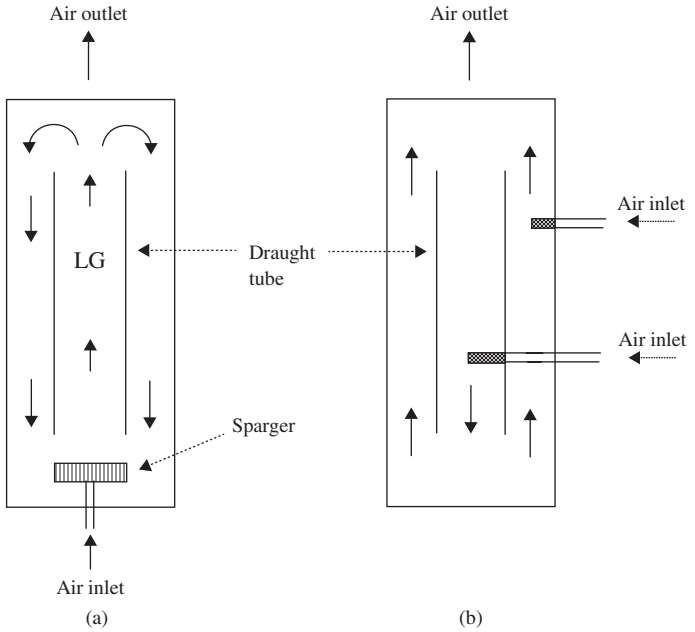

Figure 1.10 Two novel reactor designs: (a) air-lift (tower loop) and (b) deep-shaft reactors.

retained in the reactor, while the medium flows through it continuously. However, the height of the catalyst must be somewhat greater than that of the substrate solution; thus recycling of the fluid is not possible and scale-up is quite difficult.

Trickle-bed reactors *Trickle-bed reactors* are continuous gas-phase systems containing a packed-bed of heterogeneous catalyst, flowing gas and liquid phases. Providing good gas and liquid supply to biocatalysts is a major advantage of these reactors they being substantially influenced by the physical state of gas–liquid flow through the fixed bed and by the associated mass-transfer processes. The first application of this system is the trickling biological filter used for wastewater treatment. A similar system has been used for biological oxidation of ethanol to acetic acid (vinegar).

Reactors for immobilized enzymes or cells Among the newer bioreactors are immobilized enzyme or cell reactors. Immobilized biocatalysts are enzymes – cells and organelles, bound individually or in combination – that have been confined to allow their continuous reuse. Both physical and chemical techniques are used to immobilize enzymes or cells. Physical techniques tend to be less expensive and less reliable. Bioreactors for use with immobilized enzymes or cells must be constructed in such a way that the rate of movement between substrate and the biocatalyst is not rate-limiting.

Before considering methods to achieve enzyme or cell immobilization and some of the experiments conducted to date with immobilized systems, we will review some of the reasons for immobilization. When enzymes are present in solution, some enzymes will leave the reactors with the final product. This means that not only the new enzymes must be introduced to replace the lost ones, but also that enzymes must be removed from the product because they constitute undesirable impurities. Also, immobilized enzymes may retain their activity longer than those that are in solution. Most enzymes are labile under normal operating conditions, having only a very limited life. An immobilized enzyme may be fixed in position near other enzymes that are participating in a catalytic sequence, thereby increasing the catalyst efficiency for a multistep conversion.

The cost of isolating and purifying intracellular enzymes for commercial processes can so dampen profitability that their use becomes unattractive. However, advantages of isolated enzymes must be balanced against cost, depending on the nature of the conversion process. Isolated enzymes offer greater purity, which can yield higher conversion of products that are less contaminated by miscellaneous materials. Immobilizing whole cells should be considered when the extracted enzyme is unstable. Many industrial applications using continuous enzyme reactions are now carried out using immobilized microbial cells. Immobilized cells are limited in their diffusion through the cell membrane. The presence of many enzymes in the cells may also lead to side reactions, but these problems can be solved, and the enzyme systems within the microorganism can be efficiently utilized.

Reactions by immobilized cells are advantageous under the following circumstances:

- When enzymes are intracellular
- When enzymes extracted from cells are unstable during and after immobilization
- When the microbe contains no interfering enzymes (or the interfering enzymes can be readily inactivated)
- When the substrates and products are low-molecular-weight compounds

One important advantage of cell immobilization is that it gets around the washout problem of chemostat culture, providing much higher dilution rates and volumetric productivities than would otherwise be obtainable. Additional advantages are listed in Table 1.8.

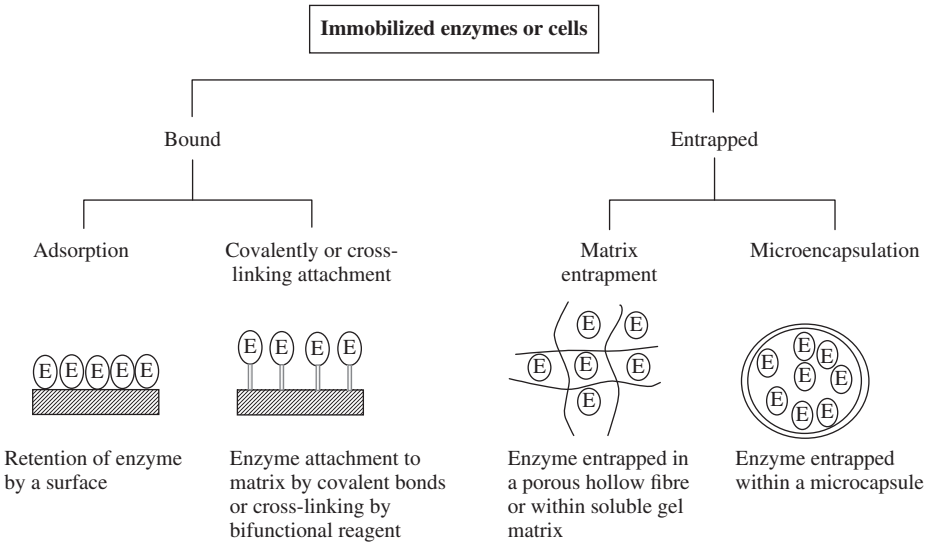

Figure 1.11 Two basic immobilization methods.

No ideal, general methods that are applicable to the immobilization of enzymes or cells of all types have been developed. Thus, it is necessary to choose suitable methods and conditions for immobilization of each system. Toxicity of immobilization reagents should also be considered in connection with the process of waste disposal. Resistance of cell wall and cell membrane to substrate and product transport would constitute a considerable shortcoming of cell immobilization.

New methods of coimmobilization of cells and enzymes are also available. The dried cells are resuspended in an aqueous solution of the enzyme and microbial cells, which are coentrapped by alginate gel formed with Ca^{2+} ions or enzyme molecules. They are covalently bound on alginates by means of carbodiimides or glutaraldehyde. Also, immobilized whole cells have been permeabilized by lytic enzymes, alcohols, or dimethyl sulfoxide, and some are now in commercial use. Figure 1.11 summarizes two basic types of immobilization methods. Basically, there are two types: those in which the catalyst is bound to a variety of carriers by adsorption or covalent attachment, and those in which the enzyme or cell is entrapped physically in a matrix or a membrane (Table 1.10).

Immobilization methods In adsorption processes, the enzyme is attached to surface-active materials such as alumina, activated carbon, clay, glass, cellulose and ion exchange resins, as shown in Table 1.11. Even for these so-called inert carriers, the binding mechanism is not simple and probably involves a combination of van der Waals (VDW) ionic and hydrogen bondings. Ion exchange resins have been shown to result in improved activity and stability characteristics. Elution of the enzyme still occurs, but it can be reduced by altering the pH, temperature, and ionic strength. The first commercially successful immobilized enzyme was prepared by adsorption, and this approach still offers advantages for large-scale industrial applications. Adhesion of cells to anion exchange resins has also been used to immobilize whole cells. One of the first attempts to immobilize cells involved *E. coli* and *Azotobacter agile*.

Table 1.10 Advantages of immobilized enzymes and cells

Enzymes	Whole cells
1. Reuse of the enzyme, which permits continuous operation	1. Reuse of the cell, which permits continuous operation
2. Increased stability	2. Unnecessary for enzyme extraction or purification
3. Enhancement of enzyme kinetic properties; greater control of catalytic process	3. Maintains intact enzyme activity and less sensitivity to changes in operating conditions
4. Enzyme-free product	4. Lower costs
5. Lower costs for expensive enzymes	5. Use of multienzyme system, which requires many enzymes and cofactor renewal
6. Use of multienzyme system	6. New medical/industrial uses
7. New medical/industrial uses	7. Reduces plant pollution problem because of continuous process (smaller volume)
8. Use of enzymes from non-GRAS* microorganisms	

*GRAS: generally regarded as safe for the purposes of the US Food and Drug Administration.

Table 1.11 Some adsorbents for the immobilization of enzymes

Process	Adsorbents
Physical adsorption	Alumina, activated carbon, silica gel, starch, clay, glass, cellulose, bentonite, collagen, titania (transition metal oxide)
Ionic binding	Cation exchangers (carboxymethyl cellulose, Amberite, Dowex 50) Anion exchangers (DEAE-cellulose, DEAE-Sephadex, Amberite IR45, Agarose)

The *covalent binding method* is one of the most commonly used techniques for enzyme immobilization through inter- and intramolecular reactions with bifunctional agents, resulting in high operational stabilities. Because of the toxicity of the coupling agents involved, however, this process is not widely used for cells. Any reactive component of the cell surface (e.g., the amino, carboxyl, thiol, hydroxyl, imidazole, or phenol groups of proteins) can be used for linking. To introduce the covalent linkage, chemical modifications of the carriers (inorganic and organic) have been used. Silica and ceramics are the major inorganic carriers. Among coupling agents, the most important are glutaraldehyde, diimido esters, toluene diisocyanate, diamines, dithiols, *p*-nitrobenzyl chloride, and succinic anhydride. Such coatings can be derivatized to aldehyde groups using glutaraldehyde, to acrylamine groups using *p*-nitrobenzyl chloride, or to carboxyl groups using succinic anhydride.

A series of enzymes such as lactate dehydrogenase, trypsin, chymotrypsin, asparaginase and lysozyme, and aldolase and glycogen phosphorylase have been modified with glutaraldehyde. In the case of organic carriers *Micrococcus* cells are immobilized on the carboxyl groups of agarose beads in a two-step process that avoids exposure of the cells

to carbodiimide. These immobilized preparations are generally difficult to handle because they are gummy or gels. They present multiple problems of high pressure drops in a packed-bed reactor and poor fluidizing properties in a fluidized-bed reactor. The method of directly entrapping enzymes or cells into polymer matrices such as polyacrylamide gel, alginate gel, carrageenan, and photo-cross-linking resin have been used most extensively. Entrapment strategies can be divided into two forms: (i) the enzyme is embedded into a polymer network and (ii) the enzyme in solution is retained by a membrane that is permeable to substrates and products. Entrapment is probably the most popular method of immobilization of microbial cells.

In another immobilization method known as microencapsulation, enzymes are entrapped in small capsules ranging from 5 to 300 μm in diameter. The enzyme is too large to move through the pores of the semipermeable membrane, but smaller substrate and reaction product molecules can move back and forth. Two membranes, cellulose nitrate and nylon, are often used, but the most promising delivery system is *liposome encapsulation*. Since liposomes differ from solid, semipermeable microcapsules in that enzyme action occurs only upon disruption of the carrier, liposomes are actually delivery devices rather than true immobilization systems. Liposomes are lipid vesicles that contain the enzyme in an aqueous environment surrounded by one or more concentric lipid bilayers. Other forms of physical entrapment processing include the use of ultrafiltration or hollow fibers to immobilize enzyme within the walls of membranes porous enough to allow the fluid to be forced through the interface, avoiding transport limitation by diffusion. Any reactions that require multienzymes or coenzymes can be immobilized in this way.

Immobilized reactors and applications Several types of immobilized enzyme reactor are known for industrial enzymes in use or under development (Tables 1.12 and 1.13). Basic construction types for immobilized reactors are presented in Figure 1.12.

In the batch-stirred reactor (Figure 1.12a), the total amount of enzyme is normally exposed to high substrate concentrations, resulting in high product concentrations. This type of reactor is thus not suitable for reactions that are subject to substrate or product inhibition.

The *continuous stirred-tank reactor* (*CSTR*; see Figure 1.12b) is started up as a batch reactor. As soon as a certain conversion has been reached, the substrate is pumped continuously into the reactor and the product is simultaneously pumped out of the reactor. This type of reactor, called a chemostat in the cultivation of cells, is not affected by pH shifts. Stirred reactors have the disadvantages of product inhibition and damage due to shearing of the catalyst.

In another type of system, the catalyst is immobilized in a bed; examples include packed-bed, fluidized-bed, and cyclonic reactors. In a *packed-bed* (*plug-flow or fixed*) *reactor* (Figure 1.12c), the substrate is converted into the product during upflow passage through a column packed with immobilized enzymes. This reactor adapts well to large-scale operation because there is a high rate of substrate conversion per unit time. Gas formation may reduce the contact surface between substrate and enzyme, however, and regulation of temperature and pH is difficult. The fluidized-bed reactor (Figure 1.12d) is theoretically attractive in terms of mass transfer. Because of the plug-flow nature of fluid movement, the immobilized enzyme particles are kept in suspension. The flow rate cannot be varied, and scale-up is quite difficult.

In *membrane reactors* (Figure 1.12e), the soluble or fixed enzymes and substrate are introduced on one side of the ultrafilter membrane. By means of a pump, the product is forced through the fiber membrane, representing a series of simple physical barriers that

Table 1.12 Applications of immobilized enzyme reactors in use and under development

Enzymes	Products	Reactors
Aminoacylase	Amino acids	Stirred tank/packed bed
α-Amylase (bacterial)	Dextrinization	Stirred tank/fluid bed
Endo/exonucleases	Nucleotides	Stirred tank/packed bed
Ficin	Soluble proteins	Packed bed (with cofactors)
Glucoamylase	Saccharification	Packed bed
Galactosidase	Raffinose hydrolysate	Stirred tank
Glucose isomerase	Fructose syrups	Packed bed/fluid bed
Glucose oxidase/catalase	Food preservation	Stirred tank
	Soft drinks	Packed bed
	Gluconic acid	Packed bed
Hydrogenases	Hydrogen by photosynthesis	Stirred tank
Invertase	Sucrose inversion	Packed bed
Lactase	Milk, whey, lactose	Tubular/packed bed
Lipases	Fatty acids	Fluid bed
Papain	Soluble proteins	Packed bed
Pectin esterase	Juice clarification	Fluid bed
Protease (microbial)	Soluble proteins	Packed bed/fluid bed/stirred tank
Rennet	Milk coagulation	Packet bed/fluid bed/open surface
Steroid esterase	Steroid modification	Stirred tank
Sulfhydryl oxidase	Flavor control in heat-treated milk	Stirred tank
Tannase	Instant tea	Packed bed
Trypsin	Soluble protein	Packed bed/stirred tank

Table 1.13 Half-lives of some immobilized enzymes

Enzyme	Support	Substrate	Temperature (°C)	$t_{1/2}$ (days)
Lactase	Porous silica or titania	5% Lactose	50	100
		Whole acid whey	50	8
		Deionized whey	40	400
		Deproteinized whey	60	8
Lactase	Zirconia-coated glass	Lactose	30	44
			50	3
	Porous titania	Whey ultrafiltrate	40	25
		Sweet whey	40	54
Glucose isomerase	Zirconia-coated glass	2.8 M glucose	50	24
			70	3
Glucose isomerase	Zirconia-coated glass	1 M fructose	50	240
			60	11
			75	1.0
			80	0.1
L-Amino acid oxidase	Porous glass	L-Leucine	37	43
Alkaline phosphatase	Porous glass	*p*-Nitrophenyl phosphate	23	55
Papain	Zirconia-coated glass	Casein	45	35
Glucoamylase	Zirconia-coated glass	Starch	40	900
			45	645
			50	100
			60	13

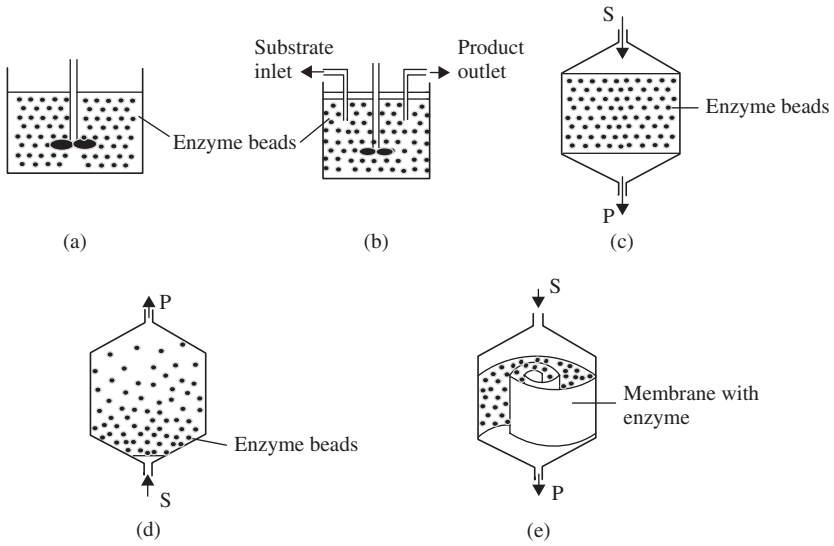

Figure 1.12 Basic immobilized enzyme or cell reactor types. (a) Batch-stirred reactor, (b) continuously fed and stirred tank reactor (CSTR), (c) packed bed or plug-flow reactor, (d) fludizied bed reactor, and (e) UF membrane/enzyme reactor.

retain macromolecules in the compartment under 0.5–5 atm pressure. Many of the commercial enzymes are depolymerases such as amylases and proteases. Membrane circulating bioreactors for depolymerases have thus many advantages and probably will be the reactors of choice for many applications. For high molecular weight substrate such as starch, a tubular reactor is recommended.

Various applications of *ultrafiltration* and *hollow fiber* systems such as the bioconversion of cheese whey into organic acids (lactate, propionate, etc.) have been well known. The main disadvantages of membrane systems are shear stress from the liquid flow, membrane adsorption of the enzyme, and fouling of the membrane, which is manifested by a progressive reduction in the permeation rate (i.e., the polarization phenomenon) and consequently limits overall reactor productivity. Many different geometries for immobilized cell reactors have also been explored, but the configurations are essentially the same as that of the enzyme reactors.

Some examples of applications of immobilized cells for the production of useful chemicals and foods are listed in Table 1.14. Here, the immobilized cell essentially serves as an immobilized enzyme catalyst, with the entire cell used to minimize treatment and processing costs involved in enzyme preparation. Immobilized enzymes have also made other important contributions to medical and analytical applications. *Immobilized enzyme electrodes* have now been constructed for many biologically important compounds (Table 1.15). An enzyme-based electrode consists of an electrochemical sensor (electrode) in contact with a semipermeable membrane of an immobilized enzyme. The enzyme acts on the substrate, and changes of substrate consumption, product formation, or cofactor concentration can be measured by means of spectrophotometer, polarimeter, and photometer. Enzyme thermistors (e.g., a palladium-coated semiconductor device) can also be used to measure ion species.

Table 1.14 Application and stability of various immobilized cells

Product	Microorganisms	Half-life (days)*	Scale
Aspartic acid	*E. coli*	120†	Industrial (1978)
		680‡	–
Malic acid	*Brevibacterium flavum*	52.5	Commercial (1977)
Citric acid	*Aspergillus niger*	–	–
Glucose	*Actinomyces missouriensis*	45†	–
Fructose	–	280‡	–
Ethanol	*Kluyveromyces maxilans*	15	Pilot scale
	Saccharomyces cerevisae	10	Pilot scale
Beer	*Saccharomyces cerevisae*	–	Commercial (Japan)
Lactic acid (from whey)	*Lactobacillus helveticus*	–	–
Propionic acid (from whey)	*Propionibacterium shermanii*	–	–
Penicillin	*Penicillium chrysogenum*	–	–
6-Amino-penicillanic acid	*E. coli*	42	–

*The enzyme activity involved in bioconversion is half its original value at optimum conditions (pH and temperature).
†Polyacrylamide.
‡Carrageenan.

Table 1.15 Examples of enzyme electrodes

Ion species	pH range for direct measurement	Concentration range (mM)
Ammonia	11–13	10^{-3} to 10^{-9}
Calcium	6–8	10^{-3} to 10^{-8}
Carbon dioxide	5	10^{-5} to 10^{-7}
Chloride	2–11	10^{-2} to 8×10^{-9}
Magnesium	–	10^{-7}
Nitrate	3–10	10^{-2} to 6×10^{-7}
Nitrate	0–2	10^{-5} to 5×10^{-10}
Potassium	3–10	10^{-3} to 10^{-9}
Sodium	9–10	10^{-3} to 10^{-9}
Phosphate	–	5×10^{-6} (min)
Sulfate	–	5×10^{-6} (min)
Metabolite	Immobilized enzyme(s)	Concentration range (mM)
Cellobiose	β-Glucosidase + glucose oxidase + catalase	0.05–5
Creatinine	Creatinine iminohydrolase	0.01–10
Ethanol	Alcohol oxidase + catalase	0.01–2
Glucose	Glucose oxidase + catalase	0.002–0.8
Lactate	Lactate 2-monooxygenase	0.005–2
Oxalic acid	Oxalate oxidase	0.005–0.5
Penicillin	β-Lactamase	0.01–500
Sucrose	Invertase	0.05–100
Triglycerides	Lipoprotein lipase	0.1–5
Urea	Urease	0.01–500

Table 1.16 Comparison of K_m and activation energy (kcal/g·mol) values of some soluble and immobilized enzymes

Enzyme	K_m (mM)*		Activation energy (kcal/g·mol)†	
	Soluble	Immobilized	Soluble	Immobilized
Glucoamylase	1.22	0.30	16.3	13.8
Alkaline phosphatase	0.10	2.90	–	–
Glucose oxidase	7.70	6.80	6.6	9.0
Papain	–	–	13.8	11.0
Lactase*	13.1	22.1	10.5	11.3

*Values from *Aspergillus niger* source.
†Values from *Kluyveromyces maxilans*.

The same type of enzyme deactivation and reaction parameter changes that occur in soluble enzyme are likely to influence immobilized enzymes. Soluble and immobilized enzymes often show the differences in kinetic constants that can be attributed to diffusion influences rather than to changes in intrinsic kinetics (Table 1.16). Stability and activity optima of immobilized enzymes are also rarely the same as soluble enzymes. In engineering, *optimal* has an economic implication that has rarely been applied to immobilized enzymes. Whenever a charged substrate reacts with an enzyme attached to a charged surface, the pH *optimum* for immobilized enzyme, for example, trypsin, was shifted down 3 full pH units from that of the soluble enzyme. High ionic strength tends to diminish these charge-interaction effects and brings the curve together (Figure 1.13). Charge-interaction effects can thus be made to work for the biotechnologist if they are

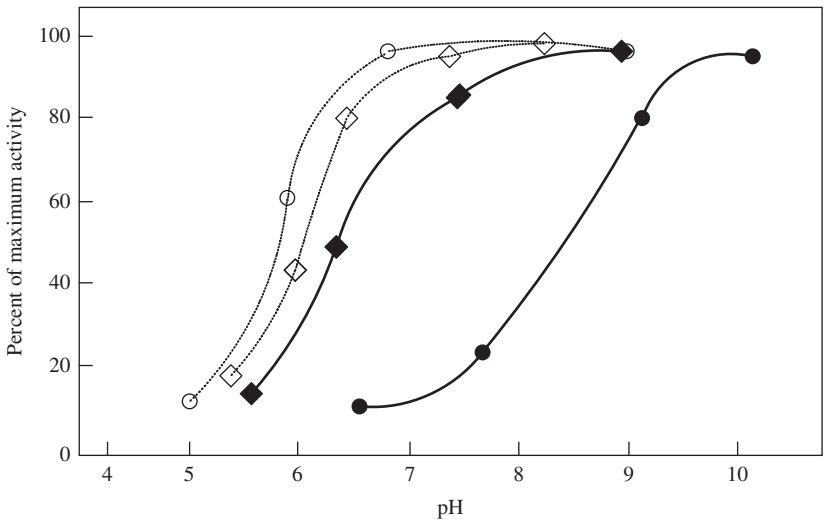

Figure 1.13 pH activity curves for trypsin (dotted) and trypsin covalently bound to ethylene–maleic acid copolymer (solid) at ionic strength of 0.06 (circles) and 1.0 (squares). Benzoylarginine ethyl ester is the substrate.

properly understood and anticipated. Further discussions on immobilized biosensors are also found in Section 1.11.

1.7.3 Mass transfer concept

There are many situations in bioprocessing where concentrations of compounds are not uniform; we rely on the mechanisms of mass transfer to transport material from regions of high concentration to regions where the concentration is low. An example is the supply of oxygen in fermenters for aerobic culture. Concentration of oxygen at the surface of air bubbles is high compared with the rest of the fluid; this concentration gradient promotes oxygen transfer from the bubbles into the medium. Another example of mass transfer is extraction of penicillin from fermentation liquor using organic solvents such as butyl acetate. When solvent is added to the broth, the relatively low concentration of penicillin in the organic phase causes mass transfer of penicillin into the solvent. Solvent extraction is an efficient downstream-processing technique as it selectively removes the desired product from the rest of the fermentation fluid.

Mass transfer is the most critical factors in the operation of a large-scale bioreactor. These are caused by *bulk flow* (*convection*) as well as *conduction* (*diffusion*) processes. For the ultimate supply of nutrients or other reactants to cells or cell products such as enzymes, diffusion processes dominate. Fick's law of diffusion states that the mass flux is proportional to the concentration gradient, that is,

$$JA\ (\text{moles/cm}^2/\text{s}) = -D_\text{A}\frac{dCA}{dX}$$
$$= (\text{cm}^2/\text{s})(\text{moles/cm}^3/\text{cm})$$

where the diffusivity D_A depends on temperature by the Arrhenius equation ($D_\text{A} = D_{\text{A}_0}e^{-E/RT}$). Diffusivities for typical biological reactants range from about $10^{-4}\,\text{cm}^2/\text{s}$ (small molecules) to $10^{-8}\,\text{cm}^2/\text{s}$ (macromolecules) in aqueous solution.

Three mass-transfer situations that occur in bioprocessing are liquid–solid mass transfer, liquid–liquid mass transfer between immiscible solvents and gas–liquid mass transfer. The overall rate of microbial growth or product formation is governed by one of many mass transfer or reaction rates, including transfer of reactant from bulk fluid to surface of microorganism, transfer across the cell membrane, transfer within the cell or organelle, and reaction within the cell or organelle. In analyzing these systems involving mass transfer, recall that events in series are governed by the slowest step, while the overall rate of processes that run in parallel are governed by the fastest rate step. Since many of the systems of interest to biologists and biochemical engineers consist of processes occurring in series, the concept of the rate-limiting step becomes particularly important.

When oxygen is required as a microbial gaseous substrate, it is frequently a limiting factor in fermentation. This is because of the very low solubility in water of oxygen as compared with other substrates and metabolites. The solubility of oxygen is only approximately 0.01 g/L (Table 1.17). Moreover, the influence of the culture medium ingredients causes the maximal oxygen content to be lower than it would be in pure water. A rapidly respiring culture will use all the oxygen available in solution within a few seconds, and *oxygen transfer* therefore has been a key problem of biochemical engineers concerned with bioreactor design. The oxygen requirement of an aerobic culture is controlled by the cell density in the reactor, the cell growth rate, and the approximate yield coefficient. The yield coefficient Y_ATP is the mass of cells formed per mole of ATP consumed for biosynthesis.

Table 1.17 Solubilities of substrates and metabolites in water at 25 °C

Substance	Solubility (g/L)
Alanine	147.0
L-Asparagine	24.6
Glucose	590.0
Glycine	21.7
Lactose	170.0
L-Leucine	22.4
Sucrose	909.0
Urea	620.0
DL-Valine	66.8
Oxygen	0.01

Table 1.18 Effect of substrate and cell yield on oxygen requirement and heat production

Microorganisms	Substrates	Cell yield, Y_s (g cell/g substrate)	Oxygen required, Y_{O_2} (g cell/g O_2)	Heat released, Y (g cell/kcal)
Bacteria	*n*-Alkanes	1.0	0.58	0.13
Yeasts	Carbohydrates	0.5	1.49	0.26
Yeasts	*n*-Alkanes	1.0	0.51	0.13

The amount of oxygen required is strongly influenced by how highly reduced the carbon substrate source is. From Table 1.18 it is seen that up to 200 g of oxygen may be required for yeast fermentation on *n*-alkane to produce 100 g of cells. Production of 100 g of cells per liter over the course of a fermentation might thus result in the replacement of the oxygen at the rate of 200 g/L per 0.01 g/L or 20,000 times. Obviously predicting oxygen transfer rates can be very important. Hence, aerobic microbial processes frequently operate under conditions of oxygen limitation; thus, there are reductions in productivity, and the costs incurred for oxygen transfer comprise a significant fraction of total operating expenditure.

The solubility of a gas in a liquid has a limited and definite value, which depends on the nature of both the gas and the liquid and on the temperature and pressure of the system. When other factors remain constant, an increase in temperature usually results in a decrease in solubility, while an increase in pressure (or partial pressure) results in an increase in solubility.

The quantitative relationship between solubility and pressure is known as Henry's law. Henry's law describes the solubility of O_2 in nutrient solution in relation to the O_2 partial pressure in the gas phase,

$$CO_2 = \frac{P_{O_2}}{H_{O_2}}$$

where CO_2 is the DO concentration, P_{O_2} is the partial pressure of oxygen in air and H_{O_2} is the Henry's law constant for the system at the appropriate temperature. As the oxygen concentration increases in the gas phase, the oxygen proportion of the nutrient solution apparently increases. The general nature of the mass transfer problem of primary concern

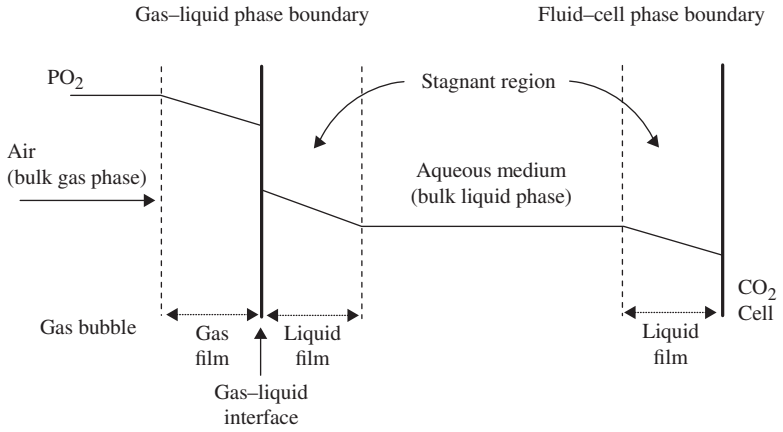

Figure 1.14 Resistance to oxygen transfer from the air bubble to a microbial cell based on the Lewis–Whitman model.

is frequently considered in the two-film model of Lewis and Whitman. A sparingly soluble gas, usually oxygen, is transferred from a source, rising through bubbles into a liquid phase containing cells. As shown in Figure 1.14, the oxygen must pass through several independent and different combinations of partial resistances: (i) resistance within the gas film to the phase boundary, (ii) penetration of the phase boundary between gas bubble and liquid, (iii) transfer from the phase boundary to the liquid, (iv) movement within the nutrient solute, and (v) transfer to the surface of the cell.

The *Lewis and Whitman model* assumes that at a gas–liquid interface, both the gas and the liquid that comprise the thin film immediately adjacent to the interface are essentially stagnant, while both the bulk gas and bulk liquid phases are in turbulent motion. Absorption results from steady state molecular diffusion processes in the two stagnant films. For fermentations carried out with single-celled microbes, the resistance in the phase boundary between gas bubble and liquid is the most important factor controlling the rate of transfer.

For most aerobic bioreactors, oxygen is transferred from air bubbles dispersed throughout the liquid medium in which the microbes are either growing or respiring. It becomes necessary to assess the rate of oxygen transfer on a unit volume basis. Oxygen transfer rates are generally predicted by

$$N_A = K_L a(C^* - C_L)$$

where N_A = volumetric O_2 transfer rate (mM O_2/L/h)
K_L = mass transfer coefficient at the phase boundary
a = specific gas–liquid interfacial area (specific exchange surface)
$K_L a$ = volumetric oxygen transfer coefficient ($h-1$)
C^* = equilibrium oxygen, concentration
C_L = DO concentration (mM/L)

The dispersion of gas bubbles in liquids is a complex process. Predicting oxygen transfer generally reduces to finding $K_L a$. This value is dependent on many variable factors (e.g., diameter, capacity, power, aeration system, aeration rate of the bioreactors) and on the density, viscosity, and composition of the nutrient, as well as the structure of the microbes,

the antifoam agent used, and the temperature. Obviously, to enhance oxygen transfer by increasing a, it is necessary either to increase gas hold-up, which is known as volume gas per volume reactor, or to reduce mean bubble diameter. The presence of dissolved inorganic and organic matter in aqueous systems markedly affects bubble size, either by influencing the coalescence characteristics or by changing the interfacial tension.

Surface-active agents such as *antiform compounds* reduce the value of $K_L a$ and cease the renewal of the bubble surface. Microorganisms themselves have a significant effect on the oxygen transfer by acting as a barrier. DO concentrations drop rapidly in the slime layer, approaching zero within 100 μm of the air–liquid interface; in other words, the film becomes anaerobic at those depths. It would certainly be useful to predict the conditions under which this will occur – that is, the conditions that will prevent the diffusion of oxygen into a region consuming oxygen (e.g., the slime layer) from keeping pace with the oxygen consumption rate. Since these processes occur in series, they are governed by the slowest step. In the design of aerobic bioreactors, we frequently use correlations of data more or less approximating the situation of interest to establish whether the slowest process step is the oxygen transfer rate or the rate of cellular utilization of oxygen (or other limiting substrate).

The maximum possible oxygen utilization rate is $X_{\mu_{\max}}/Y_{O_2}$, where X is cell density and Y_{O_2} is the ratio of moles of cell carbon formed per mole of oxygen consumed. We assume that all oxygen entering the bulk solution is rapidly consumed (Cl = 0) in this equation. If $K_L a$ Cl is much larger than $X_{\mu_{\max}}/Y_{O_2}$, the main resistance to increased oxygen consumption is microbial metabolism and the reaction is biochemically limited. Conversely, the reverse seems to be true in the mass transfer–limited mode. With unicellular bacteria, the O_2 absorption rate is constant during log growth until another substrate becomes limiting. However, in mycelial (streptomycetal and fungal) fermentations, the O_2 absorption rate decreases when O_2 becomes limiting as a result of increases of mycelium volume and viscosity.

Critical oxygen concentration (CO_{2cr}) is the value of the specific oxygen absorption rate that permits respiration without hindrance. The critical oxygen values for microorganisms lie in the range of 0.003–0.05 mmol/L. For the higher critical oxygen values (e.g., 0.02 mmol/L for Penicillium molds), oxygen mass transfer is evidently extremely important. The more important factors that can influence the total microbial oxygen demand (X_μ/Y_{O_2}) are cell species, culture growth phase, carbon nutrients, pH, substrate utilization, and biomass yield.

We have mentioned that oxygen utilization for growth is typically coupled directly to the amount of carbon source substrate consumed. Furthermore, more reduced substrates such as paraffins and methane require greater oxygen uptake by the cell than substrates such as glucose, which have approximately the same carbon oxidation state as the cell. There are four acceptable methods of determining oxygen transfer rate: the dynamic gassing-out method, the sulfite method, direct measurement of the volumetric O_2 transfer rate, and calculation from measurements of microbial growth.

1.7.4 Heat transfer concept

Energy balances allow us to determine the heating and cooling requirements of fermenters and enzyme reactors. Once the rate of heat transfer for a particular purpose is known, the surface area required to achieve this rate can be calculated using design equations.

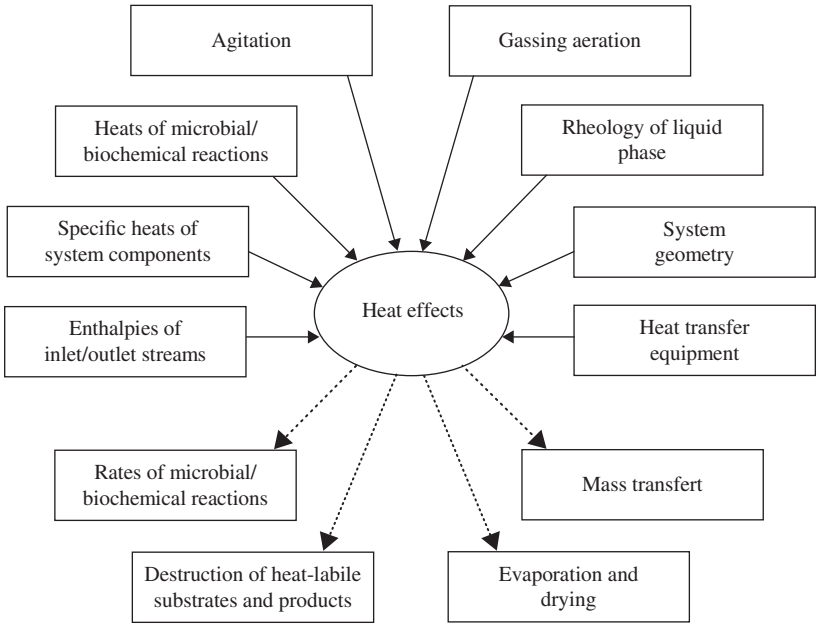

Figure 1.15 Factors contributing to and phenomena affected by heat effects in biological reactors. *Source*: Redrawn from Atkinson and Mavitura 1991.

Estimating the heat-transfer surface area is a central objective in design as this parameter determines the size of heat-exchange equipment.

The principles governing heat transfer are outlined with applications in the bioprocess design. Heat transfer is required to (i) sterilize a liquid reactor feed in a batch or continuous flow vessel (the temperature desired must be high enough to kill essentially all microorganisms in the total holding time), (ii) add heat to promote a desired reaction (as in an anaerobic sewage sludge digestor operating between 55 and 60 °C), (iii) remove excess heat generated by most microbial fermentation processes, and (iv) concentrate microbial products by drying. The first three requirements relate to cell viability and metabolism, and we will emphasize (i) and (ii). The fourth is a unit operation, drying, which is covered on engineering unit operations.

Figure 1.15 displays some important factors contributing to heat transfer requirements and phenomena affected by heat production in biological systems.

These heat effects are accounted for in an energy balance equation. In a constant pressure system with negligible changes in potential and kinetic energies, the energy balance can be expressed in terms of changes in *enthalpy* (ΔH) (i.e., the heats of chemical transformation) or phase transformation (e.g., evaporation, condensation), the sensible heat flow in mass streams, and the heat transfer to or from second fluids acting as heating or cooling devices, as follows:

$$Q_{\text{met}} + Q_{\text{ag}} + Q_{\text{gas}} = Q_{\text{acc}} + Q_{\text{exch}} + Q_{\text{evap}} + Q_{\text{sen}}$$

where Q_{met} = heat generation rate from cell growth and maintenance
Q_{ag} = heat generation rate due to mechanical agitation
Q_{gas} = heat generation rate from aeration power input
Q_{acc} = heat accumulation rate per unit volume
Q_{exch} = heat transfer rate to the surroundings or to heat exchanger coolant
Q_{evap} = heat loss rate by evaporation
Q_{sen} = sensible enthalpy gain rate of streams (exit−entrance)

The rate of heat production due to agitation and the metabolic activity of the microorganisms must be balanced by the heat loss resulting from evaporation and radiation plus heat removal by the cooling system (the jacket of the fermentor or cooling coils). Such a balance can be used to calculate Q_{met} from measurements of Q_{acc} through monitoring the initial transient temperature rise of a nearly isolated fermentor. Viscous heating and metabolic heat during microbial growth are clearly the dominant factors that can lead to heat accumulation. This heat accumulation can easily lead to problems for the fermentation. Not all fermentations are exothermic, but sometimes ΔH is endothermic, which simplifies fermentation control because the heat-producing effect of agitation tends to be balanced by the heat of fermentation for endothermic systems. Not only may agitation lead to heat accumulation, but sensitive microorganisms (either filamentous or flocculating microbes) may be damaged by the impeller. Impeller tip speed ND (rpm × impeller diameter) seems to be the key factor, where N is the rotational speed of the impeller and D is the impeller diameter.

Heat production is strongly related to the degree of reduction of the substrate. In general, hydrocarbons produce more heat than partially oxidized substrates ($Y_\Delta(CH_4) < Y_\Delta(CH_3OH) < Y_\Delta(n\text{-alkanes}) < Y_\Delta(\text{glucose})$). The yield coefficient Y_Δ is given in grams of cells per kilocalorie of energy released, Y_s indicates the ratio of grams of cells per gram of substrate utilized. At a yield coefficient of 0.5, four times as much heat is produced by cells growing on methane as by cells produced on methanol, a less highly reduced substrate (Figure 1.16). Y_Δ values also increase linearly in relation to μ, the specific growth rate of the cultures. Because of the "combustion" aspect of microbial metabolism in aerobic fermentations, the heat evolution rate correlates very well with the oxygen consumption rate for a number of microorganisms (Table 1.19). This is one important coupling between mass and heat transfer in biological systems.

Heat transfer problems that are insignificant during laboratory work may become very significant upon scale-up because heat production, which is proportional to volume of the fermentation fluid, increases more rapidly than the surface area available for jacketed heat transfer. The exchange surface available for removal of heat from the unit volume of culture decreases with scale, and this sets a maximum limit to the fermentor size. However, fermentors with internal cooling can be sized appropriately using such surface-to-volume considerations.

The heat production by power input also varies somewhat with the scale of the fermentation. Limitations on the ratio of surface area to volume can be reduced or eliminated by use of external heat exchangers. This strategy is aided by the batchwise operation of many fermentations, which mean that their heat production rates follow the batch mode. An example is provided by ethanol fermentation, in which heat release peaks at about 8 h into the fermentation and is largely complete within about 24 h. Why build an exchanger to handle the peak? Why not have two or more fermentors share a common external heat exchanger instead? If two large ethanol fermentors have a common external heat exchanger, savings in operating and capital costs should be substantial. The single

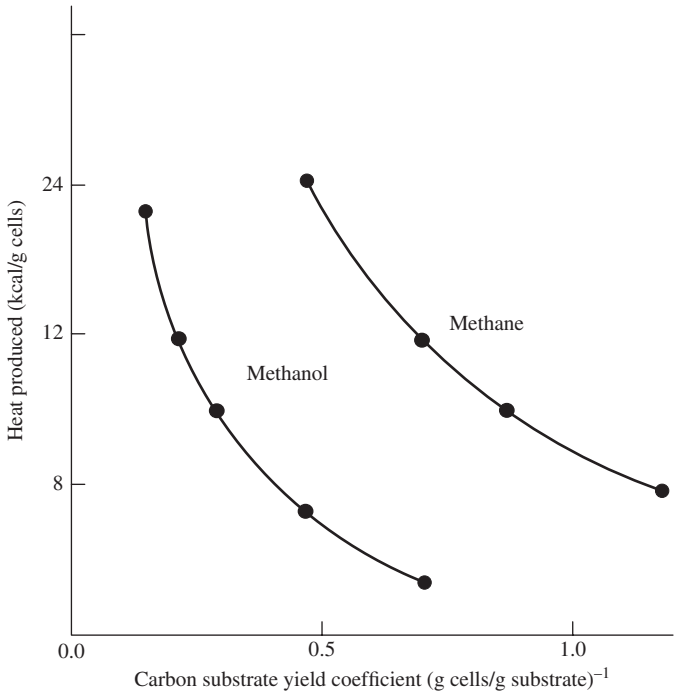

Figure 1.16 Correlation of heat production Y_Δ and biomass yield Y_s on different substrates.

Table 1.19 Heat production during the continuous culture of microorganisms using different substrates

Substrate	Y_s (g cell/g substrates)	Y_{O_2} (g cell/g O_2 consumed)	Y (g cell/kcal)
Acetate	0.36	0.70	0.21
Glucose	0.51	1.47	0.42
Maleate	0.34	1.02	0.30
Ethanol	0.68	0.61	0.18
Methanol	0.40	0.44	0.12
Isopropyl alcohol	0.43	0.23	0.074
n-Alkanes (C12–C18)	1.00	0.50	0.16
Methane	0.62	0.20	0.061

Source: Bailey, JE and Ollis, DF 1986. Biochemical Engineering Fundamentals (2nd Ed.). Reproduced with permission of McGraw Hill.

Table 1.20 Estimated operating costs for biomass production from different substrates

Substrate	Cost ($/lb cells)			Total ($/lb cells)
	Substrate	Oxygen transfer	Heat removal	
Acetate	0.167	0.0062	0.011	0.184
Alkanes	0.040	0.0097	0.014	0.054
Ethanol	0.088	0.0075	0.013	0.109
Glucose equivalent (molasses)	0.039	0.0023	0.0054	0.047
Maleate (as waste)	0.000	0.0046	0.0075	0.012
Methane	0.016	0.033	0.037	0.086
Methanol	0.050	0.012	0.019	0.081
Isopropyl alcohol	0.116	0.027	0.031	0.174

Source: Bailey, JE and Ollis, DF 1986. Biochemical Engineering Fundamentals (2nd Ed.). Reproduced with permission of McGraw Hill.

exchanger cannot service both fermentors at the same time, however. Thus, at about 24 h the flow is switched and the temperature in the first fermentor is allowed to rise slightly over the remainder of the fermentation. From the attention that mass transfer (especially oxygen transfer) and heat transfer have received in the literature, it is easy to assume that such processes must be economically dominant. More often than not, however, these costs will be important but not dominant. Substrate costs tend to dominate all other fermentation costs (Table 1.20).

1.7.5 Mass and heat transfer practice

Mass transfer and heat transfer equations are beyond the subject in this short chapter, and thus several books and references cited are suggested to read. The mass transfer theory finds applications in biotechnology in many product recovery areas. For the production of value-added products, it is impossible for the central production step to exist in isolation. The concentration of the desired product in the fermentation broths will be relatively low. Fermentation broths are complex aqueous mixtures of cells, soluble extracellular or intracellular products, and unconverted substrate. The recovery and purification of the product from the numerous undesirable contaminants which may be quite similar in size, charge, and molecular structure, may be considered to be one of the most critical aspects of industrial fermentation process. The principles referred to this section are applicable to the recovery of microbial, plant, or animal products. Product recovery is a largely undeveloped field for many of the more sensitive biological materials, especially for rDNA products. Eukaryotic gene products in prokaryotic organisms are often formed in the forms of highly insoluble inclusion bodies (IBs), frequently in inactive forms held together by disulfide bridges. Such IBs must be released, purified, and eventually converted into active proteins.

In most large-scale processes, the desired product is a metabolite that is present either intracellularly or extracellularly. *Intracellular metabolites* include nucleic acids, vitamins, enzymes, and certain antibiotics, while *extracellular metabolites* are amino acids, organic acids, alcohols, some enzymes, and most antibiotics. In either case, the product must be recovered from a solution in which a substantial amount of undesirable compounds are present. A typical unit operations approach to bioseparations can be conveniently divided into the following groups (Figures 1.17 and 1.18): cell separation/disruption, primary isolation, purification, and finishing operations such as drying, storage, and analysis. Unique

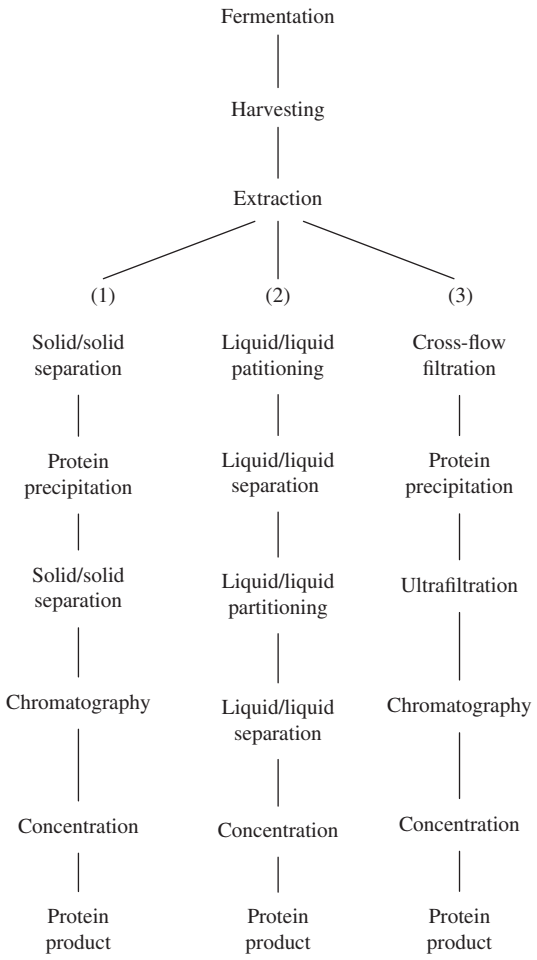

Figure 1.17 General schemes of protein recovery operation through sequences for solid–solid separation, liquid–liquid partitioning, and cross-flow filtration.

characteristics of biological fluids related to separation problems are (i) slimy gelatinous, viscous, non-Newtonian flows, caused often by the biopolymers secreted, (ii) poor filterability, poor sedimentation, and high water retention, and (iii) poor thermal stability and/or poor chemical stability as well as unfavorable forming and emulsification tendencies, hence poor mass and heat transfer properties. Fluids for which the viscosity is not a function of the rate of shear are referred to as *Newtonian fluids*, while many liquids found in bioprocesses do not exhibit this behavior and are referred to as *non-Newtonian fluids*.

Particles and molecules may be separated from a solution based on their differences in size, density, solubility, and diffusivity, which are the physicochemical properties. The size of a bacterial cell ranges from about 0.2–5 μm, and the specific gravity is very small, which makes separation extremely difficult. Separation methods include flocculation (or flotation), filtration, ultrafiltration and centrifugation (or ultracentrifugation). If an intracellular

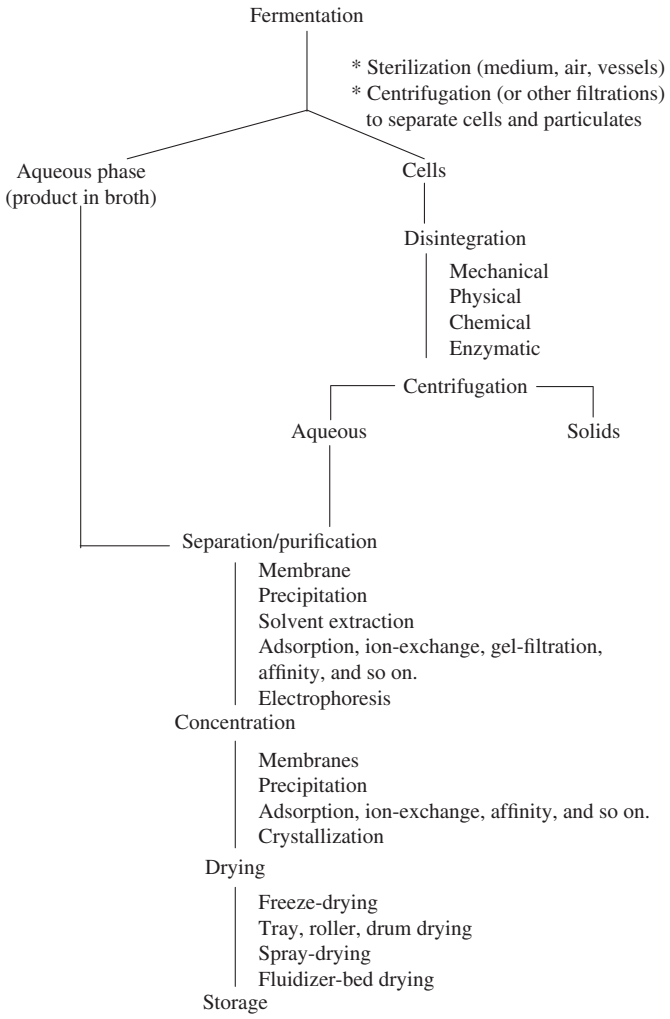

Figure 1.18 A process flow sheet for product recovery.

metabolite is to be isolated, it must be liberated from the cells by disintegration or direct extraction techniques before centrifugation. *Flocculation* is used to produce large aggregates, which settle more readily. Flocculating agents such as inorganic salts, organic polyelectrolytes or mineral hydrocolloids are often added. The flocculation of cells depends on many different factors, such as temperature, ionic strength, age of cells, surface force, and type of organism. Flotation is most readily accomplished by introducing gas bubbles into the liquid, whereupon the cells become adsorbed and rise to the layer that forms at the top, which can be removed.

Preliminary isolation can take the form of solvent extraction, sorption, precipitation or ultrafiltration. During primary separation, the concentration of the desired product increases significantly from that of the crude preparations. *Purification* usually includes

a series of adsorption/desorption, or chromatography columns for impurity removal as well as further product concentration. In finishing operations, the final volume is further reduced by centrifugation and subsequent drying of the crystallized product. Drying should not be so harsh that it damages the product, and storage conditions especially must take into account the special properties of biological matters before packaging. Analysis is also necessary not only at the end but also at various points along the way for purity requirements and bioassay.

Cell separation In the first step in process recovery, separation of cells (and solid particles) from a large volume of the culture broth is normally carried out by filtration or centrifugation. The principal separation methods based on physicochemical properties of particles and molecules are shown in Figure 1.18. Small fermentation batches can be filtered through a *plate-and-frame filter* (i.e., filter press) in which stacked flat, porous plates are used as supports for a filter (either cloth or membrane). Large samples rely on continuous filters mounted on the *rotary drum vacuum filters*, which in some cases require precoating (e.g., with diatomaceous earth) and a string or knife blade to scrape the cake from the drum. Two types of membrane filtration process – static and cross-flow – are commonly used for the convective transport of dissolved particles to the membrane surface. Under the influence of hydrostatic pressure, certain particles pass through the membrane, while other particles are retained at the membrane. The cross-flow method can reduce the tendency to clog, and there is a hundredfold increase in filtration rate compared with static flow. Three major types of filtration process are known, depending on the sizes of the particles being filtered; these are reversed osmosis, ultrafiltration and microfiltration. *Reverse osmosis* normally separates particles in the range of 0.0001–0.0001 μm or molecular weight cutoff less than 1000, while *ultrafiltration* is used for particles of 0.001–0.1 μm or molecular weight cutoff greater than 1000, and *microfiltration* serves for particles of 0.02–10 μm (Table 1.21). Ultrafiltration thus can separate compounds with molecular weights from 103 to 106 Da. However, both size (molecular weight) and shape are important, particularly for branched polymers such as polysaccharides (Figure 1.19).

Molecular diameter is also a key variable for ultrafiltration. Ultrafiltration is claimed to offer economic advantages over vacuum filtration due to the clarification it provides to the

Table 1.21 Flow characteristics of three types of pressure-driven membrane process: reverse osmosis (RO), ultrafiltration (UF), and microfiltration (MF)

Characteristics	Process		
	RO	UF	MF
Separation range (μm)	0.0001–0.001	0.001–0.1	0.02–10
MW cutoff range (Da)	$<10^3$	$>10^3$ to 10^6	$>10^5$
Retentate	Salts, macromolecules, microorganisms	Macromolecules, microorganisms	Microorganisms
Pressure (psi)	250–1000	10–100	10–100
Membranes	–	–	Cellulose esters, polyvinyl fluorides, polycarbonate, polysulfones, cellulose
Modules	–	–	Cassettes, spiral-wound bundles of tubes (1–2 cm diameter), or capillary bundles

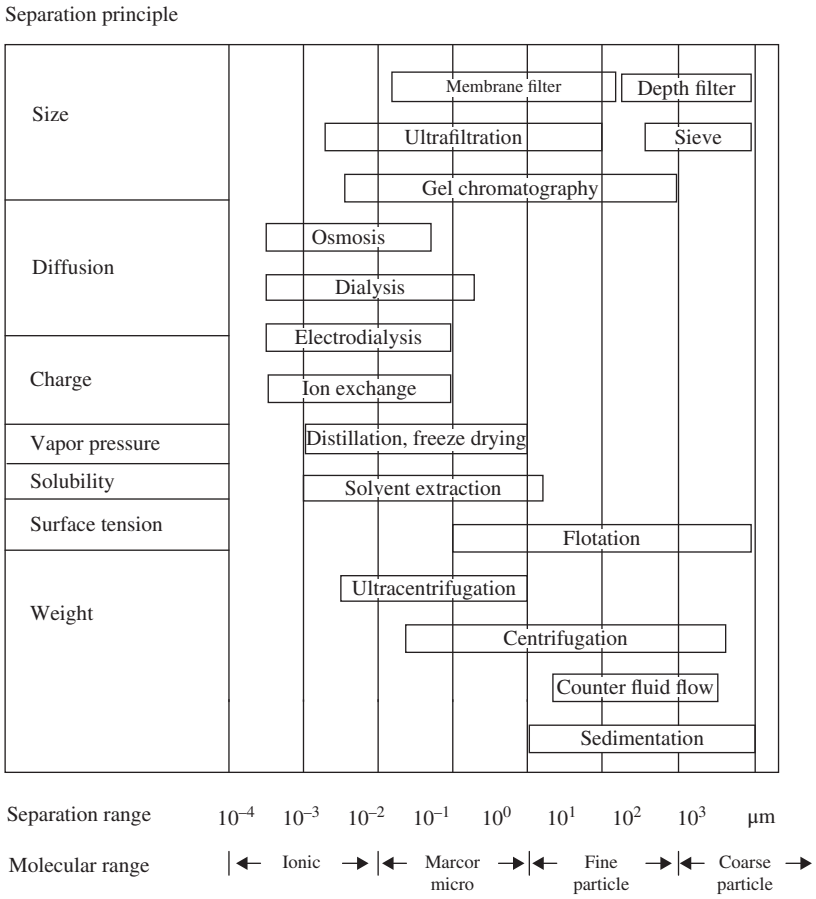

Figure 1.19 Separation methods based on physicochemical properties of particles and molecules. *Source*: Adapted from B. Atkinson and F. Mavitura 1991. Biochemical and Biotechnology Handbook, Macmillan.

fermentation broth. However, numerous parameters must be considered when selecting a filtration system. Among these are pore size and particle selectivity, cross-flow rate, cost and life span of the filter, and sterilizability of the system.

Centrifugation is used not only for achieving (fluid–particle) separation but also for enabling liquid–liquid and liquid–liquid–particle separations. For example, liquid–liquid separation is used in the manufacture of antibiotics (e.g., penicillin) by extracting the solvent from the aqueous phase by means of a two-stage continuous countercurrent extractor. The filter-and-sieve and solid-bowl centrifuges are of two distinct types. In the *filter and sieve centrifuge*, separation occurs as the particles are forced against a filter material. These centrifuges are used in great numbers for the separation of bioproducts. In the *bowl* (or *baffle*) *centrifuge* the separation occurs because of the specific gravities of the phases present, and the products are collected at the drum wall.

A wide variety of centrifuges are on the market for large-scale operations: separators (plate or nozzle) and decanters are examples. Separators have been used traditionally

for the production of baker's yeast and the clarification of the most diverse fermentation broths. A large-capacity (300 m^3/h) nozzle separator is best used for the continuous separation of bacteria. In *decanter centrifuges* (*scroll type*), sedimentation of the solid particles takes place on the rotating drum wall. Decanter centrifuges are used for the further dewatering of large single-cell proteins (yeast, algae) obtained in separators. In contrast to continuous centrifuges of other forms, *tubular bowl centrifuges* permit very high centrifugal force (g), averaging from 15,000 to 60,000 g. They thus permit the separation of very small particles and protein precipitates but use the discontinuous mode of operation, with low sludge hold-up capacity.

Cell disintegration Cells are usually disrupted after dilution of buffer to approximately 10% solids by one of the following types of methods: mechanical (milling), physical (ultrasonic, high-pressure homogenizer, freezing/thawing, drying), chemical (detergents, osmotic shock, acids, solvents, or antibiotics), or enzymatic lysis (lysozyme and other cell wall–digesting enzymes, autolysis, phages). Other means, including leaky mutants and genetically induced secretion, are also used in the production of rDNA products. The selection of a method depends principally on the nature of the cells and the cost of the method. Gram-positive bacteria and yeasts are more resistant to breakage than Gram-negative bacteria. Cell walls become more rigid, hence are better able to resist breakage, after the logarithmic stage of growth.

A *ball mill* is filled with the microorganisms together with small glass beads and subjected to high speed mixing; the action of the glass beads brings about the disintegration of the cells. One can expect a maximal breakage of 80–90% of the cells under optimum conditions. Another popular method on the industrial scale is the use of a *high-pressure homogenizer*. Cell disintegration takes place upon the application of high hydrostatic pressure followed by immediate pressure release as the cell suspension passes through a valve. To achieve good breakage (≈90%), two or three passes of the material through the homogenizer must be carried out. *Ultrasonic disintegration* is widely used in the laboratory, but it is not suitable for large-scale processes because of the high cost. Various drying methods have been used for the disintegration of cells. The mode of action is mainly due to a change in the structure of the cell wall during drying. Similarly, freezing and thawing, or heat-shock techniques can be used for the release of metabolites.

Autolysis is often used for the preparation of autolyzed yeast-flavoring ingredients. Endogenous autolytic enzymes of the yeast cells are responsible for lysis of the cell wall. Another similar technique, *plasmolysis*, is salt-induced autolysis obtained by adding a high concentration of NaCl. Osmotic shock is usually suitable for very sensitive cells such as animal cells. Detergents such as sodium dodecyl sulfate (SDS) or Triton X-100 are very effective for liberating intracellular molecules. On a small laboratory scale, investigators often rely on the enzymatic lysis of microorganisms using lysozyme or other cell wall-degrading enzymes and phage lytic enzymes.

Preliminary isolation (concentration)

Extraction Liquid extraction is based on the removal of the desired component from one of two immiscible liquids into the other, from which the components presumably may be recovered more easily. Many products can be concentrated and purified by use of a two-phase system (aqueous–organic or aqueous–aqueous). Aqueous–organic phases (amyl or butyl acetate) have been used for antibiotic recovery, but a novel aqueous–aqueous system has also been used for the purification of labile enzymes or other proteins. In solvent extraction, a two-phase system can be set up, using an organic

solvent that is immiscible with the aqueous broth. An extraction solvent should be selective (i.e., it should not dissolve unwanted components) and should have a high capacity for the solute; it should be low in volatility, relatively low in viscosity, noncorrosive, and relatively inexpensive. Selection of an appropriate extraction solvent can be particularly difficult for biological systems, which tend to be sensitive to many of the common solvents. The efficiency of the separation may be described by the distribution coefficient K as follows:

$$K = \frac{y_a}{x_a} \quad \text{at equilibrium}$$

where y_a is the mole fraction of the desired component (solute) in the extract and x_a is its mole fraction in the raffinate.

A variety of contacting patterns are possible in liquid–liquid extraction: countercurrent, fractional extraction, and cross-current extraction are examples. As a rule, the countercurrent technique offers higher driving forces, which impel the two fluids countercurrent to each other. If the solute to be removed is a weak acid or base, the distribution ratios may be markedly improved by adjusting the pH of the aqueous phase. For the purification of a typical penicillin, acidification of the broth to a pH in the range 2.0–3.0 causes protonation of the components, rendering the maximum selectivity for penicillin extraction by organic solvents. Organic solvents cannot be used for the purification of enzymes in the natural state, but aqueous two-phase systems can be readily scaled up for the recovery of the product.

Separated aqueous phases are produced by the dissolution of two incompatible polymers, such as PEG and dextran. The resulting phases are more than 75% water but rich in either PEG or dextran. The proteins are also dissolved in potassium phosphate buffer and precipitated by PEG. Cells remain in one of the phases, and the enzyme is transferred to the other phase without any loss of activity. Dextran, even its crudest form, dominates the medium makeup costs and thus must be reused. Some examples of enzymes that are purified by aqueous two-phase systems are shown in Table 1.22. However, this method is still expensive in terms of the PEG cost.

Another interesting variant of the foregoing technique, *supercritical extraction* (in which CO_2 plays a role), is used in the extraction of caffeine from coffee beans, hops for brewing, and pigments and flavor ingredients from biological materials.

Precipitation Precipitation may be brought about in many ways, such as by adding salt (e.g., ammonium sulfate or sodium sulfate) in high concentrations for salting out and adjustment of pH to the isoelectric point. Other possible additives include miscible organic solvents (ethanol, methanol, acetic acid, acetonitrile), nonionic polymers, polyelectrolytes, and polyvalent metal ions (to form a reversible protein precipitate). In concentrated salt solutions, the decrease in the solubility of proteins is a function of the ionic strength of the solution. If the desired enzyme is thermostable, contaminant host proteins may be selectively precipitated by heating at high temperatures, while the desirable thermostable enzyme remains in solution.

Biopolymers such as xanthan gum and alginate are recovered by adding divalent cation (Ca^{2+}) to form a gel precipitate. This method is thus used to form calcium alginate gels for cell immobilization. The gum polysaccharide is also directly precipitated by adding methanol or isopropyl alcohol.

Adsorption Ion exchange or adsorption with special polymer resins may be considered for the isolation of hydrophilic metabolites. Ion exchange materials are merely dissociable ion

Table 1.22 Two-phase aqueous extractions of enzymes from microbial cells*

Enzyme	Organism	Biomass concentration (%)	Phase system	Partition coefficient	Yield (%)	Purification factor
Isoleucyl-tRNA synthetase	*Escherichia coli*	20	PEG/salt	3.6	93	2.3
Fumarase		25	PEG/salt	3.2	93	3.4
Aspartase		25	PEG/salt	5.7	96	6.6
Penicillin acylase		20	PEG/salt	2.5	90	8.2
α-Glucosidase	*Saccharomyces cerevisiae*	30	PEG/salt	2.5	95	3.2
Glucose-6-phosphate dehydrogenase		30	PEG/salt	4.1	91	1.8
Alcohol dehydrogenase		30	PEG/salt	8.2	96	2.5
Hexokinase		30	PEG/salt	–	92	1.6
Glucose isomerase	*Streptomyces* sp.	20	PEG/salt	3.0	86	2.5
Pullulanase	*Klebsiella pneumoniae*	25	PEG/dextran	3.0	91	2
Phosphorylase		16	PEG/dextran	1.4	85	1
Leucine dehydrogenase	*Bacillus sphaericus*	20	PEG/crude dextran	9.5	98	2.4
Diacetate dehydrogenase	*Lactobacillus* sp.	20	PEG/salt	4.8	95	1.5
L-2-Hydroxyisocaproate dehydrogenase	*Lactobacillus confusus*	20	PEG/salt	10	94	16
D-2-Hydroxyisocaproate dehydrogenase	*Lactobacillus casei*	20	PEG/salt	11	95	4.9

*Except for pullalanase recovery, cells were disrupted by high-pressure homogenization or wet milling.
Source: Kula MR, et al. Purification of enzymes by liquid–liquid extraction. Advances in Biochemical Engineering 24, 773–778, 1982. With kind permission from Springer Science and Business Media.

pairs in which one type of charge is immobile. The separation of an antibiotic directly from the culture solution without prefiltration is an example. The total culture broth is treated with an anion exchanger in stirred columns connected in series. The use of ion exchangers for the separation of bacterial mixtures has also resulted in the development of adsorber resins without functional groups. The binding of substances to these resins takes place non-stoichiometrically by adsorption. In contrast to ion exchangers, the capacity of adsorber resins increases in the presence of salts. Whole broth processing is well known for the production of antibiotics (cycloheximide) using Amberlite (XAD) or streptomycin and novobiocin using fixed-bed ion exchange adsorbers at the pilot and process levels. This process was developed to eliminate a costly mycelial filtration, which resulted in loss of antibiotics in filter cake materials.

Purification A number of different processes are used for further purification of biologically sensitive products such as pharmaceuticals, diagnostic reagents, and research-grade enzymes. To isolate the desired compound, it will be necessary to carry out one or more chromatography steps, in sequence. The step using chromatography is the most expensive stage in product recovery.

The purification of the products by chromatography is based on the high selectivity of this technique. Separation occurs in a column, where a stationary phase (generally resin) is used to adsorb the product, which is then eluted with a mobile (liquid) phase. In chromatography, the partition coefficient α is a useful parameter. Thus in the relation

$$\alpha = \frac{q}{p_t}$$

where q is the concentration of matrix-bound protein and p_t is the concentration of protein in free solution plus the concentration of matrix-bound protein; α is affected by many factors.

The partitioning of a solute between a solid matrix packed in a column and the solvent is one of the most versatile methods for the fractionation of biological materials. The partitioning can be based on adsorption (called adsorption chromatography), molecular size (gel filtration chromatography), ionic charge (ion-exchange chromatography), selective adsorption using biospecific effectors (affinity chromatography) or molecular recognition (immunosorbent chromatography). By means of isoelectric focusing, proteins can also be purified by the use of pH gradients in association with ion exchange gels. In this process, called chromatofocusing, the proteins are separated according to their isoelectric points. The method is very good, although only small volumes can be handled.

Major advantages of chromatographic methods are characteristically high selectivity in separation and the mild reaction conditions. Table 1.23 shows various types of chromatography, which are distinguished according to the nature of the binding forces, the principle of separation, and the support materials.

In *adsorption chromatography*, separation is based on hydrophilic or hydrophobic interactions between the support materials and the biological compounds. Elution and fractionation are accomplished by higher or lower solvent systems or ionic strength. Support materials are inorganic substances such as silicates, hydroxyapatite, and alumina, or organic materials such as activated carbon and synthetic polymers (e.g., dextrans). Adsorber resins can also be used for the purification of proteins. Waters Associates and other companies make large-scale preparative systems that can handle up to 25 g or kilogram quantities of specific compounds.

In *ion-exchange chromatography*, the protein passes through a fixed bed containing ion exchange resin. An ion exchange resin is made up of an insoluble matrix to which charged groups have been covalently attached. One common cation exchange resin, carboxymethyl cellulose, often used for protein purification, is obtained by linking negatively charged carboxylmethyl groups to a cellulose backbone. Cationic (positively charged) proteins will bind to this resin by electrostatic forces. After protein has been adsorbed to the resin, the column is eluted with buffers of defined ionic strength at fixed or altered pH. Such charges in the carrier solution cause weakly bound proteins to detach from the resin first; more tightly bound molecules are induced to detach from the resin by increasing the salt gradient (ionic strength) of the eluting solution.

Ion-exchange chromatography is also used on the technical scale for the purification of antibiotics, enzymes and other proteins. In industrial applications of ion exchange, a large resin particle size is normally selected for high flow rates and for minimum pressure buildup. Finer mesh resins give higher resolution, but the increased pressure drop requires lower flow rates. Fast protein liquid chromatography (FPLC), developed by Pharmacia, permits outstanding separations of protein molecules on preparative or technical-scale columns with a volume of 300 L or more. Most typical ion-exchange resins are strongly acidic cations, weakly acidic cations, strongly basic anions, and weakly basic

Table 1.23 Different methods and characteristics of chromatographic processes

Process types	Binding nature	Separation principle	Support materials
Adsorption chromatography	Surface binding	Surface affinity	Silicates, hydroxyapatite, alumina, cross-linked dextrans, activated carbon
Ion-exchange chromatography	Ion binding	Charge	Cation exchangers (Dowex, Amberite, carboxymethyl cellulose, etc.) Anion exchangers (DEAE-cellulose, Sephadex, Sepharose, Mono Q series)
Gel filtration chromatography	Pore size	Molecular size, molecular shape	Biogel type, P and A, Sephadex and Sepharyl series
Affinity chromatography (or immunosorbent chromatography)	Biospecific adsorption/ desorption	Molecular structure	Biospecific effectors (enzyme inhibitors, monoclonal antibodies, metal chelates, protamin A, lecithins, etc.). Attached Sepharose or Sephadex series
Hydrophobic chromatography	Hydrophobic interaction	Molecular structure	Cyanogen bromide activated agarose, and so on.
Covalent chromatography	Covalent binding	Functional group (thiodisulfide exchange).	Agarose pyridine-2-yl disulfide, Sepharose–(glutathione-pyridine-2-yl disulfide) conjugate
Chromatofocusing chromatography	Net negative charge	Isoelectric point	Mono P

anions. These resins remove ions from solutions to form insoluble resin salts. Positively charged molecules are not bound by an anion exchange resin, whereas negatively charged molecules are retained.

Gel filtration chromatography is a separation technique based on the distribution of molecules between the external and internal solvents in gel particles with pores of a characteristic size. Molecules larger than this pore size cannot diffuse into the gel and pass directly through the column, while smaller molecules penetrate the gel, and their mobility is thus retarded from the solvent front. Relative molecular weight can be calculated from standard curves of known proteins. Gel filtration is used mainly to remove salts and to separate low molecular weight impurities at the industrial scale. On a small scale, however, gel filtration is used to fractionate and purify protein molecules.

Affinity chromatography uses a biospecific ligand that has been fixed covalently to an inert carrier and packed into a column to purify a molecule by highly selective adsorption and desorption. High molecular weight compounds such as enzyme inhibitors (e.g., Kunitz inhibitor for trypsin) and low molecular weight compounds such as substrate analogs (e.g., N^6-(6-aminohexyl) adenosine for dehydrogenases) can be used as group-specific adsorbents for the purification of biological compounds. Other examples

of very specific pair interactions include antigen–antibody, protein A, metal chelates, lecithins, and antibiotic bacitracin, which is a useful ligand in the purification of serine-, cysteine-, and metalloproteases from a crude mixture. The most expensive ones are protein A affinity resins, but because of the strong affinity for certain antibodies, high-yield protein A affinity, despite its cost, handles the largest, least-refined process stream (after clarification removes debris) that can deliver 99% purity in a single step. With the availability of monoclonal antibody, which is a single type of antibody produced from animal cell culture, the immunosorbent column separation form of affinity chromatography now makes it possible to purify a particular protein or other molecule with a very high specific binding constant. An example of a commercial application can be found in the purification of human leukocyte recombinant interferon produced in *E. coli*. As a result of highly specific binding, the affinity column can accept a continuous feed input until saturation is obtained. The antibody needed to make the immunosorbent column is a dominant cost and is feasible only for high value-added products.

Hydrophobic chromatography is based on an interaction between a hydrophobic matrix and corresponding hydrophilic regions in proteins and polypeptides. In aqueous solutions, protein binds to alkyl or aryl residues of the substituted agarose. Alkyl-substituted agarose is obtained from cyanogen bromide (CNBR)-activated agarose by treatment with primary amines with different chain lengths. The proteins adsorbed at a low ionic strength can be desorbed selectively with the buffer having increasing ionic strength. Since the separating power depends on the tertiary structure of the proteins, a special hydrophobic carrier must be developed or optimized, involving a high expenditure on development. The combination of hydrophobic chromatography with other chromatographies permits the production of biological compounds of high purity especially for use in the pharmaceuticals division or for diagnostic purposes.

Covalent chromatography is based on the interaction of specific groups. For example, the purification of urease is achieved by reciprocal thiol–disulfide exchange on an agarose pyridin-2-yl disulfide as water-insoluble effector. The enzyme is bound to a support through a covalent bond in the form of disulfide bridge and is eluted by low molecular weight sulfhydryl compounds such as dithiothreitol.

Chromatofocusing is a technique used for separating biomolecules according to their isoelectric points. By means of isoelectric focusing, proteins can be purified by using pH gradients in high resolution ion exchange on monobeads (e.g., Mono P, Pharmacia). Mono P is substituted with various tertiary and quaternary amines. The mechanism is based on the buffering action of the charged groups on the Mono P. A molecule has a net negative charge at a pH above its isoelectric point (pI), and any component with a net negative charge is retained on the column when the sample is applied. A polybuffer is used to titrate the column and the sample components. Titration of the Mono P results in the generation of a linear, descending pH gradient and yields focused zones of molecules with different isoelectric points. The proteins are separated close to their pI by a pH gradient. The resolving power of this technique is the most effective when sample components have a negative charge.

Recent bioprocessing separation uses disposable *membrane adsorbers* and monoliths, which function as a specialized form of tangential-flow filtration. These maintain high resolution and capacity regardless of flow rate and molecular size for shear-sensitive products such as DNA plasmids, live viruses and labile proteins. Also *expanded-bed adsorption and multimodal* (mixed mode) chromatography is used in a number of market-approved processes. Further downstream processes in bioindustry on crystallization and chromatography are discussed in Section 1.8.2.

Drying To eliminate water or solvent from a liquid material, heat must be applied to the material to be dried. However, for biological compounds it is essential to dry the final product without thermal damage. Heat transfer can be achieved either by direct contact, by convection, or by radiation. Some common dryers are commercially available for carrying out the drying with convection dryers (e.g., pulverizing, rotating, spray) and contact dryers (e.g., thin layer, chamber, drum). The freeze-drying (sublimation drying) process has gained more importance for pharmaceutical and biological products (live vaccines, serum, hormones, enzymes, vitamins). Storage operations are widely used in other process industries and therefore this process operation is not discussed in this chapter. In connection with the application of such operations dealing with biological compounds, however, two important requirements must always be stressed: first, the product should not be subjected to any harsh denaturation conditions by excessive overheating, chemical and biological contamination, or degradation; and second, the potential health hazards and dangers due to the explosive nature that is associated with dried proteinaceous dusts must be avoided.

Sterilization There are a very few sources of practical information on how to run large-scale equipment with a low probability of contamination. Thus in this section, we consider sterilization from a practical standpoint. The sterilization and prevention of contamination in fermentation is a good example of the heat transfer practice.

Sterilization is required for vessels, culture media, and air. Media sterilization is commonly achieved by moist heat in batch cycles. The design of sterilization procedures is based on the death kinetics of microorganisms. In the sterilization of vessels, however, cost-effective operation calls for the synchronous exposure to this cycle of the nutrient media, the vessel itself, the filters, and all connecting pieces and sampling devices. Typically, the *thermal death of microorganisms* is correlated by the first-order rate expression as shown in equation (1.1).

$$-\frac{dN}{dt} = -kN \tag{1.1}$$

where k is the first-order rate constant (min^{-1}), N is the number of viable organisms, and t is time of exposure to a given temperature. The decimal reduction time D is the time to reduce the microbial population by 90% or by 10-fold, $D = 2.303/k$. The rate constant k is actually dependent on temperature according to the Arrhenius equation, a form of which is shown in equation (1.2):

$$k = k_0 e^{-E/RT} \tag{1.2}$$

where E is the activation energy for thermal death of the microorganisms (kcal/g·mol), R is the gas constant (kcal/g·mol·K), T is the absolute temperature (K), and k_0 is an experimentally determined parameter (min^{-1}). E must be measured experimentally and represents the sensitivity of the microorganism to heat. All other things being equal, a large E indicates extreme sensitivity to heat, while a small E represents only slight sensitivity to heat and therefore a difficult sterilization. Typically, E ranges from 50 to 100 kcal/g·mol for vegetative cells and spores. Batch sterilization is designed on the basis of a certain percentage reduction in the number of viable cells, usually in natural logarithm or n cycles of death.

$$\ln \frac{N}{N_0} = kdt = k_0 e^{-E/RT} dt \tag{1.3}$$

In most cases, temperature is not constant; rather there is a heating up period, a constant temperature holding period (usually at 121 °C), and a cooling down period.

The sterilization level can be calculated from $k_0 e^{-E/RT}$ if an expression relating temperature to time is available.

Sterilization can be carried out by indirect heating of the medium by means of an internal or external heating steam coil, or by blowing direct steam into the medium. With direct injection of steam, the time of heating up is relatively short, but condensate accumulates within the fermentor and will increase the volume of the liquid. In indirect heating, the heating up time becomes considerably longer.

The drawbacks of the *batch sterilization* process are the extensive heating (2–3 h) and cooling (1 h) periods, while the holding period usually lasts for 30–60 min for the actual killing process at a temperature of 121 °C in a 3000 L fermentor. If the hot water obtained during cooling cannot be recovered, batch sterilization becomes very costly. Another disadvantage of batch sterilization is the extent of thermal damage to desirable components. Vitamins are destroyed, and the quality of the culture medium deteriorates as a result of undesirable browning reactions. For energy-saving reasons and to avoid undesirable reactions, *continuous sterilization* is always to be preferred to a batchwise process. Not only are savings of the order of 70–80% achieved when steam and cooling water are used, but the use of a higher temperature (135–140 °C) for a short time (5–8 min) contributes to gentle treatment of the labile components in the medium. For continuous medium sterilization, two distinct types of system exist – those employing direct steam injection into the nutrient medium, which necessitates extraclean steam, and those employing indirect heating by means of heat exchangers. There are some drawbacks to continuous sterilization – direct steam heating can add excess water to the medium; microorganisms may not spend identical amounts of time in the sterilizer; and the heat exchanger used for indirect heating or cooling can be fouled by suspended solids. In the fermentation industry, continuous sterilization is much less common than batch sterilization.

Filtration approaches may also be applied to media sterilization, but they are less reliable and not as well developed. Filtration approaches are, however, widely used in fermentation air sterilization, along with heating techniques. Air sterilization processes are required to remove microbes with dimensions as small as 0.5 μm from the vast volumes of air frequently required by aerobic processes. A fermentor having a working volume of 50 m^3 with an aeration rate of 1 volume of air per volume of liquid per minute (vvm) will need 3000 m^3 sterile air per hour. The critical importance of air sterilization on the industrial scale can be seen from these values. Among the methods available for sterilizing gases, such as filtration, gas injection (ozone), gas scrubbing, radiation (UV), and heat, only filtration and heat are used in industry. While depth filters such as glass wool filters are important historically, all filtration today associated with air sterilization relies on polymeric microporous membranes that use cartridge filters. Significant improvements in filter cartridge design have appeared within the last couple of years. Costs for air filtration depend on filter costs, pressure drop, power costs, and installation and removal costs. There is not yet an absolute filter for bacteriophage in industrial usage. Bacteriophage can cause total failure in the production of many bioingredients from microbial fermentation and of fermented foods with lactic starter cultures.

Recovery of recombinant DNA products Certain aspects of rDNA fermentation and protein recovery require careful examination not only during the initial cloning and expression, but also before process development is considered. The heterologous proteins expressed at high levels in recombinant *E. coli* often accumulate in the intracellular refractile bodies called IBs. These insoluble aggregates of highly cross-linked protein must be solubilized and renatured to obtain an active form. Methods of completely solubilizing the protein and refolding it in reducing conditions are now technically feasible but still expensive, unless

the product is high in value, like recombinant human insulin. A part of recombinant insulin and chymosin (rennin) in the IBs is present in so-called quasi-native form, in which the molecules are in the correct folded state but differ from the native form by the absence of cystine disulfide bond. Often the renaturation step used is oxidative sulfitolysis, or the dissolution of urea or guanine, a refolding and disulfide bond formation. Flow processes used in the production of recombinant chymosin from *E. coli* and *Kluyveromyces lactis* are shown in Figure 1.20.

The choice of microorganism, the available fermentation conditions, and the ability to select for secretion of the desired protein influence the choice of operations for recovery and purification of the product. Current examples of host microbes used for the production of recombinant chymosin are *E. coli* (Pfizer, USA to Chr Hansen), *K. lactis* (Gist-Brocades, Netherlands) and *Aspergillus niger* (Genencor, USA). *Aspergillus* could give the advantage of secreting high levels of enzyme. However, glycosylation, which is the addition of carbohydrate moieties to the protein, may require an extra step, namely deglycosylation.

Leader sequences from yeast proteins (pre-pro region of α-factor genes) were used in *K. lactis* to secrete prochymosin. The secretion of the desired protein should simplify

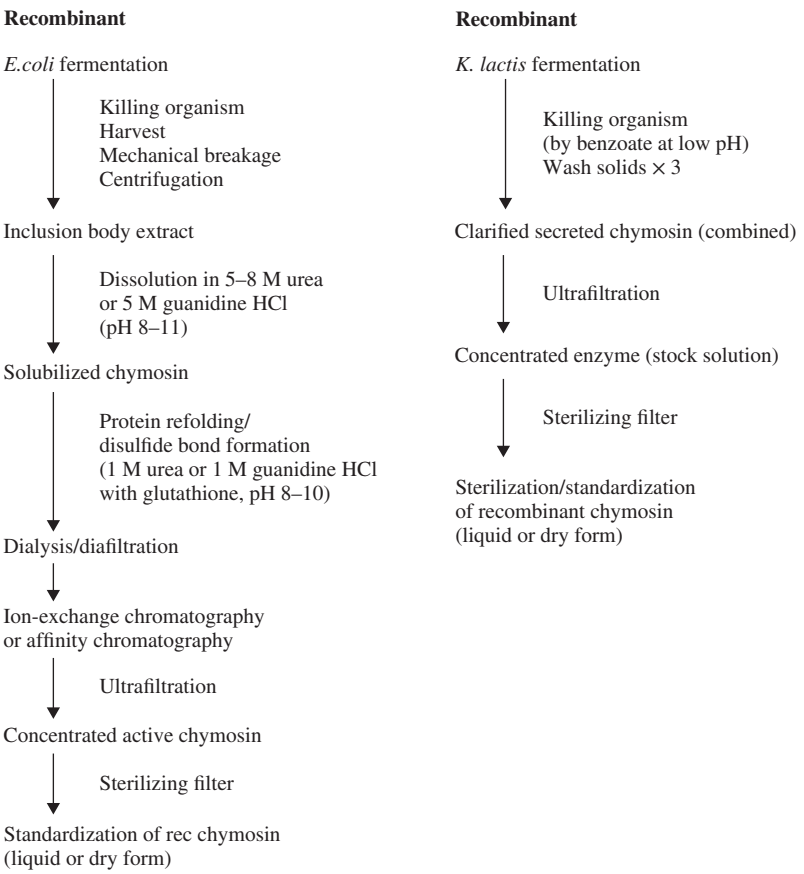

Figure 1.20 Flow processes in the production of recombinant chymosin.

downstream processing, but proteases are often excreted, which would cause severe product degradation. Prochymosin is inactive, and thus does not have proteolysis, and the prochymosin gene and a leader sequence were inserted under the control of the lactase promoter and terminator. To stabilize chymosin production, the expression vector (pUC19) was then integrated into the host chromosome at a site in the lactase promoter by recombination during DNA replication. The biochemical identity and functional properties of the recombinant chymosin were similar to those of the native chymosin. Then the production process was developed, using the existing lactase production protocol. Though a number of modifications were made to optimize the process, the general production scheme remained the same as for lactase production.

The release into the ambient atmosphere of genetically engineered microbes is not permitted in countries where such work is performed; therefore, the recombinant microbe must be inactivated before the fermentor is opened. Benzoic acid is used as a preservative for rennet products, and the low pH of the mixture needed to inactivate the cells also facilitates the autocatalytic conversion of prochymosin to chymosin. The specifications for avoiding microbial contamination of chymosin – namely, absence of any genetic information (DNA) that could be carried over, stability, and absence of β-lactamases, which inactivate the effects of antibiotics – have been met. Once the biochemical identity of recombinant chymosin had been established, the three main regulatory regimes were met, the process was carried out on a large scale, and the criteria for the demands of legislative procedures and cheese trials were verified. Furthermore, the large-scale protocol and equipment had to be suitable for use with recombinant strains. Also, the survival of the *K. lactis* strains was compared with the host strain in a number of soil and water samples. A pathogenicity study was performed with laboratory animals (e.g., mice), administering different doses by various routes, to see if the GM *K. lactis* caused any infection.

No special problems were encountered in the scale-up of the production process, since it was based on the commercial production of lactase. Besides the evidence of nontoxicity of the final product, as evidenced by acceptable levels of mutagenicity, acute oral toxicity, and allergenicity, as well as passing results from feeding trials, functional properties and cheese manufacturing trials had met. The product was then ready for commercial operation and for introduction in the market. When the genetically engineered microorganism was capable of producing chymosin on a large scale, a new source of rennet was available commercially. The recombinant microbial rennet was completely identical to calf chymosin. It is available from a virtually unlimited source, and it will eliminate the need to slaughter suckling calves. Although the absence of any health hazard to consumer had been proven, in most countries additional standards had to be met.

In addition to these legal issues, the dairy industry had to be persuaded to accept the recombinant product. Since the food industries are very conservative, this tends to be a difficult task. Once the benefits of recombinant chymosin on cheese quality and production costs are recognized, it will be a matter of time before the product is widely used. The safety issue on rDNA technology is dealt with in another chapter.

1.7.6 Scale-up and scale-down of fermentations

There are basically two ways to develop a new bioprocess: the *scale-up* and *scale-down* methods. Although the *scale-up* method is the most common one to develop a new process, drawbacks of the scale-up method exist in which the pilot plant dimension, primarily those of the reactor, may not be appropriate for the commercial process. Also, this does not include the development of a kinetic model, nor does it include simulation of the commercial-scale process. In the scale-down method, a simulation of the commercial

design exists prior to the design of the pilot plant. Simulation involves many parameters and the pilot plant is then designed to improve the estimates of the most important and least well-known parameters.

Scale-up is simply the conversion of a small-scale process developed at a laboratory to an industrial scale. The term "scale" refers to either the volume or the linear dimensions of a fermentor. It is seldom feasible to apply fermentation conditions that have worked in the laboratory directly on a commercial scale. This is because success in scale-up is evaluated on the basis of maximal yield in terms of the minimal operating cost and time. The information obtained in small units is not adequate for large-scale design, since the fluid dynamics, transport processes, and even the behavior of the cells may be changed considerably as a result of an intensification of turbulence when the size of the unit is increased. Ideally, the geometric enlargement of a reactor should be known. In the absence of this information, however, pilot plants must be constructed that subdivide the large step of this scale-up procedure between laboratory and production units to reduce the risk involved in the design of the production unit.

Most of the following information is obtained from small pilot plants; only a few results from industrial-scale plants have been published. Although many equations employing physical parameters have been derived and evaluated for use as scale-up criteria, there is no general formula because of the variation from one fermentation process to another. The most useful information concerning scale effects has been obtained using a series of geometrically similar fermentors. In reality, however, scale-up is not usually achieved with geometrically similar fermentors in laboratory, pilot plant, and industrial settings. The problem more often is do with scaling down from the production plant to carry out experiments on a laboratory scale under conditions of existing large tanks.

Scale-up is a highly interdisciplinary task, requiring the integrated use of concepts and methodologies of both biochemical engineering and microbial physiology (Figure 1.21). At the small laboratory stage, more microbiologists and biochemists are involved and data produced at this stage can be the high risks, but above the pilot plant stages, engineers are more involved and data produced at this stage can be more trusted for commercial production. This section is not intended to give an in-depth study of critical parameters; rather, it provides a general outline of scale-up procedures together with a description of the relevant techniques. The main parameters affected by scale are agitation and aeration, heat transfer, medium sterilization, and process monitoring and control. Since we have already discussed most aspects of fermentation parameters in mass and heat transfer practice, we discuss briefly the subject of agitation (or stirring) before turning to the common scale-up methods. Other biological factors (culture variability, medium components, water quality, selection of mutants, etc.) can bring different yields at different locations with similar equipment. In batchwise processing, the viscosity increases as the cell mass increases, and this is particularly important in mycelial fermentations used in antibiotics and enzymes.

Vigorous mechanical mixing of air–liquid dispersions is often necessary to obtain economic rates of biomass production, substrate consumption, or product formation. The agitation or stirring brings about homogenization of the culture and nutrients by bulk mixing, dispersion of air and immiscible liquids in the nutrient solution, and promotion of interface mass and heat transfer. All these functions require mechanical energy, which is supplied by the motion of impellers of various type and is eventually dissipated as heat. Various zones with different mixing intensity and fluid shear rate are often found in large fermentors. This nonuniform mixing leads to gas–liquid (O_2, CO_2), and liquid–solid mass transfer problems. Stagnation zones also increase lag time in process-control sensors as a result of local concentration gradients. The optimum mixer design for gas–liquid mass transfer

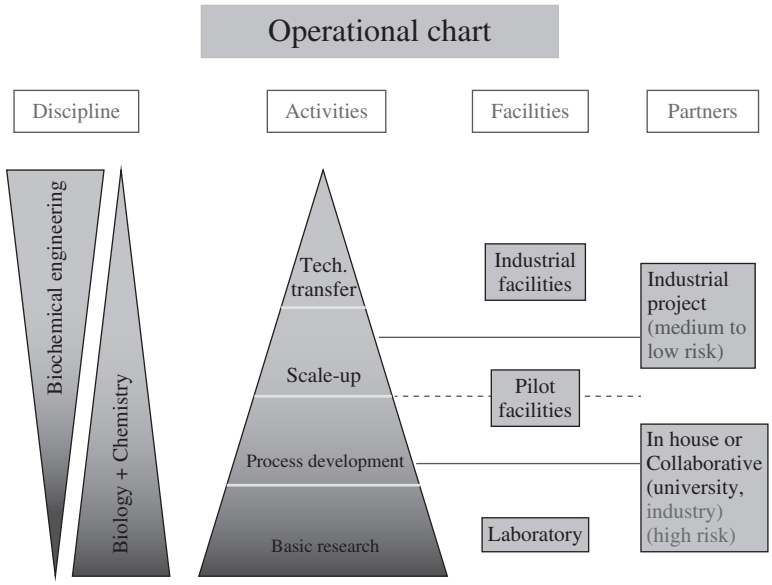

Figure 1.21 Scale-up requiring a highly interdisciplinary task, concepts and methodologies of biochemical engineers, microbiologists, and chemists.

could often turn out to be disastrous in terms of shear rate and blending of relatively fragile mycelial fermentations. In general, high-shear, low-flow impellers are inferior to low-shear, high-flow impellers. Large tanks often have high-shear rates and lower pumping capacity than small tanks (Figure 1.21).

The ability of impellers to transfer energy to a liquid is usually expressed in terms of the *power number* (*Newton's number*) N_p as follows:

$$N_p = \frac{\text{imposed force}}{\text{inertial force}} = \frac{P_o}{N^3 D_i^5 \rho}$$

where P_o = stirring power (kW)
N = stirring speed (s^{-1})
D_i = stirrer diameter (cm)
ρ = density of the medium (g/cm^3)

The power number has been correlated with the Reynolds number for several types of stirrer. The *Reynolds number* is a measure of the turbulence of the system and expresses the ratio of inertial to viscous forces in the liquid:

$$N_{Re} = \frac{ND_i^2\rho}{\eta}$$

where N = stirrer speed (s^{-1})
D_i = stirrer diameter (cm)
ρ = density
η = dynamic viscosity (g/cm · s)

The Reynolds number describes the flow only at the periphery of the stirrer. The general relationship between N_p and N_{Re} has the form:

$$N_p = K(N_{Re})^{-m}$$

where K is a constant dependent on the container geometry and the shape of the stirrer, but not dependent on the reaction size, and $m = 1$.

With laminar flow, the power required for stirring is not dependent on the density, but is correlated with fermentation parameters. In the turbulent flow range of mixing speed ($N_{Re} > 10^4$), the power number is independent of the Reynolds number.

Scale-down to a lab scale bioreactor is often necessary to obtain more consistent process performance during the scale-up. Creation and qualification of scale-down models are essential for performing several critical activities that support process validation and commercial manufacturing. A key parameter that frequently limits a scale-down aerobic fermentation model is the volumetric oxygen mass transfer coefficient (K_La), which is proportional to the rate of oxygen transfer from rising gas bubbles to the cells. This approach can be coupled with a small-scale experimental design to more accurately predict and optimize larger-scale fermentation conditions before the actual process transfer (growth and production temperature profile, feed rate, air/O2 control scheme, induction cell density, etc.).

1.7.6.1 Scale-up methods A constant aeration and agitation regime can hardly be regarded as an optimal scale-up factor, since culture requirements and rheological properties of the fermentation broth differ substantially at different periods of the fermentation. A constant oxygen transfer rate, value K_La is the most commonly used method because K_La relates well with process results such as yield and titer of the products. Neither the fermentor type nor the volume was a crucial scale-up criterion, but there was good agreement between K_La values and yields using small (6–15 L) and larger (100–3000 L) fermentors. However, the K_La value cannot be used in highly viscous non-Newtonian fermentation solutions, which have been found in many antibiotic fermentations, or under conditions of high speed stirring. The region for effective scale-up is most likely to be before foaming, mechanical damage and product inhibition can occur. Another simple method is based on the power consumed per unit volume (P/V) in aerated bioreactors. The desired prerequisite for using P/V for scale-up is a geometric similarity that is rarely attained. For geometrically similar tanks,

$$P_g = K\left(\frac{P_o^2 N D_i^3}{Q^{0.56}}\right)^{0.45}$$

where N is the rotation speed of the impeller, D_i is the impeller diameter, and K is a function of broth characteristics. However, power usually measured by wattmeter is not a true measure of power transferred to liquid; also, power requirements change over the course of

Table 1.24 Type of data required from laboratory and pilot experiments for effective scale-up into production

Oxygen uptake data related to product formation
$K_L a$ (volumetric oxygen transfer coefficient, h − 1)
Dissolved oxygen tension
Growth rate
Nutrient uptake data related to product formation
K_s (substrate specific constant)
Growth rate
Viability of environmental parameter rate constants
pH
Dissolved oxygen tension
Specific rate data
Temperature
Fluid characteristics during the course of fermentation
CO_2 effects (including back pressure)
Definition of geometry for estimation of mixing behavior
Definition of pilot plant control system
Estimation of cooling requirements

a fermentation as viscosity and aeration requirements change. Other physical methods have been tried, but impeller tip velocity representing liquid shear, mixing time, and Reynolds number of stirrers are less useful physical parameter than others.

In executing scale-up and scale-down of biological reactors, one should pay primary attention to the microbial physiological response, disregarding the geometrical similarity between the original vessel and the replica. The $K_L a$ is well related to productivity and biological parameters. The main data required from laboratory and pilot plant experiments for effective scale-up into production are shown in Table 1.24. Generally, a 1:10 ratio is used in scaling fermentation vessels, as shown in Figure 1.22. There are several advantages associated with 1:10: (i) each vessel can be used to adequately seed the next larger scale vessel; (ii) within the pilot plant, a minimum of two log orders of scale-up can be evaluated for effects or trends on process performance and control; and (iii) a maximum of one log scale-up to production is possible, to minimize scale-up risks. Scale factors in translation of pilot plant data into production are also summarized in Table 1.25.

1.7.6.2 Bioinstrumentation and computer control

Bioinstrumentation Process monitoring and control have become essential elements in the optimization of fermentation processes that call for the performance of measurements during fermentation for data analysis and subsequent control of the process. The basis of understanding and controlling a biological process is the data obtained from the biosensors and instrumentation employed. Table 1.26 lists various physicochemical sensors that have been developed for monitoring fermentation parameters. The biological parameters, with the exception of the $NADH_2$ online measurement using a fluorescent method, must all be measured outside the fermentor. We will thus concentrate on instrumentation for online physical and chemical parameters for fermentation processes. A key limitation for complete bioprocess monitoring is the inability to measure accurately and quickly many variables online.

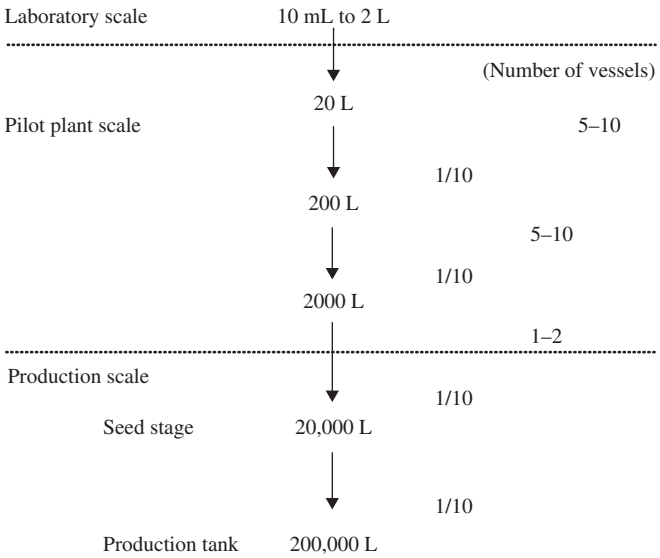

Figure 1.22 Scaling fermentation vessels by the 1:10 ratio method.

Table 1.25 Scale factors in translation of pilot plant data into production

Pilot plant	Factor	Production
Small	1. Sampling errors	Significant (nonuniformity)
Small	2. Process control response time	Large (nonuniformity)
Uniform	3. Agitation	Large (nonuniformity)
Easily controlled	4. Aeration/power input	Limitations of compressor capacity, peak load times
Difficult	5. Sterility (continuous feeds, inoculation port, valves, antifoam vessels)	Less difficult due to steam seals, jackets
Easily controlled	6. Water, steam, air quality	Difficult to control
Easily controlled	7. Heat transfer (cooling water)	Often limiting
Good	8. Reproducibility	Variable

1.7.6.3 Physical measurement The major physical process parameters that influence cellular function and process economics are temperature, pressure, agitator shaft power, impeller speed, viscosity, gas and liquid flow rates, forming, tank volume/weight ratio, and heat generation.

Temperature is the most important parameter that is measured and controlled in most fermentation processes. Depending on the dimensions of the fermentor and the desired accuracy of regulation, thermistor (semiconductor resistance thermometer), metal resistance thermometer (nickel or platinum), or thermocouple controls are most commonly used. A metal resistance thermometer, which has the advantages of stability and linearity of response, is used in most fermentors today. In many large fermentors temperature is

Table 1.26 Basic process control parameters that can be measured in fermentation processes

Physical	Chemical	Biological
Temperature	pH	Cell concentration
Pressure	Dissolved O_2	Enzyme activities
Power consumption	Exit O_2/CO_2 concentration	DNA and RNA
Agitation speed	Redox potential	NADH
Viscosity	Substrate concentration	ATP
Air–liquid flow rate	Product concentration	Protein content
Turbidity	Ionic strength	–
Volume/weight of tank	–	–
Form detection	–	–
Heat generation	–	–

controlled by cooling water or by cooling water and steam as the heating source if necessary. Pressure monitoring is important during sterilization. Diaphragm gauges are usually used to monitor pressure. Pressure can easily be controlled by using regulating valves in the exhaust air line.

For large fermentors, a wattmeter measures the total power drawn by the agitator motor. A torsion dynamometer may also be used to measure shaft power input. The online measurement of viscosity and other rheological properties of culture broths can be quite difficult, primarily because of the non-Newtonian characteristics and solids content of most fermentation broths. One possible method is to measure power consumption at several different impeller speeds. Also, a dynamic method has been proposed in which shaft power input is monitored during and after a brief shutoff in agitator drive power. Most apparent viscosity measurements are performed offline using a Couette or Brookfield viscometer. A common method for measuring the flow rates of gases (air feed, exhaust gas) is the use of variable area flowmeters such as rotameters. Large gas flow can be measured by an electrical turbine flowmeter or by laminar flow. Thermal mass flowmeters with controller are increasingly popular for measuring and controlling airflow in laboratory and pilot plant fermentors. The control of the flow of liquid media is difficult because of the need to maintain sterility. Liquid flow rates can be monitored with electromagnetic flowmeters or capacitance probes, but most of these devices are expensive. The automatic measurement of predetermined volumes has proved satisfactory. Foaming is a common problem in many fermentation processes. Foam can be detected by either a capacitance or a conductivity probe. *Foaming* is controlled by mechanical foam destruction devices using single, rotary, or multiple rotating plates, ultrasonic irradiation, or the addition of sterile antifoam agents. The silicone-based antifoam agents are the most commonly used in the fermentation industries.

1.7.6.4 Chemical measurement The pH of a fermentation is commonly monitored with a steam-sterilizable pH electrode (combined sensor) connected to a pH meter for direct visual readout. Computer-based direct digital control (DDC) has replaced the analog titrator in many recent fermentation operations. Electrodes that can be repeatedly steam-sterilized in place are available for pH, redox potential (E_h), and dissolved O_2 and CO_2 partial pressures. The partial pressure of DO in the broth is found with either galvanic (potentiometric) or polarographic (amperometric or Clark) type electrodes,

which measure the partial pressure of the DO and not the dissolved O_2 concentration. A similar CO_2 probe produced by Ingold determines pCO_2 by measuring the pH of a standard bicarbonate solution, which is separated from the fluid by a gas-permeable membrane.

Methods for the online assay of volatile components and dissolved gases are also available. Several biosensors based on coupling the action of immobilized enzymes or cells with an analytical device can detect a particular product of the biocatalytic reaction. Also enzyme thermistors can be used for the detection of heat released by the enzyme-catalyzed reaction. Some of the compounds and ion species that have been assayed by immobilized thermistors or electrodes using immobilized enzymes have been mentioned (see Table 1.15).

A concern in the use of any biosensor employing enzymes, cells, or other biochemicals is, of course, deactivation of the sensor during reactor stabilization. Also, membrane fouling by cells or medium components and external mass transport resistance can cause shifts in calibration of the sensor.

Other exhaust gases such as CO_2, O_2, CH_4 (in anaerobic methane generation), and H_2 can be indicative of fermentative metabolic status. CO_2 content in fermentation gas streams is most commonly monitored using an infrared spectrophotometer, online process gas chromatographs; process mass spectrometers are being used to simultaneously measure O_2 and CO_2 contents of the inlet and outlet air streams in fermentations. Gas chromatography can continuously analyze several compositions of exhaust gases (O_2, CO_2, CH_4, H_2) and volatile components (ethanol, acetaldehyde, etc.). A gas semiconductor system is advantageous and changes its electrical conductivity when it comes into contact with combustible gases or organic vapors of volatile substances.

1.7.6.5 Biological measurement The reliable online determination of the number of cells or their weight is still the most difficult problem facing biotechnology. Growth is normally measured by determining the increase in the amount of dry matter (biomass) or in the number of cells. These two quantities do not necessarily correlate in a constant manner. Dry weight measured by drying overnight at 105 °C is accurate and comparatively cheap, but overestimates due to foreign compounds that cannot be washed out (e.g., precipitates of insoluble salts, solid constituents of the medium) are a hazard. Wet weight can be measured by filtering the cells; but all procedures for determining cell mass require a relatively large amount of manual work and cannot be automated without considerable expenditure. Measurements of the number of total cells by microscopic counting or the Coulter counter are very labor intensive. The number of live cells can be determined by plating out the culture and occasionally by specific staining. Photometric measurements (turbidometry) rapidly and simply yield results that can be correlated with growth. However, accuracy is lower as cell density and number of air bubbles increase and as solid constituents of the nutrients interfere.

More suitable methods developed recently are the flow cytometer or fluorometer. In *flow cytometry*, the cell sample stream is irradiated by a laser or other light source such as fluorescence, and light-scattering measurements are used to obtain information on the cell size distribution. Flow cytometry can be used to detect intracellular morphology and macromolecular composition by using a specific fluorescent dye, and to take simultaneous multiple measurements on individual cells. When the culture in a fluorometer fluoresces through a fluorescent bulb, the culture light passes out through the quartz window and through a second set of filters to a photomultiplier tube. The signal from the photomultiplier is amplified and recorded. ATP levels also change rapidly as a function of cellular metabolic activity, and thus cellular ATP content can be measured ascertained by means

of a *bioluminometer*, which measures luminescence produced by a reaction requiring ATP and the enzyme luciferase. *High resolution nuclear magnetic resonance* (*NMR*) measurements of ^{31}P have been used to determine intracellular ATP, ADP (adenosine diphosphate), sugar, phosphate, polyphosphate, and pH simultaneously. It is beyond the scope of this text to describe the individual parameters in detail.

1.7.6.6 Computer applications The automatic acquisition of data, data analysis, and the development of fermentation models using computers can be applied online for improved process control and optimization. Although the application of computers to process control is widely accepted in many firms in the chemical and petroleum industries, it has not yet been used widely in biotechnology. There are two major reasons for this slowness in development: (i) it is difficult to evaluate approaches to the reduction of fermentation cost using computers because the very complexity of the fermentation processes has prevented the full understanding of biosynthesis and the regulation of metabolic formation, and (ii) suitable biosensors are not yet available in sterile systems to measure the important variables to take advantage of computer capacity.

Data that are shown in Table 3.8 can be acquired directly at the fermentor with online sensors, and large quantities of measured results can be stored in the digital form for convenient access, analysis, and display at a later time. An alarm system can also be hooked up to the data acquisition system to inform an attendant that a failure of a system part has occurred and possibly also to initiate countermeasures. In data analysis, the data measured can be combined, and quantities such as oxygen utilization rate and respiratory quotient, yield coefficient, heat balance, and productivity can be analyzed instantaneously. When biomass is not continuously measured, the biomass yield can be calculated through the O_2 uptake rate. Measurements of inlet and exit gas flow rates and composition can also be used to calculate the average volumetric mass transfer coefficient, $K_L a$. Online estimation of biomass concentration and of specific growth rates during fermentation has been a central aim of data analysis. By using mathematical models, it is also possible to improve our knowledge of the process, which in turn permits the evaluation and optimization of conditions and strategies. A large number of models exist for batch and continuous fermentation, but each model is valid only for a certain specific process; none can be used universally.

1.7.6.7 Process control There are many advantages and objectives of computer-controlled fermentation processes. In fermentations for which substrates are the major costs, monitoring and controlling of raw material addition and total energy consumption can become far more cost-efficient. Computer-aided process optimization can also maximize volumetric productivity, product concentration, and conversion yield of substrate to product. Other potential advantages of computer-aided fermentation control include quick and efficient data management, storage and reproduction. Computer control can be divided into two kinds: digital set point control (DSC) or supervisory computer control (SCC), and DDC.

In *DSC*, regulation is comparatively slow but is adequate for many biotechnology processes. The presentation of essential elements of analog regulation is particularly advantageous in the initial phase of the incorporation of process computers. The measuring, controlling and regulating devices already present can be used. In the case of failure of the computer, a return can be made directly to the conventional management of the process. It is also possible to work first with the comparatively simple software, which specifies only desired values. The environmental variables are controlled by local single-loop controllers, and the environmental controller set points come from a digital computer.

Common practice in *DDC systems* is simultaneous use of several controllers, which can offer the flexibility of manipulating more than one process input to achieve control. For example, measurements of DO level and exit gas O_2 concentration allow online estimation of $K_L a$, whereupon it becomes possible to manipulate the agitation rate and/or the gas feed rate to control DO at the desired level. DDC requires digital-to-analog converters that possess variable outputs, and new strategies and algorithms of regulation in which the process takes place continuously, while digital computers operate discretely. In other words, data acquisition and signal output take place only at discrete points in time.

The software program in the computer commands the fermentation operation. Good software development can be the major task, and these costs are a significant part of the expense of installing a process computer. Recent downward trends in hardware prices certainly reinforce this area of research. An important consideration in selecting a hardware–software system is its ability to do multitasked operations (i.e., to run programs simultaneously). There are two different types of computer program: (i) utility programs, which start up the system and create files, and language programs to permit use of high level languages (FORTRAN, BASIC, APL, COBOL, etc.), and (ii) applications programs for accomplishing particular computations and other tasks.

The application of computers to the operation and maintenance of batch and continuous reactors is essential to implement controls or to do the calculations necessary to determine the desired control strategy. However, the number of sensors available to measure such key variables as cell concentration and product concentration is quite limited. Multivariable control and dynamic optimization so far have limited utility for most fermentation processes. New and robust sensors will have to be developed before more widespread automatic control of fermentation processes is available. Recent advances in molecular genetics and cell physiology may help us to develop more accurate process models to define various process controls. Operating strategies for recombinant microbial fermentations may closely resemble those for secondary metabolites, although the biochemical processes involved and their regulation are much different. When manufacturing a recombinant protein, it is usually best to delay the expression of the product until a suitable culture growth has been caused to occur, either by adding an inducer or by depleting an inhibitor of gene expression. Early expression of the product often inhibits cell growth and may accentuate any genetic instability problems.

1.7.6.8 SCADA control system Recently systems for controlling process variables applicable to a bioprocess have become increasingly sophisticated. These systems frequently employ digital systems such as programmable logic chips (PLCs), micro-processor based software control systems, or a hybrid arrangement. Advancements in processors, communication hardware, protocols, and archival software systems have transformed the concept of data management during bioprocessing from a luxury to a necessity.

The advent of sophisticated digital systems has given the bioprocess engineer the capability to repeatedly apply the same complex series of actions to any bioprocess. This has enabled large molecule pharmaceutical manufacturing to move toward the level of reproducibility that semiconductor processing now enjoys. Additionally, the use of digital systems to implement *supervisory control and data acquisition* (*SCADA*) now allows a smoother path to satisfying the requirements of *good manufacturing practise* (*GMP*) doctrines as well as *US Food and Drug Administration* (*USFDA*) requirements.

SCADA is a kind of software application program used for process control and gather real time data from remote locations for exercising this control on equipments and conditions. The SCADA System consists of hardware and software components. The hardware

collects and feeds data into a computer that has SCADA software installed in it. The data is then processed by the computer before presenting it in a timely manner. The function of SCADA is to record and log all events in a file that is stored in a hard disk or send them to a printer. If conditions become hazardous, SCADA sounds a warning alarm.

Input/output signal hardware, network, human machine interface, controllers, database, communication and software constitute an SCADA system. There are real-time automated and integrated control systems like its cooling by the computer itself for quick responding to the process changes within the processes' own time-frame.

New Brunswick Scientific (*NBS*) has developed three next-generation BioCommand® software packages designed to enhance your ability to monitor and control your fermentation and cell culture processes through the personal computer (PC). Three distinct BioCommand® packages are offered, providing the tools needed for research, optimization, and if needed, the security and audit trails to meet the regulatory requirements. BioCommand® Track and Trend, Batch Control and Batch Control Plus SCADA software packages provide (i) automatic data logging, (ii) remote monitoring and control capabilities, (iii) ability to monitor and supervise several fermentors and bioreactors from a single PC, and so on. This SCADA system for both reusable and single-use bioprocess applications can trigger different actions automatically based on events.

1.7.7 Scale-up challenges

Although many correlations and relationships have been established for the scale-up of chemical processes, these equations are not always applicable to their bioprocessing counterparts due to the nature of living systems and the differences in the principles of unit operations and unit processes between chemical and biochemical processes. The scale-up and optimization of the complicated biochemical activities of microorganisms and animal and plant cell systems present engineering challenges that are sophisticated and difficult. Many widely used fermentation processes were successfully scaled up on the basis of a constant volumetric oxygen transfer coefficient ($K_L a$) and power consumption per unit volume (P/V). The use of traditional empirical methods, such as P/V leads to an increase in mixing and circulation times at large scale. In addition, high oxygen demands and high viscosity can cause concentration gradients in oxygen, shear and pH, which can have a significant impact on fermentation yield. Therefore, the choice of scale-up criteria is not an easy task, given the potentially sensitive and diverse responses of cells to each of the transport phenomena influenced by impeller design, system geometry, scale, fluid properties and operating parameters.

The fed-batch, which is the high cell density cultivation of microbial strains, is the preferred industrial method for increasing the volumetric productivity of such bacterial products as nucleic acids, amino acids, and heterologous recombinant proteins. This type of feeding regime avoids problems associated with catabolic regulation, oxygen limitation, and heat generation that can occur during unlimited batch processes. Importantly, the buildup of toxic concentrations of metabolic by-products via so-called "overflow" metabolic routes can also be avoided. Overflow metabolism has been reported for *S. cerevisiae* as well as for *E. coli* and occurs at glucose concentrations above 30 mg/L. For *S. cerevisiae*, overflow metabolism is known as the "Crabtree effect" and the inhibitory by-product is ethanol but produced in a similar way to acetate in *E. coli*. In batch fermentation, overflow metabolism can be avoided by the use of a slowly metabolizable carbon source such as glycerol, but the preferred method is the use of a fed-batch process where growth can easily be controlled by substrate feed rate.

The scale-up challenges are compounded when the process involves batch fermentation. Due to the typical fragility of the engineered microorganisms, large-scale fermentation vessels must be designed with the ability to (i) remove the heat buildup that results from metabolic processes; (ii) manage agitation and mixing with minimal shear damage; (iii) effectively control the highly variable liquid flow rates and turn downs that are associated with batch fermentation; and (iv) execute safeguards and sterilization techniques to guard against potential contamination.

The engineering challenges are more acute when the fermentation process is used to make low-priced commodity chemicals including alcohol, amines, acids, solvents and surfactant, and so on. Because such products are not higher-value specialty chemicals, food additives and biopharmaceuticals, their production facilities often are forced to make engineering and bioreactor design trade offs for reducing cost constraints and leaner profit margins.

One of the most commonly made mistakes during the design of large-scale fermentation processes is the failure to adequately integrate the experience, expertise, and proven techniques developed by the pilot-plant engineers, facility microbiologists, and chemists into the criteria for the overall flowsheet, equipment specifications, process and instrumentation diagrams, and waste-handling systems. During the specification of commercial-scale equipment and controls, it is crucial to study and adapt the administrative and manual tasks generated during pilot-scale operations related to closed-vessel policies, material handling, cleaning, waste handling, and other operational aspects. A well-integrated team approach, with a common project view of the need to balance cost constraints against sterility needs, is essential.

While fermentation-based syntheses were once reserved for producing high-value specialty chemicals and biopharmaceuticals, commercial-scale bioprocess facilities are already producing vaccines and therapeutic pharmaceuticals (such as Amgen's Epogen and Wyeth's Mylotarg), food products (L-phenylalanine, a building block for NutraSweet) and food-grade additives (such as the algae-derived fatty acid DHA (docosahexaenoic acid) and ARA from Martek Biosciences, which is used as a nutritional additive). The development of large-scale production of recombinant therapeutic proteins with bacterial and mammalian cell cultures, supplanting older techniques for extracting proteins from blood and making vaccines in animal tissues as well as launching a whole new category of magic-bullet medicines: monoclonal antibodies. Other specialty and commodity biochemical facilities in BP and DuPont are teaming up to commercialize bio-based butanol as a gasoline blendstock in 2007. Genomatica and DuPont Tate & Lyle Bio Products successfully produced 1,4-butanediol (BDO) on a commercial scale in 2012.

Additional challenges may arise because emerging synthesis routes often exhibit a high degree of change throughout the scale-up and design stages. Also the design team must anticipate and manage changes to the design and construction specifications to minimize costs and keep the project on schedule.

Biomanufacturing is also becoming increasingly data dependent. The IT systems that support quality and process analytics, document manufacturing results, and store records for regulators share the legacy issues of all IT systems.

When producing pharmaceuticals and food additives, biosafety issues must be considered as product contact streams are regulated by the USFDA as well as by the US Department of Agriculture (USDA) in the case of biosynthesis. The US Environmental Protection Agency (USEPA) and the National Institutes of Health (NIH) have also issued guidelines for handling many of the commercial microorganism strains. In addition, in the United

States, Toxic Substances Control Act regulations establish procedures for commercializing new or modified strains.

The genetically or pathway-engineered organisms must be out of the surrounding environment. While such improvements are worthwhile, they may necessitate changes in equipment or utilities. For example, increased metabolic rates can enhance throughput, provided the higher heat generation can be controlled within the required temperature band, and agitation and delivery systems are sufficient to deliver the needed nutrients and oxygen to the more quickly multiplying organisms. While in all commercial-scale bioprocess facilities, (i) the upstream biosynthesis operation calls for rigorous sterility requirements, (ii) the downstream portion employs specific engineering unit operations to extract and purify the target product, and appropriately dispose of all waste streams. The particular engineering requirements and challenges associated with each of these two distinct portions differ, but must be tightly integrated during process design to ensure the most-cost-effective plant operation.

During fermentation, each of multiple fermentation vessels required by a commercial-scale facility will have its own particular design and operating requirements. These include the need to introduce the fermentation broth, sterile air, and sterilized nutrients. When air lift in the vessel cannot provide sufficient mixing, the fermenter may be equipped with low-shear agitation devices. Fermentation vessels must also be designed to ensure adequate heat-removal capabilities and promote cooling as needed. Sufficient safeguards must also be in place (both through design elements and operating procedures) to guard against contamination and cell mutation by double-block and bleed valves, and steam-in-place (SIP)/clean-in-place (CIP) systems.

Meanwhile, the variable flow rates associated with different stages of the cells metabolism and growth cycle, and the required cleaning cycles have tremendous design implications for process parameters including flow and pressure. All of these factors complicate the internal geometry in terms of baffles and agitators of the vessel, as well as the number, location and type of tank nozzles and ports needed.

Commercial-scale fermentation vessels must also be equipped with a variety of advanced instruments, sensors, and transmitters to monitor everything from pressure, level, and temperature inside the fermenter to pH, DO, and nutrient levels in the fermentation broth. The appropriate number and location of the analytical instruments and in-process checks must also be reconciled against capital and operating cost constraints, and sterilization concerns.

When the desired product ends up in the fermentation broth (excreted from the microorganisms or within the cell body), cells are destroyed; the product is separated and purified; and the dead cell bodies, unreacted carbohydrate feedstock/nutrients and by-products are removed, concentrated and neutralized before disposal. The dead cell bodies and other solid waste, and the high biological oxygen demand (BOD) aqueous streams produced throughout the process must be disposed of properly. The specific handling and disposal requirements are ultimately dictated by the biosafety classification of the microorganisms in the waste stream. Residual high-BOD aqueous waste streams are typically treated in onsite aerobic or anaerobic digesters. While aerobic digestion is economically applied for BOD up to 10,000 ppm, anaerobic digestion is generally used from 8,000 ppm and up.

Several issues that further deserve particularly close attention during the design and construction of large-scale bioprocess facilities are (i) the extreme sensitivity of the modified fragile organisms creates unique design and operating challenges, particularly when it comes to maintaining close control over all of the critical operating parameters within vessels; (ii) contaminants by phage infections and mutations within the bioengineered organism population, can lead to the disposal of the valuable fermentation batch and an

immediate shutdown for sterilization and cleanout. Designing systems that adequately filter airborne contaminants and bacteria, and remain dried (to avoid entrained condensate carrying bacteria through filters) requires design rigor that is crucial for facilities operating in hot, humid climates; (iii) commercial-scale fermentation facilities handle enormous volumes of water and steam (with varying composition and temperature) from fermentation, purification, evaporation and cleaning systems and thus recycle of water and energy reuse must be maximized; (iv) bioprocesses require removal of large volumes of water, and thus considerable operating savings can be realized by opting for today's highly efficient separations technologies, such as evaporation with mechanical vapor recompression and multiple-effect evaporators; (v) proper design of the waste-disposal facilities can also help to contain operating costs by further drying using standard press, plate, belt or drum dryers before being sent to a landfill for disposal.

The biosafety classification of the microorganism used in the fermentation process will determine the level of containment that is required for operations such as sampling, offgas venting and waste disposal to minimize the potential for biohazard risk to personnel and the environment. For the successful scale-up, the identification and modification of a suitable organism, followed by prudent pilot-scale studies certainly is crucial to success with bio-based manufacturing. A scale-up strategy that combines integrated teamwork with solid engineering efforts can minimize costly rework and delays, and help today's promising manufacturing routes based on renewable feedstocks to achieve their full commercial-scale potential on time and budget.

Summary

Effective interaction between biotechnologists and bio(chemical)engineers has been the key factor for both the technological innovation and the commercial success of the industrial microbiological processes. The primary objective of bioengineering practice is to develop processing systems that economically transform raw materials into marketable products using fermentation and downstream or product recovery processing.

The microbial and processing engineering factors that affect the commercial process are productivity and conversion efficiency, which are in turn controlled by the physical phenomena occurring in the reactor through mass and heat transfer capacities. Process constraints and limitations imposed by the instabilities of biological materials and by such new technologies as immobilized reactors, rDNA and hybridoma require the prevention of product denaturation and high expenditures on development.

When fermentation is complete, it is necessary to recover the desired end product; this involves separation of the cells and other particulates from the fermentation broth, but purification of biomolecules or metabolites with or without cell disruption may be required, as well. Such operations are referred to as downstream processing. Purification usually involves a series of adsorption/desorption or chromatography columns, and principles and applications in industrial scale were discussed. In finishing operations, the final volume is further reduced and the product is stored until end use. But at various points along the way, analyses are needed to facilitate the implementation of quality and safety considerations.

Recently systems for controlling process variables applicable to a bioprocess have become increasingly sophisticated employing digital systems such as PLCs, microprocessor based software control systems, or a hybrid arrangement. This has enabled large molecule pharmaceutical manufacturing to move toward the level of reproducibility that semiconductor processing now enjoys.

1.8 Molecular thermodynamics for biotechnology

Thermodynamics is a fundamental discipline that enables us to understand how energy is handled by living organisms. Many of the concepts are better understood by considering that the laws of thermodynamics are based on the stochastic behavior of large sets of molecules. However, cells are nonequilibrium systems in which information plays an essential role and thus, several nonstandard concepts should be explained why these very ordered systems proliferate actively in our planet despite their apparent disregard of the second law of thermodynamics. The *second law of thermodynamics*, commonly known as the law of increased entropy, states that in all energy exchanges, if no energy enters or leaves the system, the potential energy of the state will always be less than that of the initial state.

Until 1950, thermodynamics was not a prominent field in biotechnology, probably due to lack of data with respect to biomolecular properties, thermodynamic equilibrium positions, energy efficiency relations and the complexicity of biological systems. Although most of the bioprocesses were not optimized as chemical processes, bioprocess techniques with recent developments in rDNA technology have increased significantly the application of thermodynamics in various fields of biotechnology (Table 1.27). The knowledge of the cell as a highly efficient cell factory in catalyzing/noncatalyzing reactions and minimizing energy consumption and side-reaction products has lead to a reassessment of the principles in many classical chemical engineering principles. The improved understanding of molecular thermodynamic opens up an immense field of new possible applications for the chemical industry and biotechnology. All biotechnology-related business is now using biological information and techniques at the molecular level toward the discovery of new products and new processes. Thus, it has become important that physical, thermodynamic, and structural information are imperative to gain a deeper sense of the functional properties of biological macromolecules. The recent efforts in this field have resulted in models, simulation methods, and tools that allow not only solving basic science problems, but also contribute substantially to industrial research and development. Modified cells with tailor-made expression systems are now utilized for large-scale commercial production of chemicals, enzymes, and biopharmaceuticals.

As molecular thermodynamic can be applied to various fields, it is not easy to cover all, but only two important subjects on biotechnology: (i) Protein stability and protein folding and (ii) Downstream processes on crystallization and chromatography will be emphasized.

1.8.1 Protein folding and stability

Proteins, polymers of different amino acids joined by peptide bonds, serve many important physiological functions and have unique structural properties of proteins, especially on

Table 1.27 Applications of molecular thermodynamics in various fields of biotechnology

• Equilibrium studies in downstream processing
• Bioprocess optimization
• Biomolecule properties
• Protein stabilization
• Metabolite and biomass production
• Cell transport
• Agriculture and food production
• Environment protection and biofuels

stability and folding. Protein structures are stabilized by noncovalent intramolecular interactions between amino acid side chains. Protein complexes are also formed by specific noncovalent intermolecular interactions. All biological processes depend on proteins being stable and in the appropriate folded conformation. It is important to know how proteins fold into their biologically active states, and how these active states are stabilized. A primary goal of protein engineering, rational drug design, and biopharmaceutical production is the development, production, and storage of stable proteins with full functionality. Although there have been rapid advances in structural biology and relating structure to biochemical function and mechanism, knowledge of protein structure alone does not ensure accurate prediction of stability, function, and biological activity. The complete characterization of any protein requires stability determination and the forces which lead to stability and correct folding. Protein stability is intimately connected with *protein folding* – proteins have to be folded into their final active state (and maintain it) to be stable. Protein folding is the process by which newly synthesized polypeptide chains acquire the three-dimensional structures necessary for biological function. The correct *three-dimensional structure* is essential to function, though some parts of functional proteins may remain unfolded.

Thermodynamic studies As shown in Figure 1.23, an important factor to be considered during protein folding is whether the process is thermodynamically or kinetically controlled. The information required for the proper folding is available in the linear polypeptide chain, but stable 3D structure of proteins can be affected by *miscoding* (error in protein synthesis) and/or *misfolding* (error in protein folding). Many theories on protein folding and stability have been proposed on theoretical and experimental studies, but the last step in Figure 1.23 as to whether protein folding is controlled by thermodynamics or kinetics is still unknown. Among many, two models of protein folding are currently being confirmed. In the diffusion collision model by Karplus and Weaver (1994), nucleation occurs at different regions of polypeptide chain forming microstructures that diffuse and coalesce to form substructures with the native conformation. In the nucleation-condensation model, the secondary and tertiary structures of the protein are made at the same time. Recent studies have shown that some proteins show characteristics of both of these folding models.

The folding of proteins is probably known as one of the most complex processes in biochemistry as it occurs in three important steps. The initial step occurs very quickly within about 0.01 s, followed by the formation of the molten globule in about 1 s, and then the final step forms the native protein structure in a much slower step within 500–2500 s. Its understanding implies the characterization from the unfolded state of all conformational changes such as intermediate and transition states that separate the unfolded polypeptide from its fully folded and active form. The situation is also rendered more complicated because most of the proteins are made up of several domains all having their own thermodynamic and kinetics parameters of folding.

Counter pressures are also exerted by the possible existence of disulfide bridges, which have to be appropriately paired through oxidative processes driven by the disulfide bonds generation machinery implicating oxido-reductases and isomerases as well as by the existence of the cis and trans possible configuration of the peptide bonds immediately preceding the prolyl residues. This cis–trans isomerization is dependent on a ubiquitous class of foldases known as peptidyl-prolyl cis–trans isomerases. Current theories of protein folding clearly indicated that the formation of stable protein structure after synthesis is a thermodynamically controlled process. Protein stability depends in the free energy change between the folded and unfolded states which is expressed by the following equation:

$$-RT \ln K = \Delta G = \Delta H - T\Delta S$$

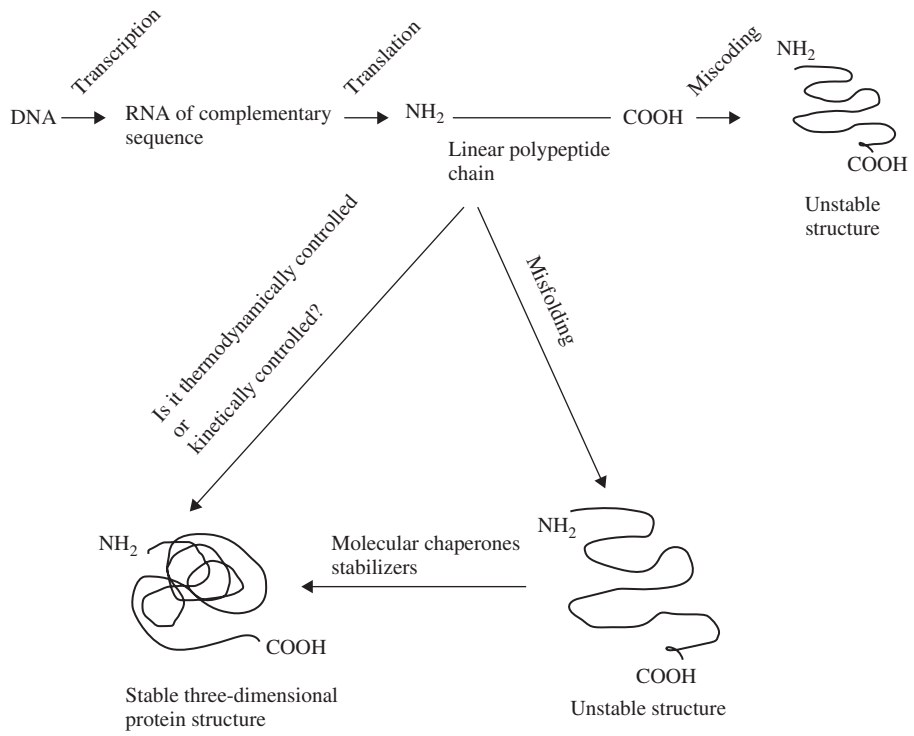

Figure 1.23 Different steps involved in synthesis of stable protein. *Source*: With kind permission from Springer Science+Business Media: Biotechnology and Bioprocessing Engineering, 8, 2003, 9–18, SN Gummadi, Fig. 2.

where R represents the Avogadro number, K, the equilibrium constant, G, the free energy change between folded and unfolded, H, the enthalpy change and S, the entropy change from folded to unfolded. The enthalpy change, H, corresponds to the binding energy (dispersion forces, electrostatic interactions, VDW potentials and hydrogen bonding), while hydrophobic interactions are described by the entropy term, S. Proteins become more stable with increasing negative values of G, as the free energy of the unfolded protein (G_U) increases relative to the free energy of the folded or native protein (G_U). In other words, as the binding energy increases or the entropy difference between the two states decreases, the folded protein becomes more stable. The folded conformation of a domain is apparently in a relatively narrow free energy minimum, and substantial perturbations of that folded conformation require a significant increase in free energy. Measured by free energy, the maximum occurs when $S = 0$, while that measured by the equilibrium constant occurs when $H = 0$. These maximum stabilities can occur at quite different temperatures, but both are used in different situations. Regardless of which one is used, however, the stability of the folded state decreases at both higher and lower temperatures.

While factors such as binding interactions do obviously play a part in stabilizing the protein, they cannot account for a very significant portion of stabilization effects since similar phenomena occur in the unfolded state, the hydrophobic effect is probably the major stabilizing effect. The thermodynamics of protein stability is modeled quite well by the

Energy landscape theory, where the energy of a protein is a function of the topological arrangement of the atoms. The description of complex systems such as proteins requires more than such simplistic models. Each value in this surface describes the protein in a specific conformation, and there is an energy landscape for each state of the protein (e.g., neutral, charged, folded, intermediate, or unfolded).

The thermodynamic behavior of proteins as determined in various temperature-jump experiments is best described by stretched exponentials as opposed to Arrhenius's law, where the rate coefficient decreases with increasing speed as the temperature is reduced as follows,

$$\kappa(T) = \kappa_0 \exp\left(-\left(\frac{E}{kT}\right)^2\right)$$

This behavior corresponds to that of what are described as glasses or spin-glasses, which undergo a transition in which transition temperature depends on the characteristic observing time.

The random-energy model by Bernard Derrida (1981) correlates well with the rough energy landscape diagram for proteins. The random-energy model described the misfolding protein states on the energy landscape, with the misfolding minima acting as "traps" that slow down the protein molecules folding process; these traps become successively more difficult to escape as the temperature is lowered. The robustness of the protein native structure to conformational change is a consequence of the funneled nature of the energy landscape of a minimally frustrated protein. The geometry of this landscape cannot be significantly changed by the modification of a few isolated residues. Random heteropolymers on the other hand, have an energy landscape consisting of multiple funnels with each on leading to a different structure, making them more inclined to conformational change as a result of sequence modification.

Molecular chaperons Failure to fold into native structure produces inactive proteins that are usually toxic. To avoid these toxic species, cells appear to invest in a complex network of special proteins, known as *molecular chaperons*, which use ingenious mechanism to prevent protein aggregation and promote efficient folding. As protein molecules are highly dynamic, constant surveillance of chaperon is essential to ensure protein homeostasis (proteostasis). The folding process often begins cotranslationally, so that the N-terminus of the protein begins to fold, while the C-terminus portion of protein is still being synthesized by the ribosome. All cells have thus evolved various measures to cope with the presence of misfolded proteins. The two main alleviatory strategies employed by cells are probably either to prevent the misfolded proteins from aggregation and refold them to the native state by the help of molecular chaperons or to target them for degradation in case of unattainable native state. Specialized proteins called *chaperons* assist in the folding of many proteins. The chaperones are major prokaryotic and eukaryotic proteins, with the function of helping in the folding of nascent polypeptide chains, helping refolding of denatured proteins, and preventing aggregation of surface-exposed hydrophobic parts of proteins, having problems with folding. Chaperones help the proteins to fold, so they increase the speed of folding, by stabilizing unstable intermediates of the appropriate polypeptide chain, and decreasing the activation-energy barriers during folding. They do not change the thermodynamics of folding, that is, the ratio of folded and unfolded polypeptides. They only influence the kinetics of gyration in that they are often correlated with the enzymes. However, sometimes they are very similar to them, but sometimes are very different, as they are not too specific for the ligands, they help to fold, the substrates are very large, and their large-scale functions make them key-molecules of the cells. Mostly, they recognize

hydrophobic surfaces on the proteins, and prevent them from aggregation. Besides this function, chaperones can play an important role in signal transduction, in the maintenance of the organized state of the cytoplasm and other intracellular compartments, in the motions inside the cell, and some other vital functions of the cells.

Sometimes they are called *stress proteins*, or *heat-shock proteins* (*Hsps*), because their synthesis increases (in most of the cases) after various forms of cellular stress, such as heat, cold, detergents, increase of ionic strength, changes in pH, toxic agents. However, the termini are not equivalent to each other, as some of the chaperones' level does not change upon stress.

Recent studies on certain neurodegenerative diseases such as *Alzheimer*, *transmissible bovine spongiform encephalopathy* (*BSE*), commonly known as *mad cow*, variant *Creutzfeldt–Jakob disease* in humans, hemolytic anemia, *Parkinson's disease*, and so on, arises due to *protein misfolding*. This suggests that knowledge on protein folding and unfolding is very important. The process of protein folding, while critical and fundamental to virtually all of biology, still remains a mystery. *Macromolecular crowding* may be important in chaperone function. The crowded environment of the cytosol can accelerate the folding process, since a compact folded protein will occupy less volume than an unfolded protein chain. However, crowding can reduce the yield of correctly folded protein by increasing protein aggregation. Crowding may also increase the effectiveness of the chaperone proteins such as *GroEL*, which could counteract this reduction in folding efficiency.

Folding an isolated or expressed globular protein is a major biotech problem and often limits the commercialization of protein products. Many proteins, especially large ones, do not refold efficiently after being unfolded or, when they are over expressed in bacteria, form IBs that represent fairly homogenous, insoluble aggregates of the expressed protein.

More information on the various types and mechanisms of a subset of chaperones that encapsulate their folding substrates can be found in the *chaperonins*. Chaperonins are characterized by a stacked double-ring structure and are found in prokaryotes, in the cytosol of eukaryotes, and in mitochondria. Other types of chaperones are involved in transport across membranes, for example, membranes of the mitochondria and ER in eukaryotes. New functions for chaperones continue to be discovered, such as assistance in protein degradation, bacterial adhesion activity, and in responding to diseases linked to protein aggregation (e.g., *prion*).

Major chaperon classes Chaperons are usually classified by their molecular mass (*Hsp40*, *Hsp60*, *Hsp70*, *Hsp90*, *Hsp100*, and the *small Hsps*) in Table 1.28. They are known to be involved in many proteome-maintenance functions like *de novo* folding, refolding of stressed-denatured proteins, oligomeric assembly, protein trafficking and assistance in proteolytic degradation. The known chaperons involving in *de novo* protein folding and refolding are Hsp70s, Hsp90s, and chaperonins (Hsp60s) that are multicomponent molecules in promoting folding through ATP and cofactor-regulated binding and release cycles. These chaperons typically recognize hydrophobic amino acid side chains exposed by nonnative proteins that may functionally cooperate with *ATP-independent chaperons* (e.g., small Hsps) functioning as "*holdases*."

The *Hsp70 chaperones*, which are essential in all eukaryotes, assist a large number of protein-folding processes, including *de novo* folding of polypeptides, refolding of misfolded proteins, solubilization of protein aggregates, degradation of proteins, translocation of proteins across membranes, assembly and disassembly of oligomeric complexes, and the regulation of stability and activity of certain natively folded proteins. In the ATP-dependent mechanism, *de novo* folding and refolding is promoted by kinetic

Table 1.28 The heat shock protein family of molecular chaperons

Chaperons	Protein family	Prokaryotes/eukaryotes	Function/structure
Hsp70	Dnak	Hsc73 (cytosol) BIP (endoplasmic reticulum) SSc1(mitochondria) Ct Hsp70 (chloroplasts)	ATP-dependent stabilization/Solved
Hsp60	GroEL	TRiC (cytosol) Hsp60 (mitochondria) Cpn60 (chloroplasts)	ATP-dependent protein folding /Solved
Hsp90	HtpG	Hsp90 (cytosol) Grp94 (endoplasmic reticulum)	ATP-driven reaction & maturation of steroid hormone receptors/Solved
Hsp100	–	ClpA, ClpX, HsIU	ATP-independent/Solved
Small Hsps (12–43 kDa)	–	–	ATP-independent/Some solved

partitioning ($K_{fold} > K_{on} > K_{agg}$), where folding, association and aggregation constants, respectively. Chaperon binding (or rebinding) to hydrophobic regions of a nonnative protein transiently blocks aggregation. The ATP-dependent reaction cycle of Hsp70 is regulated by chaperones of the Hsp40 family and nucleotide-exchange factors. Binding and release by Hsp70 is achieved by the allosteric coupling of a conserved amino-terminal ATPase domain with a carboxy-terminal peptide binding mechanism. Hsp70 system, a central player in protein folding and proteostasis control has proven effective in preventing toxic protein aggregation in disease models.

Chaperonins belong to a large class of molecules (about 800–900 kDa) that function by globally enclosing substrate proteins (up to about 60 kDa) for folding. Chaperonins are divided into two groups of common evolutionary origin: *group I chaperonins* are mostly found in prokaryotes, mitochondria, and plastids and *group II chaperonins* in archaea and the eukaryotic cytosol. Group I chaperonins, such as *E. coli GroEL*, are homo-oligomers with seven 57 kDa subunits per ring and the two rings contact each other in a 2:1 subunit arrangement, that is, one subunit of one ring interacts with two subunits in the other. Group II chaperonins, such as TRiC/CCT in the eukaryotic cytosol or the thermosome in archaea, are homo- or hetero-oligomers with eight or nine subunits of 57–61 kDa per ring, and their rings are exactly in register with 1:1 interring subunit contacts. For refolding of misfolded proteins, group I chaperonins cooperate with a homoheptameric cochaperone of 10 kDa subunits (*GroES* in *E. coli*), which closes the folding chamber like a lid by interacting with the apical domains. In contrast, group II chaperonins do not cooperate with a lid-forming cochaperone but instead have an insertion in the apical domain, which functions as a built-in lid. The *GroEL/GroES complex* in *E. coli* is the best characterized chaperonin complex, in which GroEL is a double-ring 14mer with a greasy hydrophobic patch at its opening and GroES is a single-ring heptamer binding to GroEL in the presence of ATP transition state analogs of ATP hydrolysis.

Hsp90 forms a proteostasis hub that controls numerous important signaling pathways in eukaryotic cells. Hsp90s like Hsp60s and Hsp70s can bind to misfolded proteins and

prevent their aggregation, but their main essential function is believed to be the interaction with a defined set of proteins in a native or near-native state. Many of these proteins are transcription factors and protein kinases involved in the control of cell homeostasis, proliferation, differentiation, and apoptosis. The *Hsp100/Clp family* of chaperones belongs to the superfamily of AAA+ domain-containing ATPases associated with various cellular activities. The AAA+ domain is characterized by sensor 1 and sensor 2 sequence motifs consisting two subdomains. Most AAA+ proteins form oligomers with ATP bound close to the interface between subunits, and the neighboring subunit contributes the so-called arginine finger for ATP hydrolysis. Hsp100 proteins contain one or two AAA+ domains arranged in hexameric rings with a central pore through which substrate proteins can be threaded. Many Hsp100 proteins (*ClpA*, *ClpX*, *HslU* in *E. coli*) associate with *ring-forming peptidases* (*ClpP*, *HslV*), unfold proteins, and feed them into the proteolytic chamber. ClpB in *E. coli* and its relatives in lower eukaryotes (*Hsp104*) and plants (*Hsp101*) do not associate with peptidases but cooperate with the Hsp70 system to dissolve protein aggregates. The molecular mechanism of Hsp100 chaperones is best understood for *ClpX*. Two crystal structures of this covalently linked ClpX protein provide key insights into the conformational changes associated with the ATPase cycle and suggest a mechanism by which ATP hydrolysis is coupled to substrate translocation.

The Hsp100/Clp family of chaperones also belongs to the superfamily of AAA+ domain-containing ATPases associated with various cellular activities. The AAA+ domain is characterized by sensor 1 and sensor 2 sequence motifs and consists of two subdomains. Two crystal structures of this covalently linked ClpX protein provide key insights into the conformational changes associated with the ATPase cycle.

Many Hsp100 proteins (ClpA, ClpX, HslU in *E. coli*) associate with ring-forming peptidases (ClpP, HslV), unfold proteins, and feed them into the proteolytic chamber. ClpB in *E. coli* and its relatives in lower eukaryotes (Hsp104) and plants (Hsp101) do not associate with peptidases but cooperate with the Hsp70 system to dissolve protein aggregates. The molecular mechanism of Hsp100 chaperones is best understood for ClpX.

Small heat-shock proteins (*sHsps*) also exist in all three domains (Archaea, Bacteria, and Eukarya) and possess molecular chaperone activity by binding to unfolded polypeptides and preventing aggregation of proteins *in vitro*. These are a family of stress-inducible molecular chaperones that range in size from 12 to 43 kDa and that form oligomers consisting 9–50 subunits. The ability of sHsps to form oligomers contributes to their thermal stability and ability to avoid denaturation in response to high temperatures. At denaturing temperatures, sHsps can prevent the aggregation of proteins by binding to, and forming a stable complex with, folding intermediates of their substrate proteins. In some cases, sHsps can also promote renaturation of unfolded polypeptides. All proteins of this family contain the so-called *α-crystallin domain* (*αC domain or ACD*), a region of ∼90 residues. This domain is considered an important hallmark of sHsps, independent of their origin and nature. sHsp shows extensive sequence variation and evolutionary divergence. Many sHsps have been shown to act in an ATP-independent fashion to bind up to an equal weight of nonnative protein to limit aggregation and to facilitate subsequent refolding by ATP-dependent chaperones.

In addition to chaperones, other enzymes are also involved in proper folding of some proteins within the cell. *Protein disulfide isomerases* (*PDI*) are involved in the proper formation of some disulfide bonds. Another set of such enzymes are the peptidyl prolyl *cis–trans* isomerases (PPI). PDI catalyze formation of disulfide bonds (—S—S—), that act like "staples" in a protein structure and these can often form in a complex pathway, for example, BPTI (bovine pancreatic trypsin inhibitor). Disulfide bond formation usually requires an oxidative environment (periplasm in bacteria, ER in eukaryotes), while the

cytoplasm is usually a reducing environment. *PPI* are ubiquitous enzymes – isomerases or rotamases that catalyze the cis–trans isomerization without breaking bonds. In proteins most peptidyl bonds are trans (omega =torison along the C—N bond = 180) and this conformation is heavily favored in both denatured or folded forms. However, in extended chains, the peptide bond preceding a proline can be either in trans or in cis forms, with the trans form only slightly more favored than the cis form. In folded proteins, on the other hand, only about 7% of all prolyl-peptide bonds are cis. The ER is an important site for protein folding in eukaryotes. About 1/3 of all proteins in eukaryotes fold within the ER, especially all secretory and membrane proteins. ER is especially rich in chaperones, such as BIP, which belongs to the Hsp70 family.

Much progress has been made in understanding the molecular chaperons. Concerning the molecular mechanism, a clear picture is emerging for Hsp60s and Hsp100s. For Hsp100s, it is not clear how stalled substrates are dealt with and whether dissociation of the hexamer is an option or part of the mechanism. The picture is less clear for Hsp70s, Hsp60s, and sHsps.

Summary

All biotechnology-related business is now using biological information and techniques at the molecular level toward the discovery of new products and new processes. Thus, physical, thermodynamic, and structural information are thus imperative to gain a deeper sense of the functional properties of biological macromolecules. Molecular thermodynamic can be applied to various fields such as equilibrium studies in downstream processing, bioprocess optimization, biomolecule properties, protein stabilization, metabolite and biomass production, cell transport, agriculture and food production, and environment protection and biofuels. The recent efforts in this field have resulted in models, simulation methods, and tools that allow not only solving basic science problems but also contributing substantially to industrial research and development. Among many fields, protein stability and protein folding and downstream processes on crystallization and chromatography are very important in industrial applications. Protein stability is also important for many reasons in an understanding of the basic thermodynamics of the process of folding and increased protein stability may be a multibillion dollar value in food and drug processing, and in biotechnology and protein drugs. The process of protein folding, while critical and fundamental to virtually all of biology, remains a mystery. Moreover, perhaps not surprisingly, when proteins do not fold correctly (i.e., "misfold"), there can be serious effects, including many well-known diseases and failure to fold into native structure produces inactive proteins that are usually toxic. Incorrectly folded prion proteins must be able to deform their correctly folded analogs and to change their spatial structure. They transfer their own incorrect shape to the healthy proteins. When incorrectly folded, the protein has many regions containing β-sheets, structures and has a tendency to self-assemble into larger aggregates. These amyloids cannot be broken down and thus form deposits in the brain's tissue (http://phys.org/news/2013-08-deadly-molecular-mechanism-prion-protein.html#jCp). Proteins fold through intermediate states that have exposed hydrophobic surfaces, and when the intermediates are present at high concentration, they have a strong tendency to aggregate. To avoid these toxic species, cell appears to invest in a complex network of special proteins, known as molecular chaperons, which use ingenious mechanism to prevent protein. The cell contains large protein oligomers called molecular chaperone that permits proteins to fold in protected environments aggregation and promote efficient folding. Besides this function, chaperones can play an important role in signal transduction, in the

maintenance of the organized state of the cytoplasm and other intracellular compartments, in the motions inside the cell, and some other vital functions of the cells.

1.8.2 Downstream processes on crystallization and chromatography

Downstream processing, which refers to the recovery and purification of biosynthetic products from natural sources such as animal or plant tissue or fermentation broth, consists of different unit processes. These depend on the product characteristics, purity level, and product localization in the cells (intracellular) or extracellular secreted into the medium. In addition to mechanical separations, the techniques used are vacuum drying, membrane filtration, precipitation, crystallization, chromatography, and lyophilization as discussed in the previous section. Downstream processing and analytical bioseparation both refer to the separation or purification of biological products, but at different scales of operation and for different purposes. Downstream processing implies manufacture of a purified product fit for a specific use, generally in marketable quantities, while analytical bioseparation refers to purification for the sole purpose of measuring a component or components of a mixture, that deal with sample sizes as small as a single cell. Downstream purification has the potential to become a critical bottleneck in large-scale production of expensive bioproducts.

Crystallization *Crystallization* process can be used to purify a substance or to remove water and salts in other to obtain a solid intermediate or product, and in drug substance formulation. Understanding the effects of the combinations of protein–protein, protein–solvent, and protein–solute interactions can provide insight into areas such as cellular processes, disease origins, and protein processing. The nature of a crystallization process is governed by both thermodynamic and kinetic factors, which can make it highly variable and difficult to control. Factors such as impurity level, mixing regime, vessel design, and cooling profile can have a major impact on the size, number, and shape of crystals produced.

Crystallization is the process of formation of solid crystals precipitating from a solution, molten material or more rarely deposited directly from a gas. Crystallization is also a chemical solid–liquid separation technique, in which mass transfer of a solute from the liquid solution to a pure solid crystalline phase occurs. Crystallization is an aspect of precipitation, obtained through a variation of the solubility conditions of the solute in the solvent, as compared to precipitation due to chemical reaction.

The crystallization process consists of two major events, *nucleation* and *crystal growth*. *Nucleation* is the step where the solute molecules dispersed in the solvent start to gather into clusters on the nanometer scale that become stable under the current operating conditions. These stable clusters constitute the nuclei, but when the clusters are not stable, they redissolve. Therefore, the clusters need to reach a critical size to become stable nuclei. Such critical size is dictated by the operating conditions (temperature, supersaturation, pH, salt, solvents, surface active agents, etc.). The difference between the actual value of the solute concentration at the crystallization limit and the theoretical (static) solubility threshold is called *supersaturation* and is a fundamental factor in crystallization dynamics. Supersaturation is the driving force for both the initial nucleation step and the following crystal growth, both of which could not occur in saturated or undersaturated conditions. It is at the stage of nucleation that the atoms arrange in a defined and periodic manner that defines the crystal

structure. The *crystal growth* is the subsequent growth of the nuclei that succeed in achieving the critical cluster size. Nucleation and growth continue to occur simultaneously while the supersaturation exists. Depending upon the conditions, either nucleation or growth may be predominant over the other, and as a result, crystals with different sizes and shapes are obtained. Once the supersaturation is exhausted, the solid–liquid system reaches equilibrium and the crystallization is complete, unless the operating conditions are modified from equilibrium to supersaturate the solution again.

Many compounds have the ability to crystallize with different crystal structures, a phenomenon called *polymorphism*. Each polymorphism is in fact a different thermodynamic solid state and crystal polymorphs of the same compound exhibit different physical properties, such as dissolution rate, shape (angles between facets and facet growth rates), melting point, and so on. For this reason, polymorphism is of major importance in industrial manufacture of crystalline products.

Protein crystallization, which has been mostly applied in protein structure analysis, has been recognized in principle as a method of protein purification. Within a crystal, protein molecules form a regular lattice able to exclude other proteins as well as misfolded protein molecules of the same type. Therefore, as routinely applied to small molecules, crystallization can also be used as a cheap and scalable purification procedure. The feasibility of protein purification by crystallization has been demonstrated for an industrial lipase or the model protein ovalbumin. However, the only biopharmaceutical routinely crystallized at industrial scale and with excellent recovery yields is insulin. Insulin is a small and extraordinarily stable peptide able to refold easily into its native structure even after exposure to organic solvents. It is crystallized late in the purification sequence where most of the impurities have already been removed. Additional benefits of protein crystallization from a formulation perspective are the higher stability of crystalline proteins in comparison to protein solutions, making crystalline formulations an attractive alternative with potentially longer shelf life, and the possibility to control delivery of a protein by making use of crystal dissolution kinetics. Many challenges of thermodynamics are in the design of a crystallization process on determinations of crystallization window, solubility curve, metastable zone, nucleation zone, nucleation, and growth kinetics as well as growth habit.

Chromatography The product purification process that is done to separate contaminants that resemble the product very closely in physical and chemical properties is expensive to carry out and requires sensitive and sophisticated equipment. This stage contributes a significant fraction of the entire downstream processing expenditure by using affinity, size exclusion, RPC, crystallization, and fractional precipitation especially for the therapeutic proteins. In principle, the separation methods exploit the differences in physiocochemical, thermodynamic, or molecular properties between the target product and the contaminants. The separation is based on differential partitioning between the mobile and stationary phases. Subtle differences in a compound's partition coefficient result in differential retention on the stationary phase and thus changing the separation.

Chromatographic thermodynamics is the study of how to explain molecular separation behaviors in chromatographic methods thermodynamically. Large-scale chromatography operations continue to occupy a key position in the overall strategy for the downstream processing and purification of protein products for therapeutic use. In commercial manufacturing, a requirement exists to increase the scale of the chromatography operations, which are typically developed and optimized in small-scale experiments. Also, there are a broad range of next-generation sorbents and membranes commercially available

for process chromatography. These new chromatography media are often characterized by significantly improved performance compared to their classical chromatographies with higher dynamic binding capacities, higher operational flow rates, and specific and distinctive retention mechanisms. Nevertheless, traditional media based on agarose or polymers continue to be routinely used for new method development due to their proven suitability for protein purification in numerous Food and Drug Administration (FDA) or European Medicines Agency (EMA) approved applications.

The major drawback of chromatographic procedures is the high cost of adsorption media, which can cost more than US$10,000 per liter of *Protein A* resin. Major breakthroughs to replace Protein A that are readily acceptable to regulatory authorities are unlikely in the short term to dramatically lower overall costs. However, recently modified manufacturing processes for anion and cation exchangers have significantly improved their performance. It is a matter of time before alternative to traditional chromatography for protein and antibody becomes available in industrial scale.

To quantify molecular thermodynamic properties and operating conditions, qualitative concepts based on the substantial experience can assist in translating the data into quantitative tools for designing separation methods and final process design. Physicochemical properties of biomolecules important in chromatography separation are molecular size, charge, pI, hydrophobicity, and affinity. Besides these, interactions of biomolecules among themselves in different environments are the key parameters in their partitioning in different phases. For example, the *osmotic second virial coefficient* (*SVC*) is a thermodynamic property of dilute protein solution, which characterizes pair-wise protein self-interactions. This SVC has been used to model and explain thermodynamically a number of separation techniques like crystallization, precipitation, aqueous two-phase separation, folding/refolding, and aggregation. However, some separation methods are very complex for modeling to obtain many physicochemical and thermodynamic parameters as input. Selection and design of downstream processing operations for biomolecules have thus been impossible in a systemic manner due to the lack of fundamental knowledge on the thermodynamic properties of the molecule to be separated.

Separation in ion-exchange chromatography occurs because of charge differences between product and contaminant molecules. As the net charge varies with the solution pH, the elution profile depends solely on operational pH and ionic strength. For a given operation condition, charge density than the net charge is the parameter affecting protein-partitioning behavior in ion-exchange chromatography. The separation principle of size-exclusion chromatography is based on molecular size, but concentration dependence of retention is a nonnegligible parameter at high concentration of biomolecules. The other chromatography techniques such as *hydrophobic interaction chromatography* (*HIC*) and *RPC* exploit the variable hydrophobic nature of biomolecules. HIC is based on the reversible interaction between the hydrophobic patches on the biomolecules and the mildly hydrophobic stationary phase at high salt concentration. Retentions of biomolecules in this system depend on the type and concentration of salt and density and type of hydrophobic ligand in the stationary phase.

The term *hydrophobicity* covers average *surface hydrophobicity*, location and size of hydrophobic patches on the biomolecule surface (named as surface hydrophobicity distribution). The basic retention process in RPC is basically the same as in HIC, but RPC matrices are generally more hydrophobic than HIC matrices; thus elution is done by organic solvents than aqueous solutions. The other affinity chromatography that is used in pharmaceutical protein purification is based on the biospecific affinity between the ligand attached on the stationary phase and the biomolecules in the liquid phase.

Summary

Downstream processing has a major impact on the economic performance of biochemical manufacturing processes, that may cost up to 80% of the total production costs; that is why a thorough and reliable development of separation processes is essential for the scale-up of economic processes for complex biochemical mixtures. Crystallization as an aspect of precipitation, obtained through a variation of the solubility conditions of the solute in the solvent can be useful to crystallize from downstream intermediates, in particular from crude homogenate, resulting in high product purity and scale-up to several kilograms per batch. Some products such as an IgG antibody were able to crystallize with a yield of 98%. This type of development enables the downstream processes, whereby time-consuming and expensive chromatographic steps can be replaced by the use of crystallization. In addition, protein crystallization has the potential to become a new economic tool for the stable formulation and storage of biopharmaceuticals.

Although new chromatography media are often characterized by significantly improved performance compared to their classical predecessors on higher dynamic binding capacities, higher operational flow rates, and specific and distinctive retention mechanisms, traditional media (e.g., based on agarose or polymers developed in the 1970s or even before) continue to be routinely used for the development of new methods due to their proven suitability for protein purification in numerous FDA or EMA approved applications. In these cases, the potential of modern ion exchangers and novel mixed-mode or multimode sorbents still remains poorly exploited. As a result, significant additional costs in production may occur and result in severe economic consequences.

1.9 Protein and enzyme engineering

As shown in the previous chapter, mutagens including ionizing radiations (e.g., X-rays, γ-rays), nonionizing radiations (e.g., UV light – which is strictly speaking not a mutagen) and various chemicals (e.g., sodium bisulfite, hydroxylamine or methoxylamine, nitrosoguanidine, benzene, ethidium bromide, etc.) have been used in the past to deliberately produce random mutations in the DNA of organisms in attempts to improve them – a process known as strain improvement. The mutants have then been screened and selected for improvements such as an increased yield. This is quite a success story since the yield of penicillin from industrial strains of the organism is millions of times higher than the original wild-type mold isolated from a moldy melon bought in a market. The success of this method depends on screening techniques to identify mutations that result in the desired properties. Both approaches should be used in parallel as regions of the molecule identified by *RM* may be used as targets for further rounds of site specific modification. *Site-directed mutagenesis* (*SDM*) will replace tedious traditional mutation procedures but this technique requires rDNA technology and also needs detailed knowledge of the three-dimensional structure of the enzyme. Since this information requires much research effort to obtain the data and this technique is an expensive undertaking, only improvements which are big enough to justify expensive R&D expenditures should be carried out. *Computer-aided molecular modeling* could provide a three-dimensional image of structures which will allow their manipulation in real time and visualization of molecular parts. A schematic diagram of protein engineering techniques is shown in Figure 1.24. Comparison of rational design (SDM) and directed evolution (RM) are shown in Figure 1.25.

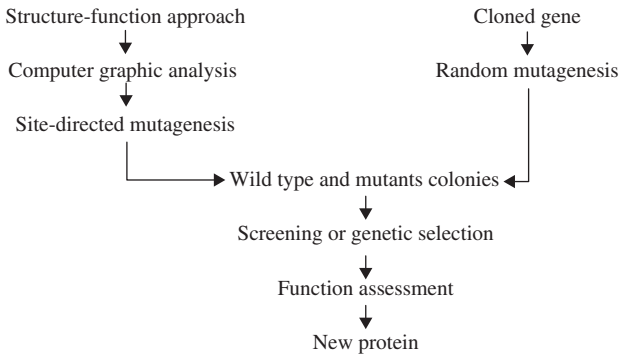

Figure 1.24 A schematic diagram of protein engineering techniques.

The desired result from those techniques never gave any guarantee (it was of the "hit-and-miss" nature) because mutations are random and most of them are disadvantageous to the organism. Nowadays, rDNA Technology offers ways of speeding up the process to make it far more specific. One of the rDNA technologies is *protein engineering*, which is the process of developing useful or valuable proteins. It is a young discipline involving many disciplines such as biology, protein chemistry, structural chemistry, and enzymology to define and exploit the relationship between protein structure and catalytic function. Two general techniques, *rational design* and *directed evolution*, for protein engineering are not mutually exclusive and are often applied. In the future, more detailed knowledge of protein structure and function, as well as advancements in high-throughput technology will greatly expand the capabilities of protein engineering.

In rational protein design, the scientist uses detailed knowledge of the structure and function of the protein to make desired changes. This technique is more specifically termed *in vitro mutagenesis* or *SDM*. This technique involves the alteration of single or a small number of known amino acids in a protein through the use of short oligonucleotide primers coding for the change so that its catalytic or other properties can be modified. This approach may also be applied to other proteins to modify antigenicity, digestivility, and the nutritional value. This generally has the advantage of being inexpensive and technically easy, since SDM techniques are well developed. However, its major drawback is that detailed structural knowledge of a protein is often unavailable, and even when it is available, it can be extremely difficult to predict the effects of various mutations. *Computational protein design algorithms* seek to identify novel amino acid sequences that are low in energy when folded to the prespecified target structure. While the sequence-conformation space that needs to be searched is large, the most challenging requirement for computational protein design is a fast, yet accurate, energy function that can distinguish optimal sequences from similar suboptimal ones.

Main methods in protein engineering are to: (i) isolate the gene coding for the protein/enzyme via mRNA and its conversion into cDNA, (ii) sequence the gene, (iii) decide on mutation that will "improve" the enzyme normally based on study of the enzyme's three-dimensional structure and amino acid sequence as well as the computer modeling, (iv) use SDM to produce the desired change in codon(s) in the gene and subsequently the amino acid sequence of the enzyme molecule, and (v) test new enzyme for improvement.

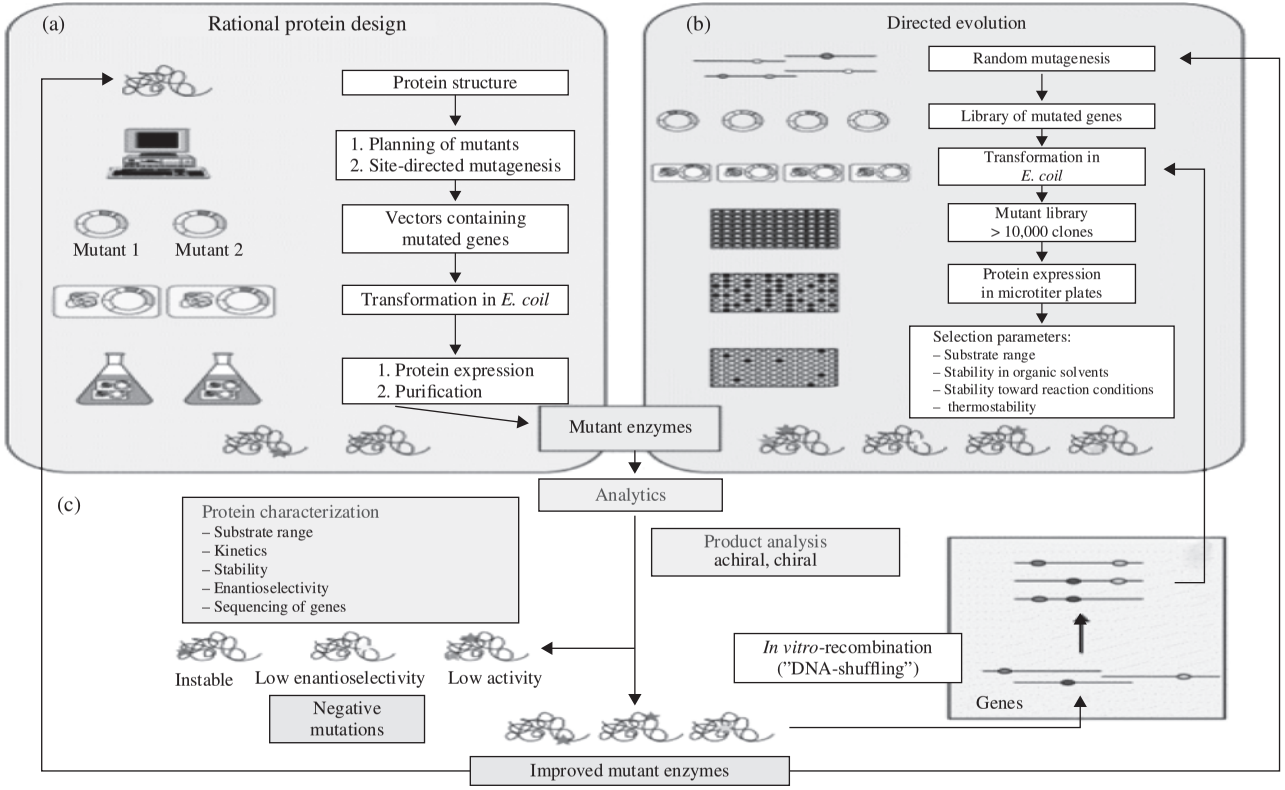

Figure 1.25 Comparison of rational design (site-directed mutagenesis, SDM) and directed evolution (random mutagenesis, RM). (a) In rational design, mutants are created on the basis of their protein structure and then prepared by SDM; after transformation in the host, the variant is expressed, purified and analyzed for desired properties. (b) Directed evolution starts with the preparation of mutant gene libraries by random mutagenesis (RM). They are then expressed in the host. Proteins libraries are usually screened in microtiter plates using several parameters. Protein characterization and product analysis sort out desired and negative mutants. *In vitro* recombination by DNA shuffling, for example, can be used for further improvements. (c) Both protein engineering approaches can be repeated or combined until biocatalysis with desired properties are generated. *Source*: Bornscheuer and Pohl 2001. Reproduced with permission of Elsevier.

There are now several methods for carrying out SDM, including polymerase chain reaction (PCR)-based ones. The following one, called the *single-primer method* is based on the use of the phage vector M13.

Methods of SDM using M13 phage are to (i) isolate required enzyme gene, via mRNA and its conversion into cDNA, (ii) sequence the DNA of the gene (in order to decide on change required for primer in stage 5), (iii) splice gene into M13 vector dsDNA and transduce *E. coli* host cells, (iv) isolate ssDNA in phage particles released from host cells, (v) synthesize an oligonucleotide primer with the same sequence as part of the gene but with altered codon (mismatch/mispair) at desired point(s), (vi) mix oligonucleotide with recombinant vector ssDNA, (vii) use DNA polymerase to synthesize remainder of strand. (Oligonucleotide acts as a primer for the DNA synthesis). Then add ligase to make join between primer and new strand permanent dsDNA molecule, and (viii) transform *E. coli* cells and allow them to replicate recombinant vector molecule. DNA replication is semiconservative, therefore two types of clone are produced each of which excretes phage particles containing ssDNA: type 1 containing the wild-type gene (i.e., unaltered) and type 2 containing the mutated gene. Ratio of the two types should be 1:1 but is not usually because *E. coli* "edits out" some of the mismatches, (ix) select mismatch clones bearing the mutation, and (x) if gene expression is required, use these clones to extract mutated DNA and insert it into an expression vector system with appropriate promoter, and so on, to produce the modified gene product ("designer protein").

Directed evolution In directed evolution, *RM* is applied to a protein, a selection regime is used to pick out variants that have the desired qualities, and further rounds of mutation and selection are then applied. This method mimics natural evolution that generally produces superior results to rational design. An additional technique known as *DNA shuffling* mixes and matches pieces of successful variants in order to produce better results. This process mimics the recombination that occurs naturally during *sexual reproduction*. Directed evolution has a great advantage in that it requires no prior structural knowledge of a protein, nor is it necessary to be able to predict what effect a given mutation will have. The results of directed evolution experiments are often surprising in that desired changes are often caused by mutations that were not expected to have that effect. However, they require high-throughput, which is not feasible for all proteins. Large amounts of rDNA must be mutated and the products screened for desired qualities. The sheer number of variants often requires expensive robotic equipment to automate the process. A typical directed evolution experiment involves three steps: (i) *Diversification*: The gene encoding the protein of interest is mutated and/or recombined at random to create a large library of gene variants. Techniques commonly used in this step are *error-prone PCR* and DNA shuffling, (ii) *Selection*: The library is tested for the presence of mutants (variants) possessing the desired property using a screen or selection and isolated high-performing mutants by hand, while selections automatically eliminate all nonfunctional mutants, and (iii) *Amplification*: The variants identified in the selection or screen are replicated manyfold, and sequence their DNA in order to understand which mutations have occurred. These three steps are termed a "round" of directed evolution. Most experiments involve more than one round and at the end of the experiment, all evolved protein or RNA mutants are characterized using biochemical methods.

Directed evolution can be performed in living cells (*in vivo* evolution) or may not involve cells at all (*in vitro* evolution). *In vivo* evolution has the advantage of selecting for properties in a cellular environment, which is useful when the evolved protein or RNA is to be used in living organisms, but *in vitro* evolution is more often versatile in the types of selections that can be performed. *In vitro* evolution experiments can generate larger libraries

because the library DNA need not be inserted into cells, the currently limiting step. Most directed evolution projects seek to evolve properties that are useful to humans in an agricultural, medical, or industrial biocatalysis.

For the examples of engineered proteins, a protein with a novel fold, known as *Top7*, has been designed as well as sensors for unnatural molecules using computational methods. The engineering of fusion proteins has yielded *rilonacept* (IL-1 Trap), a pharmaceutical for the treatment of cryopyrin-associated periodic syndrome. Another computational method, IPRO (iterative protein redesign and optimization) successfully engineered the switching of cofactor specificity of *Candida boidinii* xylose reductase to increase or give specificity to native or novel substrates and cofactors. Computation-aided design has also been used to engineer complex properties of a highly ordered nano-protein assembly. A protein cage, *E. coli* bacterioferritin (EcBfr), which naturally shows structural instability and an incomplete self-assembly behavior by populating two oligomerization states is the model protein in this study.

Although many overproduced genetically engineered enzymes are commercially available, not many protein-engineered proteins are commercially available. Commercially marketed enzyme and protein are (i) *subtilisin* protease (from *Bacillus amyloliquefaciens*), which is the principal enzyme in the detergent enzyme preparation, Alcalase. This has been aimed at the improvement of its activity in detergents by stabilizing it at even higher temperatures, pH and oxidant strength, and (ii) The engineered fusion protein is *Rilonacept*, also known as IL-1 Trap (marketed by Regeneron Pharmaceuticals under the trade name Arcalyst), which is an IL-1 inhibitor for the treatment of cryopyrin-associated periodic syndrome. Further discussions on the engineered enzymes are in the Enzyme section in Part II.

Summary

Protein engineering, as the process of developing useful or valuable proteins, is a young discipline with much research taking place in the understanding of protein folding and recognition for protein design principles. Two general techniques for protein engineering, "rational" protein design and directed evolution are not mutually exclusive and both are often applied. Protein engineering aims at modifying the sequence of a protein, and hence its structure, to create enzymes with improved functional properties such as stability, specific activity, inhibition by reaction products, and selectivity toward nonnatural substrates. In the future, more detailed knowledge of protein structure and function, as well as advancements in high-throughput technology, may greatly expand the capabilities of protein engineering. New developments include advanced computational design, development of new and useful biocatalysts, and integration of functional biological parts with fabricated devices and construction of next generation biopharmaceuticals. Protein engineering also continues to provide valuable and fundamental understanding of natural protein construction and function that in turn will inevitably improve our ability to generate the next generation of novel proteins.

1.10 Genomics, proteomics, and bioinformatics

Genomics is to characterize the genome sequence of a number of model organisms that brought about a revolution in biology and medicine. Although the genetic blueprints of a number of model organisms are now available, the big challenge in deciphering the function

of the encoded proteins and how these contribute to the functioning of the organism as an entity remains. Functional genomic research uses the so-called post-genomic technologies to unravel the function of recently identified genes. The genomics researches include a comprehensive range of gene cloning, gene discovery/characterization, gene expression and protein expression, and characterization.

Functional genomics attempts to make use of the vast wealth of data produced by genomic projects such as genome sequencing projects to describe gene (and protein) functions and interactions and to answer questions about the function of DNA at the levels of genes, RNA transcripts, and protein products. The simple goal of functional genomics is to understand the relationship between an organism's genome and its phenotype. Unlike genomics, which is the static aspects of the genomic information such as DNA sequence or structures, functional genomics focuses on the dynamic aspects such as gene transcription, translation, and protein–protein interactions. A key characteristic of functional genomics studies is their genome-wide approach to these questions and generally involved in high-throughput methods rather than a more traditional "gene-by-gene" approach. The promise of functional genomics is to expand and synthesize genomic and proteomic knowledge into an understanding of the dynamic properties of an organism at cellular and/or organismal levels. This would provide a more complete picture of how biological function arises from the information encoded in an organism's genome. Understanding how a particular mutation leads to a given phenotype has important implications for human genetic diseases and this can eventually result in the discovery of a treatment or cure.

Functional genomics includes function-related aspects of the genome itself such as mutation and polymorphism analysis, as well as measurement of molecular activities. The latter comprise a number of "-omics" such as *transcriptomics* (gene expression), *proteomics* (protein expression), and *metabolomics*. Functional genomics uses mostly multiplex techniques to measure the abundance of many or all gene products such as mRNAs or proteins within a biological sample to quantitate the various biological processes and improve our understanding of gene and protein functions and interactions. Meanwhile, *Comparative genomics* is the study of the relationship of genome structure and function across different biological species or strains. Comparative genomics is an attempt to take advantage of the information provided by the signatures of selection to understand the function and evolutionary processes that act on genomes.

At the RNA level (*transcriptome profiling*), *microarrays* measure the amount of mRNA in a sample that corresponds to a given gene or probe DNA sequence. Probe sequences are immobilized on a solid surface and allowed to hybridize with fluorescently labeled "target" mRNA. The intensity of fluorescence of a spot is proportional to the amount of target sequence that has hybridized to that spot, and therefore to the abundance of that mRNA sequence in the sample. Microarrays allow for identification of candidate genes involved in a given process based on the variation between transcript levels for different conditions and shared expression patterns with genes of known function. *SAGE* (*serial analysis of gene expression*) is an alternate method of gene expression analysis based on RNA sequencing rather than hybridization. SAGE relies on the sequencing of 10–17 base pair tags, which are unique to each gene. These tags are produced from poly-A mRNA and ligated end-to-end before sequencing. SAGE gives an unbiased measurement of the number of transcripts per cell, as it does not depend on prior knowledge of what transcripts to study (as microarrays do).

At the protein level, *protein–protein interactions*, a *yeast two-hybrid* (*Y2H*) screen tests a "bait" protein against many potential interacting proteins ("prey") to identify physical protein–protein interactions. This system is based on a transcription factor,

originally GAL4, whose separate DNA-binding and transcription activation domains are both required in order for the protein to cause transcription of a reporter gene. In a Y2H screen, the "bait" protein is fused to the binding domain of GAL4, and a library of potential "prey" (interacting) proteins is recombinantly expressed in a vector with the activation domain. In vivo interaction of bait and prey proteins in a yeast cell brings the activation and binding domains of GAL4 close enough to result in the expression of *a reporter gene*. It is also possible to systematically test a library of bait proteins against a library of prey proteins to identify all possible interactions in a cell. New methods include protein microarrays, immunoaffinity chromatography followed by mass spectrometry (MS), dual polarization interferometry, microscale thermophoresis and experimental methods such as phage display and computational methods.

DNA microarray technology *DNA microarray technology* (also known as DNA arrays, DNA chips or biochips) shown in Figure 1.26 represents one of the latest breakthroughs and major achievements in experimental molecular biology. Although this

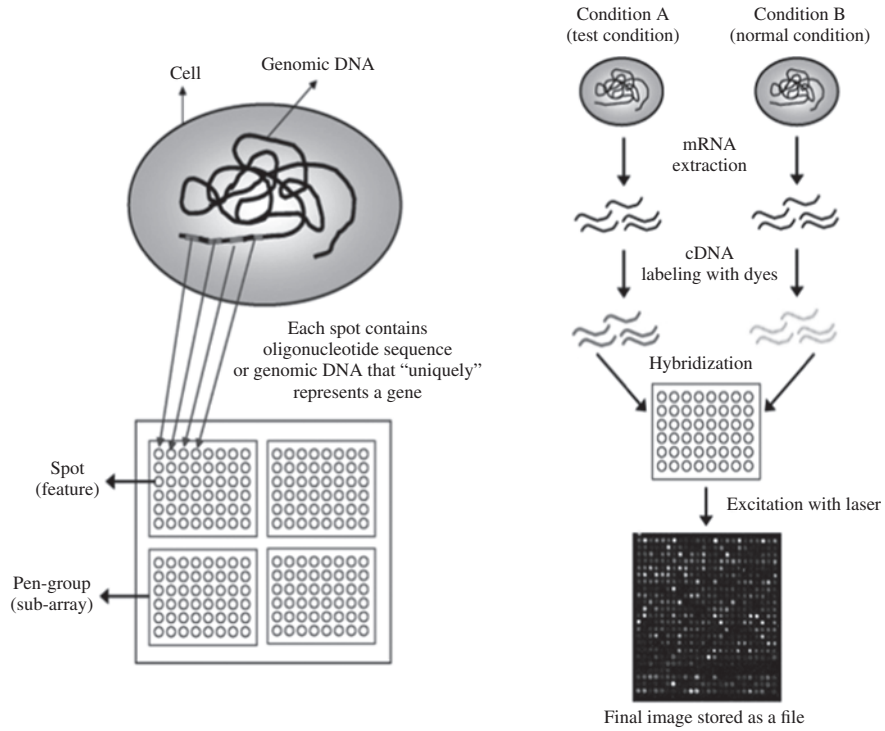

Figure 1.26 (a) A microarray may contain thousands of "spots." Each spot contains many copies of the same DNA sequence that uniquely represents a gene from an organism. Spots are arranged in an orderly fashion into pen-groups. (b) Schematic of the experimental protocol to study differential expression of genes. *Source*: M. Madan Babu (2004) Computational Genomics: Theory & Application (ed. Richard P. Grant); Taylor & Francis ISBN: 978-1904933014; www.mrc-lmb.cam.ac.uk/ … /microarray/figure1236.ppt. Reproduced with permission.

novel technology in which labeled nucleic acids could be used to monitor the expression of nucleic acid molecules attached to a solid support, the first article describing the application of DNA microarray technology (Brown and Botstein, 1999) to expression analysis was published in the scientific literature in 1995 by Dr. Patrick Brown at Stanford University (http://hhmi.org/science/genetics/brown.htm). Such widespread adoption of DNA microarray technology in both industry and many academic research laboratories was largely due to its aptitude to provide researchers the opportunity to quickly and accurately perform simultaneous analysis of literally thousands of genes in a massively parallel manner, or even entire genome of an organism, for example (Bacteria, Yeast, Virus, Protozoa, Mouse, or Human), in a single experiment, hence providing extensive and valuable information on gene interaction and function. DNA microarrays can be used to detect differences in the levels gene expression in different populations of cells on a genome-wide level. DNA microarray technology may be defined as a high-throughput and versatile technology used for parallel gene expression analysis for thousands of genes of known and unknown function, or DNA homology analysis for detecting polymorphisms and mutations in both prokaryotic and eukaryotic genomic DNA. Each identified sequenced gene on the glass, silicon chips or nylon membrane corresponds to a fragment of genomic DNA, cDNAs, PCR products, or chemically synthesized oligonucleotides of up to 70mers and represents a single gene. Usually a single DNA microarray slide/chip may contain thousands of spots each representing a single gene and collectively the entire genome of an organism. Each DNA spot contains picomoles (10^{-12}moles) of a specific DNA sequence, known as *probes* (or *reporters or oligos*). These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (also called antisense RNA) sample (called *target*) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target.

Microarray technology has evolved from Southern blotting, where fragmented DNA is attached to a substrate and then probed with a known DNA sequence. The first reported use of this approach was the analysis of 378 arrayed lysed bacterial colonies each harboring a different sequence, which were assayed in multiple replicas for expression of the genes in multiple normal and tumor tissue. This was expanded to analysis of more than 4000 human sequences with computer driven scanning and image processing for quantitative analysis of the sequences in human colonic tumors and normal tissue and then to comparison of colonic tissues at different genetic risk. These early gene arrays were made by spotting cDNAs onto filter paper with a pin-spotting device. The use of miniaturized microarrays for gene expression profiling was on a complete eukaryotic genome (*S. cerevisiae*) on a microarray in 1997.

The core principle behind microarrays is hybridization between two DNA strands, the property of complementary nucleic acid sequences to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs. A high number of complementary base pairs in a nucleotide sequence means tighter noncovalent bonding between the two strands. After washing off of nonspecific bonding sequences, only strongly paired strands will remain hybridized. Fluorescently labeled target sequences that bind to a probe sequence generate a signal that depends on the hybridization conditions (such as temperature), and washing after hybridization. Total strength of the signal, from a spot (feature), depends upon the amount of target sample binding to the probes present on that spot. Microarrays use relative quantization in which the intensity of a feature is compared to the intensity of the same feature under a different condition, and the identity of the feature is known by its position.

There are three basic types of samples that can be used to construct DNA microarrays, two are genomic and the other is transcriptomic, that measures mRNA levels. What makes them different from each other is the kind of immobilized DNA used to generate the array and, ultimately, the kind of information that is derived from the chip. The target DNA used will also determine the type of control and sample DNA that is used in the hybridization solution. Determining the level, or volume, at which a certain gene is expressed is called microarray expression analysis, and the arrays used in this kind of analysis are called "expression chips."

Basic steps in performing a DNA microarray experiment are: (i) The first step in the process of microarrays is the preparation of the target DNA, which can be done from the genomic source, or expressed sequence tags. (ii) The PCR is then used to amplify this DNA. (iii) This amplification step is vital in many applications and array formats. (iv) The DNA sequences are printed onto the microscope slides robotically, in a specific grid pattern. There are a number of methods for producing the microarray slides, and these are mainly done using robotic systems. They have their own advantages and disadvantages.

The organism is grown in two different conditions (a reference condition and a test condition). RNA is extracted from the two cells, and is labeled with different dyes (red and green) during the synthesis of cDNA by reverse transcriptase (RT). Following this step, cDNA is hybridized onto the microarray slide, where each cDNA molecule representing a gene will bind to the spot containing its complementary DNA sequence. The microarray slide is then excited with a laser at suitable wavelengths to detect the red and green dyes. The final image is stored as a file for further analysis (www.mrc-lmb.cam.ac.uk/ … /microarray/figure1236.ppt). To determine which genes are turned on and which are turned off in a given cell, one must first collect the messenger RNA molecules present in that cell. One then labels each mRNA molecule by using a RT enzyme that generates a complementary cDNA to the mRNA. During that process fluorescent nucleotides are attached to the cDNA. The tumor and the normal samples are labeled with different fluorescent dyes. Next, the labeled cDNAs must be placed onto a DNA microarray slide. The labeled cDNAs that represent mRNAs in the cell will then hybridize – or bind – to their synthetic complementary DNAs attached on the microarray slide, leaving its fluorescent tag. A special scanner is then to measure the fluorescent intensity for each spot/areas on the microarray slides. If a particular gene is very active, it produces many molecules of messenger RNA, and thus, more labeled cDNAs, which hybridize to the DNA on the microarray slide and generate a very bright fluorescent area. Genes that are somewhat less active produce fewer mRNAs, thus, less labeled cDNAs, which results in dimmer fluorescent spots. If there is no fluorescence, none of the messenger molecules have hybridized to the DNA, indicating that the gene is inactive. One often uses this technique to examine the activity of various genes at different times. When co-hybridizing tumor samples (red dye) and normal sample (green dye) together, they will compete for the synthetic complementary DNAs on the microarray slide. As a result, if the spot is red, this means that specific gene is more expressed in the tumor than in normal (up-regulated in cancer). If a spot is green, then it means that gene is more expressed in the normal tissue (down regulated in cancer). If a spot is yellow, then that means that specific gene is equally expressed in normal and tumor.

Proteomics investigates the structure and function of proteins, the principal constituents of the protoplasm of all cells. The term proteomics was coined to make an analogy with genomics, the study of the genes. The *proteome* is the entire complement of proteins including the modifications made to a particular set of proteins, produced by an organism or system. While proteomics generally refers to the large-scale experimental analysis

of proteins, it is often specifically used for protein purification and *MS*. In many ways, proteomics runs parallel to genomics: Genomics starts with the gene and makes inferences about its products (proteins), whereas proteomics begins with the functionally modified protein and works back to the gene responsible for its production. The sequencing of the human genome has increased interest in proteomics because while DNA sequence information provides a static snapshot of the various ways in which the cell might use its proteins, the life of the cell is a dynamic process. This new data set holds great new promise for proteomic applications in science, medicine and most notably in pharmaceuticals.

After studying of genomics and transcriptomics, proteomics is considered the next step in the study of biological systems. It is much more complicated than genomics mostly because while an organism's genome is more or less constant, the proteome differs from cell to cell and from time to time. This is because distinct genes are expressed in distinct cell types, meaning that even the basic set of proteins which are produced in a cell needs to be determined. In the past this was done by mRNA analysis, but this was found not to correlate with protein content. It is now known that mRNA is not always translated into protein, and the amount of protein produced for a given amount of mRNA depends on the gene it is transcribed from and on the current physiological state of the cell. Proteomics thus confirms the presence of the protein and provides a direct measure of the quantity present. Not only does the translation from mRNA cause differences, but many proteins are also subjected to a wide variety of chemical modifications after translation. Many of these *post-translational modifications* are critical to the protein's function. One such modification is *phosphorylation*, which happens to many enzymes and structural proteins in the process of *cell signaling*. The addition of a phosphate to particular amino acids – most commonly serine and threonine mediated by serine/threonine kinases, or more rarely tyrosine mediated by tyrosine kinases – causes a protein to become a target for binding or interacting with a distinct set of other proteins that recognize the phosphorylated domain.

Ubiquitin is a small protein that can be affixed to certain protein substrates by enzymes called E3 ubiquitin ligases. Determining which proteins are poly-ubiquitinated can be helpful in understanding how protein pathways are regulated. This is therefore an additional legitimate "proteomic" study. In addition to phosphorylation and ubiquitination, proteins can also be subjected to methylation, acetylation, glycosylation, oxidation, and nitrosylation. Some proteins undergo all of these modifications, often in time-dependent combinations, that illustrate the potential complexity. Even cell may make different sets of proteins at different times, or under different conditions, any one protein can undergo a wide range of post-translational modifications, thus a "proteomics" study can become quite complex very quickly.

Applications of genomics and proteomics Analysis of different levels of gene expression in healthy and diseased tissues by proteomic approaches is as important as the detection of mutations and polymorphisms at the genomic level and may be of more value in designing a rational therapy. Protein distribution/characterization in body tissues and fluids, in health as well as in disease, is the basis of the use of proteomic technologies for molecular diagnostics. Proteomics will play an important role in medicine of the future which will be personalized and will combine diagnostics with therapeutics. Important areas of application thus include cancer (oncoproteomics) and neurological disorders (neuroproteomics) and crop improvement. A combinatorial approach of accelerated gene discovery through genomics, proteomics, and other associated -omic branches of biotechnology is proving to be an effective way to speed up the crop improvement programs worldwide. In the near future, swift improvements in -omic databases are becoming critical and demand immediate attention

for the effective utilization of these techniques to produce next-generation crops for the progressive farmers.

The markets for proteomic technologies are difficult to estimate as they are not distinct but overlap with those of genomics, gene expression, high-throughput screening, drug discovery, and molecular diagnostics. The largest expansion will be in bioinformatics and protein biochip technologies. The main objective and promise of Functional Genomics is to foster in-depth understanding of the processes that make up a living organism, which in turn will lead to new biomedical and pharmacological applications as well as biosynthetic and biotechnical developments.

The continuously increasing knowledge and data basis in the "-omics" field offers new prospects for the development of disease- or pathogen-specific biomarkers. Molecular diagnostics using such biomarkers provides the opportunity for early recognition of diseases combined with high sensitivity. However, the development of reliable diagnostic and prognostic markers and their validation is still a challenge. Next-generation sequencing genomic data are generated better, faster, and cheaper and different systems and technologies are emerging; both throughput and accuracy are the main targets for improvement. Next-generation sequencing opens the door to new diagnostic and prognostic applications as well as for food and agriculture including pathogen detection, and so on. Food proteomics is one of the most dynamic and fast-developing areas in food science in different safety aspects, such as food authenticity, detection of animal species in the food, and identification of food allergens and pathogens.

One of the most promising developments to come from the study of human genes and proteins has been the identification of potential new drugs for the treatment of disease. This relies on genome and proteome information to identify proteins associated with a disease, which computer software can then use as targets for new drugs. For example, if a certain protein is implicated in a disease, its 3D structure provides the information to design drugs to interfere with the action of the protein. A molecule that fits the active site of an enzyme, but cannot be released by the enzyme, will inactivate the enzyme. This is the basis of new drug-discovery tools, which aim to find new drugs to inactivate proteins involved in disease. As genetic differences among individuals are found, researchers expect to use these techniques to develop personalized drugs that are more effective for individuals. Proteomics is also used to reveal complex plant-insect interactions that help identify candidate genes involved in the defensive response of plants to herbivory.

Parallel analysis of the genome and the proteome facilitates discovery of post-translational modifications and proteolytic events, especially when comparing multiple species (comparative proteogenomics). Comparative proteomic analysis can reveal the role of proteins in complex biological systems, including reproduction. For example, treatment with the insecticide triazophos causes an increase in the content of brown planthopper (*Nilaparvata lugens* (Stål)) male accessory gland proteins (Acps) that can be transferred to females via mating, causing an increase in fecundity (i.e., birth rate) of females. To identify changes in the types of accessory gland proteins (Acps) and reproductive proteins that mated female planthoppers received from male planthoppers, researchers conducted a comparative proteomic analysis of mated *N. lugens* females. The results indicated that these proteins participate in the reproductive process of *N. lugens* adult females and males.

Limitations of genomics and proteomics studies Proteomics typically gives us a better understanding of an organism than genomics. First, the level of transcription of a gene gives only a rough estimate of its *level of expression* into a protein. Second, many proteins experience *post-translational modifications* that profoundly affect their activities; for

example, some proteins are not active until they become phosphorylated. Third, many transcripts give rise to more than one protein, through alternative splicing or alternative post-translational modifications. Fourth, many proteins form complexes with other proteins or RNA molecules, and only function in the presence of these other molecules. Finally, protein degradation rate plays an important role in protein content. Proteomics experiments conducted in one laboratory are not easily reproduced in another. A more common way to determine post-translational modification of interest is to subject a complex mixture of proteins to electrophoresis in "two-dimensions," (so-called *two-dimensional gel electrophoresis*), which simply means that the proteins are electrophoresed first in one direction, and then in another, which allows small differences in a protein to be visualized by separating a modified protein from its unmodified form. Recently, another approach has been developed (*PROTOMAP*) that combines SDS-PAGE (polyacrylamide gel electrophoresis) with shotgun proteomics to enable detection of changes in gel-migration such as those caused by proteolysis or post-translational modification. For proteomic study, more recent techniques such as *matrix-assisted laser desorption/ionization* (*MALDI*) have been employed for rapid determination of proteins in particular mixtures and increasingly *electrospray ionization* (*ESI*). The proteomic networks contain many *biomarkers* that are proxies for development and illustrate the potential clinical application of this technology as a way to monitor normal and abnormal cell development for guided development of clinical diagnostics by computer modeling.

New *fluorescence two-dimensional differential gel electrophoresis* can also be used to quantify variation in the 2D DIGE process and establish statistically valid thresholds for assigning quantitative changes between samples. Proteome analysis of *Arabidopsis peroxisomes* has been established as the major unbiased approach for identifying new peroxisomal proteins on a large scale.

Bioinformatics is the application of computer technology to the management and analysis of biological data. The result is that computers are being used to gather, store, analyze, and merge biological data. This field has developed out of the need to understand the code of life, DNA. Massive DNA sequencing projects have evolved and added to the growth of the science of bioinformatics. DNA codes for genes that code for proteins, which determine the biological makeup of humans or any living organism. Bioinformatics is an interdisciplinary research area, which is the interface between the biological and computational sciences. The ultimate goal of bioinformatics is to uncover the wealth of biological information hidden in the mass of sequence, structure, literature, and other biological data and obtain a clearer insight into the fundamental biology of organisms and to use this information to enhance the standard of life for mankind. It is being used now and in the foreseeable future in the areas of molecular medicine to help produce better and more customized medicines to prevent or cure diseases, it has environmental benefits in, identifying waste cleanup bacteria and in agriculture it can be used for producing high yield low maintenance crops. These are just a few of the many benefits bioinformatics will help develop.

Biological databases are archives of consistent data that are stored in a uniform and efficient manner. These databases are from a broad spectrum of molecular biology areas in which primary or archived databases contain information and annotation of DNA and protein sequences, DNA and protein structures and DNA and protein expression profiles. Secondary or derived databases contain the results of analysis on the primary resources including information on sequence patterns or motifs, variants and mutations, and evolutionary relationships. Information from the literature is contained in bibliographic databases, such as. It is essential that these databases are easily accessible and that an intuitive query system is provided to allow researchers to obtain very specific information

on a particular biological subject. The data should be provided in a clear, consistent manner with some visualization tools to aid biological interpretation.

Specialist databases for particular subjects such as *EMBL database* for nucleotide sequence data, *UniProtKB*/*Swiss-Prot protein database* and *PDBe* for 3D protein structure database. *SRS* (*sequence retrieval system*) is also a powerful, querying tool provided by the *EBI* (*The European Bioinformatics Institute*) that links information from more than 150 heterogeneous resources. The EBI provides a wide range of biological data analysis tools that fall into the following four major categories: (i) *similarity searching tools*, (ii) *protein function analysis*, (iii) *structural analysis*, and (iv) *sequence analysis*. Commonly used software tools and technologies in this field include *Java*, *XML*, *Perl*, *C*, *C++*, *Python*, *R*, *MySQL*, *SQL*, *CUDA*, *MATLAB*, and *Microsoft Excel*.

Metagenomics Metagenomics is the genomic analysis of unculturable microorganisms by direct extraction and cloning of DNA from an assemblage of microorganisms to discover new genes and make functional predictions. The development of next-generation DNA-sequencing technologies such as pyrosequencing systems have become the standard for ribosomal RNA identification (i.e., 16S, 18S, etc.) that do not require cloning or PCR amplification has allowed the large-scale metagenomic sequencing of environmental habitats such as aquatic environment, coral reefs, deep sea thermal vents, fermented food, animal, and human gut and cattle feces. Metagenomics has broad implications for human health and disease, animal production, and environmental health and has opened up a creative wealth of data, tools, technologies, and applications that allow us to access the majority of organisms that we still cannot access in pure culture (an estimated 99% of microbial life). Novel genes and gene products discovered through metagenomics include the first bacteriorhodopsin of bacterial origin; novel small molecules with antimicrobial activity; and new members of families of known proteins, such as an $Na^+(Li^+)/H^+$ antiporter, recA, DNA polymerase, and antibiotic resistance determinants.

Summary

Advancements in Genomics and Proteomics, together with integrating myriad disciplines from structural biology to bioinformatics, have brought tremendous changes in biotechnology, drug discovery, molecular diagnostics, practice of medicine, and food/agriculture in the post-genomic era – the first decade of the 21st century.

After genomics and transcriptomics, proteomics is the next step in the study of biological systems. It is more complicated than genomics because an organism's genome is more or less constant, whereas the proteome differs from cell to cell and from time to time. Distinct genes are expressed in different cell types, which means that even the basic set of proteins that are produced in a cell needs to be identified. In the past, this phenomenon was done by mRNA analysis, but it was found not to correlate with protein content. It is now known that mRNA is not always translated into protein, and the amount of protein produced for a given amount of mRNA depends on the gene it is transcribed from and on the current physiological state of the cell. Proteomics confirms the presence of the protein and provides a direct measure of the quantity present. A combinatorial approach of accelerated gene discovery through genomics, proteomics, and other associated -omic branches of biotechnology is proving to be an effective way to speed up the crop improvement programs worldwide.

1.11 Biosensors and nanobiotechnology

1.11.1 Biosensor

Biosensor is an analytical device in which a biologically derived sensing element is in intimate contact with a physiochemical transducer to give an electrical signal (Figure 1.27). The three basic components are biological element, transducer, and electronic component. The *sensitive biological element* can be derived from biological materials such as tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, and nucleic acids, and so on. Specific molecular recognition is a fundamental prerequisite, based on affinity between complementary structures such as enzyme-substrate, antibody-antigen and receptor hormone, and this property in biosensor is used for the production of concentration–proportional signals. To prepare biosensors, enzymes, cells and tissues, proteins, and nucleotides must be immobilized on the surface of the silicas, quartz, metals, carbons, semiconductors, and polymers. Immobilization can be done by simple *adsorption* (not normally used), *entrapment* (nonreactive gel, redox gel, conducting polymer, sol–gel network), *entrapment with cross-linking* (glutaraldehyde linking, polyethyleneglycol, polyethyleneimene) and *covalent coupling* to surface (direct), organosilanes, avidin-biotin anchors, and supporting membranes. Biosensor's selectivity and specificity highly depend on biological recognition systems connected to a suitable transducer. The main objectives are to develop rapid, specific, reliable, inexpensive, and compact devices.

Biosensors have been developed during the last two decades for environmental, industrial, and biomedical diagnostics (Table 1.31), but they are largely used in the biomedical area due to the largest market opportunity. Table 1.30 shows various applications of biosensors in different fields. Other applications are in food industries to detect contaminants, adulterants, nutritional compositions, evaluate product freshness, and monitoring quality control during the fermentation process. The market for biosensors application areas analyzed includes point-of-care testing, home diagnostics, environmental monitoring, research laboratories, process industry, and security and bio-defense. There are several applications of biosensors in food analysis. In food industry optic coated with antibodies are commonly used to detect pathogens and food toxins. The light system in these biosensors has been fluorescence, since this type of optical measurement can greatly amplify the signal. A range of immuno- and ligand-binding assays for the detection and measurement of small molecules such as water-soluble vitamins and chemical contaminants (drug residues), such as sulfonamides, have been developed for use on *SPR* (*surface plasmon resonance*) *sensor* systems, often adapted from existing enzyme-linked immunosorbant assay (ELISA) or other immunological assay. However, biosensor sector in the agri-food sector is very conservative

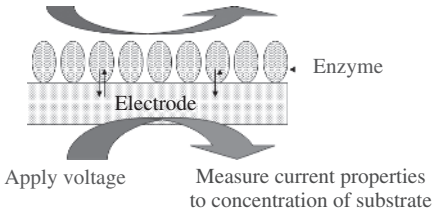

Figure 1.27 Principle of electrochemical biosensors. *Source*: J.F. Rusling, Department of Pharmacology, University of Connecticut Health Center.

Table 1.29 History of commercial biosensors

1962	First and most widely used commercial electrochemical glucose biosensor based on glucose oxidase (Dr. Leland C. Clark)
1975	Commercial glucose biosensor (Yellow Springs Inst.) and First microbe-based biosensor, first immunosensor
1976	First bedside artificial pancreas (Miles)
1980	First fiber optic pH sensor for *in vivo* blood gases (Peterson)
1982	First fiber optic-based biosensor for glucose
1983	First surface plasmon resonance (SPR) immunosensor
1984	First amperometric biosensor (glucose oxidase for glucose detection)
1987	Blood-glucose biosensor (MediSense ExacTech)
1990	Surface plasmon resonance (SPR) based biosensor (Pharmacia BIACore)
1992	Handheld blood biosensor (i-STAT)
1996	Launching of Glucocard
1998	Blood-glucose biosensor launch (LifeScan FastTake; Roche Diagnostics)
2008	HLAB-2020 biosensor for detection of *E. coli* 0157:H7 and food allergens (Hanson Technologies) Lipid analyzer ICA-LG 400 (enzyme electrode)for measuring cholesterol, triglycerides, and phospholipids (Toyo Jozo)
2011	Biacore Q biosensor (label free SPR) for vitamin and food contaminant (General Healthcare, USA)
Current	Nanobiosensors using quantum dots, nanoparticles, nanowire, nanotube, and so on.

Table 1.30 Applications of biosensors in different fields

Clinical/medical
- Glucose monitoring in diabetes patients.
- Analysis of electrolytes: Na^+, K^+, Cl^-, Ca^{2+}, pH.
- Analysis of metabolites: glucose, cholesterol, L-Lactate, urea.
- Drugs and neurotransmitters, and so on.

Bioprocess control and food analysis
- Alcohols, amino acids, sugars, carbon dioxide.
- Oxygen, pH, cell concentration, and so on.
- Routine analytical measurement of folic acid, biotin, vitamin B12, and pantothenic acid as an alternative to microbiological assay.
- Detection of pathogens, toxic metabolites such as mycotoxins, and so on.
- Determination of drug residues in food, such as antibiotics and growth promoters, particularly meat and honey.
- Measurement of fish freshness.

Environmental and agricultural
- Detection of heavy metals, pesticides, herbicides, organophosphate BOD, and so on.

Drug development
- Drug discovery and evaluation of biological activity of new compounds.
- Scientific detection of crime and warfare agents.
- Remote sensing of airborne bacteria to counter bioterrorist activities.

and the high cost of many commercial biosensors is not going to be an economically viable alternative.

The global market for biosensors in 2012 is estimated to US$8.5 billion and projected to reach US$16.8 billion by 2018 (http://www.marketresearch.com/Industry-Experts-v3766/Biosensors-Global-Overview-6846583/).

Modes of transduction include (i) *amperometric sensors* that monitor currents generated when electrons are exchanged between a biological system and an electrode, (ii) *potentiometric sensors* (measure the accumulation of charge density at the surface of an electrode), (iii) *optical biosensors* (correlate changes in concentration, mass, or number to direct changes in the characteristics of light), and (iv) other *physicochemical sensors* (monitor biological interactions through changes in enthalpy, ionic conductance, and mass). In *electrochemical biosensor* (Figure 1.27), many chemical reactions produce or consume ions or electrons, which in turn cause some change in the electrical properties of the solution, which can be sensed out and used as measuring parameter by (i) amperometric biosensor, (ii) conductimetric biosensor, and (iii) potentiometric biosensor. In *glucose biosensor*, glucose reacts with glucose oxidase (GOX) to form gluconic acid as well as two electrons and two protons. Glucose mediator reacts with surrounding oxygen to form H_2O_2 and GOX. This GOX can react with more glucose; thus, higher the glucose content, higher the oxygen consumption. The glucose content can be detected by the Pt-electrode.

Recently, arrays of many different detector molecules such as *electronic nose devices* have also been applied. The pattern of response from the detectors is used to fingerprint a substance. In the *Wasp Hound* odor-detector, the mechanical element is a video camera and the biological element is five parasitic wasps that have been conditioned to swarm in response to the presence of a specific chemical. Current commercial electronic noses, however, do not use biological elements. Many optical biosensors based on the phenomenon of SPR are evanescent wave techniques, that utilize a property of gold and other materials. A thin layer of gold on a high refractive index glass surface can absorb laser light, producing electron waves (surface plasmons) on the gold surface. SPR sensors operate using a sensor chip consisting of a plastic cassette supporting a glass plate, one side of which is coated with a microscopic layer of gold.

Piezoelectric sensors utilize crystals that undergo an elastic deformation when an electrical potential is applied to them. An alternating potential (AC) produces a standing wave in the crystal at a characteristic frequency. This frequency is highly dependent on the elastic properties of the crystal, such that if a crystal is coated with a biological recognition element, the binding of a (large) target analyte to a receptor will produce a change in the resonance frequency, which gives a binding signal. In a mode that uses surface acoustic waves (SAW), the sensitivity is greatly increased. This is a specialized application of the quartz crystal microbalance as a biosensor.

Biosensors found in various medical fields show much promise in diagnosing medical conditions and detecting genetic disorders, environmental monitoring of pollution and detection of hazardous chemicals, and in food analysis. Currently, one-shot glucose monitors are one of the most popular commercially available biosensor monitors. Similar handheld devices are being used as well to detect pesticides and pollutants in air and water. Currently, GM microorganisms play an increasingly significant role in improving the capacity of biosensors. Electrochemical and optical types of transducers have been widely employed in microbial biosensors, although bioluminescence and fluorescence methods have been highlighted lately. The *microbial fuel cell* (*MFC*), which has been mainly applied in *BOD biosensors*, is a promising technology with respect to their applications in environmental monitoring and food analysis, including measurement of a variety of

common pollutants, products in fermenting processes, antibiotic residues, and toxins in food.

Applications for food contaminants Although biosensors are not commonly used for food microbial analysis, they have great potential in the detection of microbial pathogens and their toxins in food. They enable fast or real-time detection, portability, and multipathogen detection for both field and laboratory analyses. Several applications have been developed for microbial analysis of food pathogens, including *E. coli* O157: H7, *Staphylococcus aureus*, *Salmonella*, and *Listeria monocytogenes*, as well as various microbial toxins such as staphylococcal enterotoxins and mycotoxins. Biosensors have several potential advantages over other methods of analysis, including sensitivity in the range of nanogram per milliliter for microbial toxins and <100 colony-forming units per milliliter for bacteria. Fast or real-time detection can provide almost immediate interactive information about the sample tested, enabling users to take corrective measures before consumption or further contamination can occur. Miniaturization of biosensors enables biosensor integration into various food production equipment and machinery. Potential uses of biosensors for food microbiology include online process microbial monitoring to provide real-time information in food production and analysis of microbial pathogens and their toxins in finished food.

Biosensors can also be integrated into *Hazard Analysis and Critical Control Point* programs, enabling critical microbial analysis of the entire food manufacturing process. Hanson Technologies (USA) developed HLAB-2020 biosensor for detection of *E. coli* 0157:H7 and food allergens and also introduced *Safe Vegetable Screening System* (*HSVS-1000*) for the ultra rapid detection of pathogens in fresh-cut produce without culturing. The elimination of culturing associated with many hours of delay in waiting for results represents a major advancement in food safety screening. The HSVS-1000 can simultaneously screen for multiple pathogens, allergens and toxins, including *E. coli* O157:H7 and Salmonella, in 2 h or less utilizing large sample sizes.

Many transducers used to detect food-borne pathogens are mostly electrochemical (amperometric), optical (luminescence), SPR, mass/acoustic (piezoelectric), and mass (catntilever), but require more work to prove their use with actual food samples. One such approach is the use of *antibody sandwich assays*, whereby antibodies specific for types of bacteria interact with the bacteria in the sample, a secondary antibody conjugated to a signal-generating moiety either by itself or with a substrate. While fiber optic biosensors that can detect fluorescence, amperometric biosensor method using a *horseradish peroxidase* (*HRP*)-labeled secondary antibody and HRP substrate to generate the signal can be used to *E. coli* 0157:H7 within 1 h after receiving a milk sample, biosensor methods that incorporate a PCR by immobilizing an oligonulceotide specific for a gene of *E. coli* 0157:H7 onto a mass/acoustic (piezoelectric) transducer were used in food samples.

Bacteriophage can also be used for the direct detection of *E. coli* in an array-format electrochemical (conductometric) assay. Several multiplexing biosensors using disposable microarray formats or PCR were tried to detect *E. coli* 0157:H7, *S. typhimurium*, and *Legionella pneumophila*, and so on. PCR tests rely on the power of DNA polymerase to selectively amplify genetic fragments of any agents to make billions of copies, which can then be observed by one of several different methods. However, ELISA and PCR assays even with automation systems take time to run, that are usually labor intensive.

Analysis of food contamination is not only vital for the consumer protection, but also aid the global trade process and barrier. For toxins, pesticide and verterinary drug residues, physicochemical methods such as *liquid chromatography* (*LC*)*–tandem mass spectrometry* (*MS*/*MS*) are used to confirm the presence of these compounds, but these methods are

expensive, complicated to operate, and time-consuming. Current methods in this area have limitations; thus, future directions should optimize performance, develop portable biosensors for onsite monitoring, combine genetic and DNA approaches, nanotechnology, and phage-based biosensors for food-borne pathogens. Several applications of biosensors in food analysis are optic coated with antibodies that are commonly used to detect pathogens and food toxins. The light system in these biosensors has been fluorescence, since this type of optical measurement can greatly amplify the signal. The use of portable biosensors, such as commercial glucose biosensors on the market, will revolutionize medical diagnosis and environmental monitoring by allowing point-of-care and onsite testing. Few portable optical, *SPR* biosensors are already used in (i) detection of antibiotic residue in milk as well as toxins in shellfish, (ii) elctrochemical (conductometric) biosensor system for bacterial concentration measurements in dairy products, and (iii) optical (flurorescence) array biosensor for botulinum neurotoxins (BoNTs). The form of analyte may range from macromolecule to a microelement and heterogeneous distribution of analyte in the food has made the situation worse for the analyst. In most cases, the analyst needs to separate the analyte from the food before detection.

Advances made until now in biosensor applications for the detection of food contaminants are quite varied, but advantages of biosensors are significant compared with other conventional methods such as *radioimmunoassay*, *enzyme-linked immunosorbent assay*, *fluorescence immunoassay*, and *luminescence immunoassay*. The main areas of development should include multiplexing, the ability to simultaneously analyze a sample with the potential of unrelated pathogens, toxins, pesticides, or drug residues. Chapter 9 in the first edition has described the details of biosensor for foods.

1.11.2 Nanobiotechnology and nanobiosensor

Nanobiotechnology is the application of *nanotechnology* to life sciences including the application of nanoscale tools to biological systems and the use of biological templates to create nanoscale products. Nanotechnology that focuses on the understanding and control of matter at approximately 1–100 nm (a nanometer is a billionth of a meter) has become one of the most promising scientific fields of research in recent decades and has the potential to provide new solutions on medical and food industries, for example, for disease-treatment delivery methods and biosensors for pathogen detection. Table 1.31 shows applications of nanobiosensors underway or commercialized in various fields.

Nanobiotechnology applications are broad: nanomedicine, nanobiosensors (biochips), nanofluidics, molecular self-assembly, intelligent drug delivery systems, and nanomachines, that become the convergence of engineering and molecular biology.

The application of nanotechnology to biosensor design and fabrication promises to revolutionize diagnostics and therapy at the molecular and cellular level. *Nanobiosensors* are based on a combination of nanotechnology with varying biosensing techniques with the aim of improving existing clinical practices and rapid detection of bacteria and viruses. The nanobiosensors are equipped with immobilized bioreceptor probes such as antibodies and enzyme substrate, and laser excitation is transmitted to photometric system in the form of optical signal (fluorescence).

Nanobiosensors will first revolutionize the future of disease diagnosis and other biomedical applications. The potential for monitoring *in vivo* biological processes within single living cells, for example, the capacity to sense individual chemical species in specific locations within a cell, will also greatly improve our understanding of cellular function, thereby revolutionizing cell biology. Existing nanoprobes have already demonstrated the capability of performing biologically relevant measurements inside single living cells.

Table 1.31 Applications of nanobiosensors underway or commercialized for biomedicals, environments, cosmetics, packagings, and food analysis

Types	Applications
Biomedicals	
Submicron fiber-optic nanotubes	Monitoring pH, nitric acid
SPOT-NOSED on nanoelectrode	Diagnose diseases at early stages (with a layer of olfactory proteins)
Nanosphere lithography (NSL)	Detect streptavidin (1 pm concentrations) with Ag nanoparticles
Antibody based piezoelectric	Anthrax, HIV hepatitis
Optical with mouse anticytochrome	Detect cytochrome C in a single cell *c* antibodies
Multiple biomarker system	Vista's NanoBioSensor™ (NBS)
Cosmetics	
TiO_2 + coating	Sun protecting ream
Lipid nanoparticles	Q10 cream
Nanoencapsulation	Tip-top up (omega 3 bread)/canola active oil
Food packaging/composite/others	
Nanofood packaging	Film (Bayer)
Nano clay particle	Beer bottle (Nano Co. Inc)
Nano cleaning	Emulsion disinfectant
Nano catalytic devise	Protect oil breakdown (www.oilfresh.com)
Nano feed	Nanoselenium for chicken (Attair Nanobiotech Ltd)
Carbon nanotubes in food packaging	Antimicrobial effects on *E. coli*, and so on.
Food additives/contaminants	
Carbon nanofibers (CNF)	Detection of food pathogens
Carbon nanotubes (MWCNTs-chitosan)	Detection for organophosphate insecticide
Acetylcholinesterase (AChE) on multiwall carbon nanotubes-chitosan	Detection of organophosphate and caramate pesticides
Magnetic nanoparticles conjugated with specific antibodies using interdigitated array microelectrode	Detection of *E. coli* O157:H7
Direct-charge transfer (DCT) nanowire Immunosensor	Detection of many pathogens including *Bacillus cereus*, and *Vivbrio parahaemolyticus*.
Nanoporous silicon-based DNA biosensor	Detection of *E.coli* and *Salmonella*
Quantom dot (QD) nanobiosensors based on FOF1-ATPase	Detection of virus
Amperometric penicillin nanobiosensor using multi-walled carbon nanotubes (MWCNTs), hematein, and β-lactamase	Detection of penicillin in milk
Animal husbandry/Aquaculture	
Adhesin-specific nanoparticles	Removal of pathogens from livestock
Nanoparticles	Detection of toxins in feed, drug residues,
Nanoparticles/platinum-alumina cryogel	Prevention of diseases spread in animals
Nanowires	Detection of pollutants in aquaculture

Source: Adapted from Li YH and Tian X. 2012. Sensors (Basel), 12, 2519–2153.

A fiber-optic nanosensor basically is a nanoscale probe that consists of a biologically or chemically sensitive layer that is covalently attached to an optical transducer. Various kinds of nanomaterials, such as *gold nanoparticles* (*GNPs*), *carbon nanotubes* (*CNTs*), *magnetic nanoparticles*, and *quantum dots*, are being gradually applied to biosensors because of their unique physical, chemical, mechanical, magnetic and optical properties, and markedly enhance the sensitivity and specificity of detection. GNPs show a strong absorption band in the visible region due to the collective oscillations of metal conduction band electrons in strong resonance with visible frequencies of light, which is called SPR. There are several parameters that influence the SPR. It is well known that well-dispersed solutions of GNPs display a red color, while aggregated GNPs appear a blue color. Based on this phenomenon, a GNPs-based biosensor to quantitatively detect the polyionic drugs such as protamine and heparin was established.

Despite the fact that biosensors are promising devices when it comes to fast and easy detection of analytes, their use has not yet been established in clinical routine to replace immunoassay techniques. Numerous publications and patents dealing with nanobiosensors have been published, but only a small part of the work reported was performed using real samples. The problem of nonspecific binding arising from complex sample matrices is yet to be solved. Biosensors based on *molecular switches* offer a promising tool to solve this problem, because only the analyte binding is able to generate a signal response and exclude cross-reactivity.

Biosensors are, in fact, artificial molecular switches that mimic the natural ones, which direct chemical responses throughout the cell. They help to build biologically based sensors for the detection of chemicals ranging from drugs to explosives to disease markers. All creatures from bacteria to humans have been monitoring their environments in order to survive with biomolecular switches, made from RNA or proteins. Molecular switches have been reported for optical and electrochemical transduction principles, which are currently the most commonly used transduction principles for biosensors. As disease-related marker profiles are still under investigation, specifications regarding a suitable biosensor instrument can hardly be given at the moment. However, almost certainly such a device should permit multiplex analysis to determine a marker profile in a sample in one measurement cycle. Therefore, a biosensor array would be required. At least as long as these profiles are not established, it could be useful to keep these arrays flexible. This would be supported by a packaging strategy in which each biosensor element is integrated in a single, array-compatible housing, allowing user-defined combination. This would make the arrays potentially adaptable to the respective application and hence make the underlying biosensor instrument more versatile. *RNA biochip* as biosensor elements that can be used to detect toxic metals such as cobalt, drug compounds such as theophylline, and natural compounds such as cyclic AMP and cyclic GMP in a single assay are under testing. The latest knowledge in nanobiotechnology and enzyme engineering will eventually lead to have low-cost, easy-to-operate in homes and doctor's office to diagnose patients and detect contaminants and biological attacks.

Food nanotechnology is an emerging area, and tens of millions of dollars are being spent in a global race to apply nanotechnologies in food production, processing, and packaging. Most applications of nanotechnology are currently in food packaging to improve plastic materials bareers, and incorporation of functionally bioactive components in packaging materials to extend shelf-life, improve food safety, and improve taste. Nanotechnology analysts estimated that about 150–600 nanofoods and 400–500 nanofood packaging applications are already on the markets (www.scribd.com/doc/9197096/Nano-food). The main areas of application include food packaging and food products containing nano-sized or nanoencapsulated ingredients and additives. CNTs used in food packaging

exhibited powerful antimicrobial effects on *E. coli*. A simple method for immobilization of acetylcholinesterase (AChE) on multiwall carbon nanotubes (MWCNTs)-chitosan composite was proposed to detect organophosphate and caramate pesticides. Magnetic nanoparticles conjugated with specific antibodies have been used to detect *E. coli* O157:H7 using interdigitated array microelectrode in ground beef samples. A direct-charge transfer (DCT) nanowire immunosensor could be used to detect many pathogens including *Bacillus cereus*, and *Vivrio parahaemolyticus*. A nanoporous silicon-based biosensor product is produced by a company that is aimed at detecting the bacteria *E. coli* and *Salmonella* by analyzing their DNA. Quantom dot (QD) nanobiosensors based on F0F1-ATPase have been tried to detect avian influenza or other virus; fluorescence intensity of orange and green QD biosensors with and without virus can be used to detect virus when the ADP is added to initialize reaction. Another example is an amperometric penicillin biosensor with enhanced sensitivity; it was successfully developed by coimmobilization of *MWCNTs*, hematein, and β-lactamase on glassy carbon electrode using a layer-by-layer assembly technique. This nanobiosensor offered a minimum detection limit of 50 nm for penicillin V, much lower than conventional methods.

Summary

As biosensor technology advances, biosensors are now being developed for detection of microbial pathogens, cancer detection, and monitoring. In addition, biosensor technology is being applied to food analysis such as food pathogens, allergens, food and biomaterial quality testing, and basic research on molecular interactions. Biosensors offer several advantages over other analytical methods including rapid and even real-time measurements, high sensitivity, selectivity, and specificity even when a complex or turbid sample matrix is used. As the technology advances, producing lab-on-a-chip devices, these self-contained portable instruments will allow measurements outside the laboratory, in the field or at the bedside. Biochip technologies could offer a unique combination of performance capabilities and analytical features of merit, and allow simultaneous detection of multiple biotargets. Nanosizes of the probes minimize sample requirement and reduce reagent and waste requirement. For medical applications, this cost advantage will allow the development of extremely low cost, disposable biochips that can be used for in-home medical diagnostics of diseases without the need of sending samples to a laboratory for analysis.

Health aspects of nanotechnology and nanoparticles are described together in Section 1.13.

1.12 Quorum sensing and quenching

Quorum sensing (*QS*), which involves a regulation of genes in response to population density, is a cell-to-cell signaling mechanism used among various species of bacteria. In QS, bacteria release chemical signal molecules called autoinducers into the surrounding environment. When the number of cells is low, the concentration of autoinducers is also low, and the bacteria seem benign. However, when a certain threshold of cell density is reached, the autoinducers are internalized into the cell and the population of cells responds to the environment. The type of this response vary among different species of bacteria, and can vary from emitting fluorescent light, formation of biofilms, initiation of sporulation, production of antibacterial agents, motility, or conjugation (Table 1.32). A significant portion of bacterial genome (4–10%) and proteome (about 20%) can be influenced by QS.

Table 1.32 Examples of bacterial quorum sensing controlled processes and traits

Microorganism	Major signal molecules	Regulatory system	Benefits/traits
Vibrio fischeri	HAI-1, CAI-1 AI-2	LuxLM/LuxN LuxP/LuxQ	Bioluminescence emission, *V. harveyi* Symbiosis
Bacillus subtlis	ComX CSF (PhrC) PhrA, -E, -F, -K, -H	ComP/ComA Rap proteins	Competence, sporulation, biofilm formation, antibiotic production
Myxococcus xanthus	A-signal C-signal	SasSRN	Fruiting body formation or sporulation
Pseudomonas aeruginosa	3O-C12-HSL C4-HSL	LasI/LasR RhlI/RhlR OscR (orphan)	Biofilm formation, virulence factors
Staphylococcus aureus	AIP-I, AIP-II, AIP-II, AIP-IV	AgrC/AgrA	Biofilm formation, virulence factors
Streptococcus mutans	CSP (ComC) XIP (ComS)	ComD/ComE ComR	Bacteriocins, biofilm formation, competence
Streptococcus pneumoniae	CSPs	ComD/ComE	Competence, fratricide, biofilm formation, virulence

Source: Adapted from Li YH and Tian X. 2012. Sensors (Basel), 12, 2519–2153.

QS was first described in *Vibrio fischeri*, a luminescent marine bacterium. *V. fischeri* can be free-living or grow in light organs of their symbiotic hosts such as squid and fish. *V. fischeri* generate the light that has similar intensity to that of the moon and starlight above, protecting the host from predators by making them invisible to predators below. Thus, in this circumstance, the purpose of QS is symbiosis. In this bioluminescent system shown in Figure 1.28, (i) *LuxI* gene (encodes an autoinducer synthase (LuxI) produces the autoinducer *N-homoserine lactone* (*HSL*), (ii) HSL exits the cell and re-enters freely against gradient when external concentration reaches a threshold value, (iii) HSL binds to the *LuxR* gene (a transcription factor), (iv) HSL-LuxR complex binds upstream of the *LuxL*CDABE operon, and (v) increased transcription of luciferase system as well as the exponential increases in *LuxI* synthesis.

Another example of microorganism that exhibits QS mechanism is *Staphylococcus aureus*. Like in the case of *Vibrio fischeri*, *S. aureus* responds to its environment in population-dependent manner. In this case, however, the QS mechanism is involved with the pathogenesis of *S. aureus*. *S. aureus* found in approximately 30% of the adult population are part of normal flora of human intestinal tract. However, when these organisms or their extracellular products breach the epithelial layer, serious diseases can result. QS enhances the bacteria's pathogenicity by producing virulence factors. Also, it helps with formation of biofilms, groups of interacting cells, and enhances pathogenicity because biofilms are often resistant to antibiotic therapy and clearance by hosts.

Another microorganism that utilizes quorum sensing for its pathogenesis is *Pseudomonas aeruginosa*. *P. aeruginosa*, an opportunistic pathogen often isolated in nocosomial infections, secretes virulence factors which allow for its pathogenicity

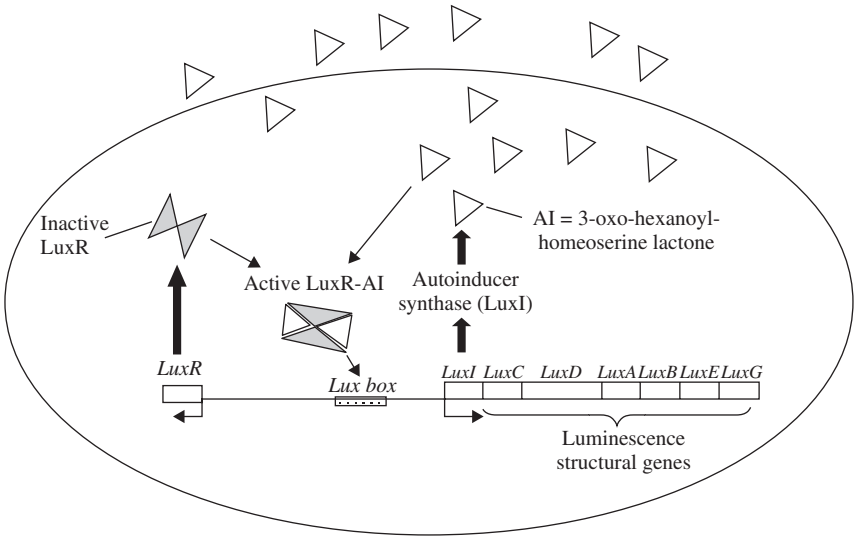

Figure 1.28 Model of quorum sensing in *Vibrio fischeri*. *Source*: Popham DL and Stevens AM 2006. Bacterial quorum sensing and bioluminescence, pp. 201–215. In Tested Studies for Laboratory Teaching, Vol. 27 (MA O'Donnell, ed.). Proceedings of the 27th Workshop/Conference of the Association for Biology Laboratory Education (ABLE). Reproduced with permission.

and the QS regulates gene expression of some of these extracellular factors. In addition, the QS plays a role in a development of biofilms, which greatly enhances the bacteria's pathogenicity.

As the QS system regulates expression of some virulence factors and contributes to the pathogenicity of bacteria, the QS is a possible candidate for an antibacterial drug target. As a result, there are numerous researches going on to use this system to reduce pathogenesis of bacteria.

Active research is underway to (i) discover new drugs that inhibit QS, so-called *quorum quenching* (*QQ*), thus generating a new type of antibiotic drug for the infectious pathogens, and (ii) inhibit QS system, to prevent food pathogens and food spoilages.

The extensive use of antibiotics brought the problem of emergence of drug resistance of pathogens to the medical society. The problem of drug resistance among pathogenic bacteria is facing more difficulties with the emergence of multidrug resistance strains and resistance even to the most developed drugs. Inevitably, an alternative to antibiotics is a necessity. Inhibiting QS is ideal as QS is not directly involved with survival of the pathogen. It does not seem to put much survival pressure on microorganisms to develop resistance against this type of inhibitors.

One system that was suggested as a potential antibacterial drug target is *two-component signal transduction system*, that is made up of two parts: a *sensor kinase* and a *response regulator*. The first part, a sensor kinase, responds to an environmental signal with autophosphorylation; the response regulator then interacts with the kinase to regulate the gene expression.

Among many genes that are regulated, genes that are involved in virulence and biofilms are also controlled. Blocking this system will thus work as an antibacterial drug.

Furthermore, this system works in a different method from conventional antibiotics, and this could provide alternatives to the drug-resistance problem.

Biofilm and quorum sensing The QS plays an important role in the development of biofilms. Formation of biofilms contributes to the pathogenecity of bacteria because it can assist in colonization by other pathogens, or can increase resistance to conventional antibiotics. For example, *P. aeruginosa* uses the quorum sensing to form biofilms in the lungs of cystic fibrosis patients and causes a major problem in treating chronic patients. It was discovered that mutations of *lasI* system of quorum sensing results in structurally altered biofilms. The biofilms contribute to the persistence and severity of *P. aeruginosa* (Kalia and Purohit, 2011). It also contributes to increased virulence because the presence of biofilms provides a chance for secondary pathogens to colonize the patients. With such roles that biofilms play in virulence of pathogens, it is clear that inhibition of biofilms will result in the reduced pathogenicity. Consequently, as a method to inhibit biofilms, the inhibition of the QS is being suggested. In a recent research, QS inhibitors were shown to increase *P. aeruginosa*'*s* susceptibility to antibiotics both *in vitro* and *in vivo* (Brackman et al., 2011).

Autoinducers The autoinducers involved in QS system differ in Gram-positive and Gram-negative bacteria. The molecule is known as oligopeptides in Gram-positive bacteria. The oligopeptides signals can be processed with side chain modifications. For instance, in *Staphylococcus* species, thiolactone rings are added to the oligopeptides. Also, in *B. subtilis*, isoprenyl groups are added to the oligopeptides. In Gram-negative bacteria, the QS uses *acylhomoserine lactones* (*AHLs*) as signaling molecules. The specificity of AHLs is determined by the differences in the length, degree of saturation, and substitution of the acetyl side chains. By understanding the mechanisms of autoinducers' antagonists, potent QS inhibitors can be designed.

Inhibitors To develop the inhibitors against the QS, it is essential to know what properties will result in the most ideal QS inhibitors. First, an ideal *quorum sensing inhibitors* (*QSI*) should be chemically stable and not be easily degraded by the host. It should also have low-molecular-mass and should significantly downregulate the expression of genes controlled by QS. This downregulation has to be highly specific. In other words, it should only downregulate the virulence-related genes regulated by QS, and not affect the expression of gene related to bacterial growth or survival. If the inhibitor negatively affects the expression of such genes, the survival pressure will push the pathogens to develop resistance against the inhibitors.

Three strategies can be applied for inhibition of QS. The first method is to block signal production. The second strategy is to inactivate signal molecules, which can be achieved in two ways: either use antagonist of autoinducers or degrade signals that are already created. The last method is to target the signal receptor.

Nanofactories Engineered biological nanofactories were suggested as a method to control on and off the QS (Fernandes et al., 2010). In other words, to inhibit pathogenesis, they can be used either to keep the QS off or turn on the QS even when the amount of bacteria is below the threshold level. This suggests that these nanofactories can induce the QS, and subsequently pathogenesis when the amount of bacteria would normally be insufficient to cause illness. The pathogens can then be detected and be cleared by the body's immune system. This "biological nanofactory" is made up of antibody for targeting and a fusion protein that produces autoinducer when bound to the target microorganism.

Summary

While some bacteria are communicating with neighboring cells (quorum sensing), others are interrupting the communication (QQ), thus creating a constant arms race between intercellular communication. QS in food microbiology and biotechnology became very important in the relationship between QS and food spoilage, enzyme and toxin production in food spoilage, and biofilm formation in food processing industries. Also this knowledge in the inhibition of biofilm formation and other QS interference mechanism naturally present in food and transgenic plants expressing the QS signal inactivating enzymes could be an alternative to control food spoilage and the potentially hazardous food-borne bacterial contamination.

Recent evidence extends the role of QQ to detoxification or metabolism of signaling molecules as food and energy source. While QQ has been explored as a novel anti-infective therapy targeting, quorum sensing evidence begins to show the development of resistance against QQ.

As antibiotic-resistant bacteria become a global threat to public health, novel therapeutics represent an important area of current scientific research. QS is a key regulatory system that controls the expression of virulence determinants, thus making QS an effective target for novel drug design as well as agricultural and industrial applications. QQ provides a strategy to disrupt QS, and in turn attenuates virulence determinants. The future of QS research lies in the discovery of additional communication signals, and thus QQ as a promising anti-infective strategy can be developed based on information obtained from QS studies for biotechnological and pharmaceutical applications.

1.13 Micro- and nano-encapsulations

Micro- or nanocapsules are small liquid, solid, gas, or living element coated by another substance in order to protect and separate the materials. *Micro-* or *nanoencapsulation* is a process in which tiny particles or droplets are surrounded by a coating to give small capsules with many useful properties. In its simplest form, a micro- or nanocapsule is a small sphere with a uniform wall around it. Core material can be released from micro- or nanocapsules when their walls burst under outside pressure, melt under heat influence, burn under high temperature, degrade under the influence of light, dry out, dissolve in a solvent or the core material is released gradually through the permeable wall of the microcapsule. Those capsules can be of various forms such as emulsion, suspension or powder with a variety of core materials, wall thicknesses, and particle sizes. Under the microscope single micro- or nanocapsules, clustered micro- or nanocapsules, multicore micro- or nanocapsules can be distinguished. Micro- or nanocapsules are often of spherical form, but micro- or nanocapsules of irregular forms are also known. The products obtained by a process of enclosing micron-sized particles of solids or droplets of liquids or gasses in an inert shell are called microparticles, microcapsules, and microspheres which differentiate in morphology and internal structure. When the particle size is below 1 μm, they are called *nanoparticles*, *nanocapsules*, *nanospheres*, and particles in diameter between 3 and 800 μm are known as microparticles, microcapsules, or microspheres (Figure 1.29).

Despite the similarity of both encapsulation, many micro- or nanocapsules bear little resemblance to these simple spheres. The core may be a crystal, a jagged adsorbent particle, an emulsion, a suspension of solids, or a suspension of smaller micro- or nanocapsules even with multiple walls.

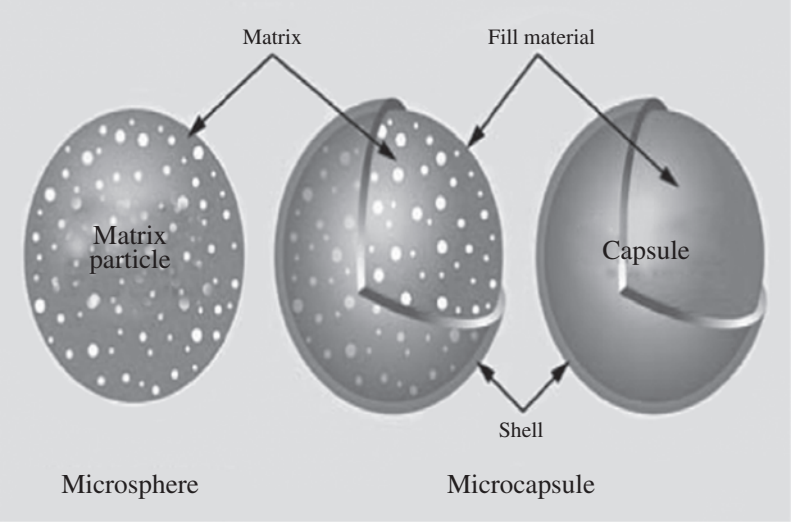

Figure 1.29 Particle size and morphology of microsphere and microcapsule.

Technologies of micro- and nanoencapsulation are currently providing solutions to complex issues such as sustained release and targeted delivery. The cell microencapsulation technology involves immobilization of the cells within a polymeric semipermeable membrane that permits the bidirectional diffusion of molecules such as the influx of oxygen, nutrients, growth factors, and so on that are essential for cell metabolism and the outward diffusion of waste products and therapeutic proteins. At the same time, the semipermeable nature of the membrane prevents immune cells and antibodies from destroying the encapsulated cells considering them as foreign invaders. The main motive of cell encapsulation technology is to overcome the existing problem of graft rejection in tissue engineering applications and thus reduce the need for long-term use of immunosuppressive drugs after an organ transplant to control side effects. Many varieties of both oral and injected pharmaceutical formulations are also microencapsulated to release over longer periods of time or at certain locations in the body. Aspirin tablets that can cause peptic ulcers and bleeding are often produced by compressing quantities of microcapsules that will gradually release the aspirin through their shells to avoid adverse effects such as gastric irritation or stomach damage by the drug.

The areas of applications are broad including food industry, pharmaceuticals, cosmetics, electronics, photography, agriculture, chemical industry, textile industry, graphic industry, biotechnology, and so on. However, micro- and nanoencapsulation technologies aiming at food and biotechnology are:

1. Biocatalysis in food processing and biotechnology on immobilization and microencapsulation of living cells, bioactive substances and functional food ingredients for controlled and effective delivery into food systems (e.g., antioxidant, anti-inflammatory, antimicrobial, anticancer compounds, enzymes, probiotics, etc.);
2. Improvement of quality, nutritional value, safety, and shelf-life of food (oxidation stability, etc.);

3. Design and utilization of food-grade micro- and nanoemulsions for delivery of nonpolar functional components (bioactive lipids, hydrophobic drugs, etc.);
4. Isolation, chemical modification, and purification of biopolymers (specifically cationic/anionic oligosaccharides and hydrophobic derivatives);
5. Biodegradable food-packaging materials (mainly cellulose and starch-based materials, biodegradable polymer films), and surface modification;
6. Polymer characterization by LC techniques and rheology;
7. In the case of food manufacturing, the current state of nanoencapsulation applications is not clear, though some products are already on the market. Fundamental research is ongoing for effective transfer of knowledge into innovation and to deal with considerations regarding risks, regulatory aspects, and consumer skepticism. Little is known about the unintended consequences of nanoparticles and how the particles interact with the environment and human body. No general conclusion can be made on the safety of nanofood and food contact materials incorporated with nanomaterials.

According to WHO, new data and measurement approaches are needed to ensure safety of products using nanotech can be properly assessed. Nanobiotechnology can easily fall into the premade trap of genetically modified foods (GMFs).

1.13.1 Microencapsulation

Microcapsules can be classified into three categories according to their morphology: (i) mononuclear (core–shell) capsules containing the shell around the core, (ii) polynuclear capsules which have many cores enclosed within the shell, and (iii) matrix types which are distributed homogeneously into the shell materials. In addition to these three morphologies, microcapsules can also be mononuclear with multiple shells, or they may form clusters of microcapsules.

Microparticles or microcapsules consist of two components, namely core material and coat or shell materials. Core material contains an active ingredient while coat or shell material covers or protects the core materials. Different types of materials such as active pharmaceutical ingredients, proteins, peptides, volatile oils, food materials, pigments, dyes, monomers, catalysts, pesticides, and so on, can be encapsulated with different types of coat materials such as ethylcellulose, hydroxyl ethylcellulose, carboxymethylcellulose, sodium alginate, poly-lactic-glycolic acid (PLGA), gelatin, polyesters, chitosan, and so on. To a large degree, the selection of the appropriate coating material dictates the resultant physical and chemical properties of the microcapsules. The coating material should be able to form a film that is cohesive with the core material, chemically compatible and nonreactive with the core material; it should also be able to provide the desired coating properties on strength, flexibility, impermeability, optical properties, and stability.

Most common applications of microencapsulation are in food ingredients as most flavorings are volatile. Encapsulation of these components can thus extend the shelf-life of products by retaining within the food flavors. Some ingredients are encapsulated to mask the organoleptic properties like taste or flavors of the products and even to last longer, as in chewing gum. The amount of encapsulated flavoring required is substantially less than liquid flavoring, as liquid flavoring is lost and not recovered during chewing. Flavorings that are comprised of two reactive components can be encapsulated individually, that add to the finished product separately so that they do not react and lose flavor potential prematurely. Some flavorings must also be protected from oxidation or other reactions caused by exposure to light.

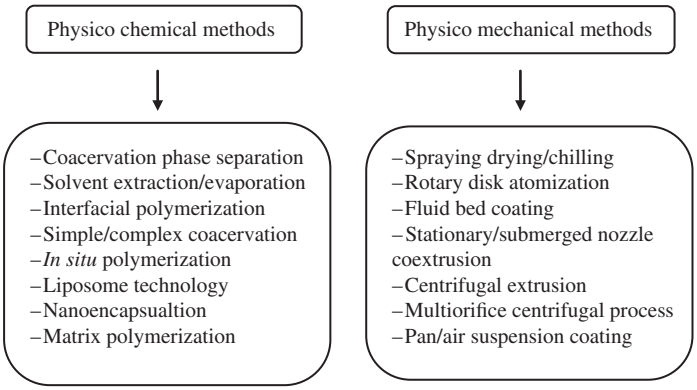

Figure 1.30 Manufacturing techniques of microcapsules.

Many factors are to be considered when selecting the encapsulation process and also many different techniques are available for the encapsulation of core materials. In general, microencapsulation processes are usually categorized into (i) Physicochemical methods and (ii) physical methods (Figure 1.30). However, these labels can be somewhat misleading, as some processes classified as mechanical might involve or even rely upon a chemical reaction, and some chemical techniques rely solely on physical events.

1.13.1.1 Physicochemical methods Capsules for carbonless paper and for many other applications are produced by a chemical technique called complex coacervation. This method takes advantage of the reaction of aqueous solutions of cationic and anionic polymers such as gelatin and gum arabic. The polymers form a concentrated phase called the complex coacervate. The coacervate exists in equilibrium with a dilute supernatant phase. As the water-immiscible core material is introduced into the system, thin films of the polymer coacervate coat the dispersed droplets of core material. The thin films are then solidified to make the capsules harvestable. In *interfacial polymerization* (*IFP*), which is another chemical method of microencapsulation, the capsule shell is formed at or on the surface of the droplet or particle of dispersed core material by polymerization of the reactive monomers. After a multifunctional monomer is dissolved in the core material, this solution is dispersed in an aqueous phase. When a reactant to the monomer is added to the aqueous phase, polymerization quickly occurs at the surfaces of the core droplets, forming the capsule walls.

IFP can be used to prepare bigger microcapsules, but most commercial IFP processes produce smaller capsules in the 20–30 μm diameter range for herbicides and pesticide uses, or even smaller 3–6 μm diameter range for carbonless paper ink. *In situ* polymerization is a chemical encapsulation technique very similar to IFP. The distinguishing characteristic of *in situ* polymerization is that no reactants are included in the core material. All polymerization occurs in the continuous phase, rather than on both sides of the interface between the continuous phase and the core material, as in IFP. Examples of this method include *urea-formaldehyde* (*UF*) and *melamine formaldehyde* (*MF*) *encapsulation systems*. Polymer–polymer incompatibility, also called phase separation, is generally grouped with other chemical encapsulation techniques. This method utilizes two polymers that are soluble in a common solvent, and yet do not mix with one another in the solution. The polymers

form two separate phases, one rich in the polymer intended to form the capsule walls, the other rich in the incompatible polymer meant to induce the separation of the two phases. The second polymer is not intended to be part of the finished microcapsule wall, although some may be caught inside the capsule shell and remain as an impurity.

Solvent evaporation technique is currently used by companies including the NCR Company, Gavaert Photo - Production NV, and Fuji Photo Film Co., Ltd. to produce *microcapsules*. Three phases including core, coat material and *liquid manufacturing vehicle* (*LMV*) are present. The microcapsule coating is initially dissolved in a volatile solvent, which is immiscible with the LMV phase. A core material is dissolved in the coating polymer solution. The core coating material mixture is dispersed with agitation in the LMV phase to obtain the appropriate size microcapsule. The mixture is then heated to evaporate the solvent for polymer. Once the core material is dispersed in the polymer solution, the polymer shrinks around the core. In case the core material is dissolved in the coating polymer solution, a matrix type microcapsule is formed. After all the solvent for the polymer is evaporated, the liquid vehicle temperature is reduced to ambient temperature with agitation and then the microcapsules can be used in the suspension form, coated onto substrates or isolated as powders. The core materials may be either water soluble or water insoluble materials. A variety of film forming polymers can be used as coatings.

Liposome microencapsulation has been used mostly in pharmaceutical applications to achieve targeted delivery of vaccines, hormones, enzymes, and vitamins. However, technology has evolved in recent years to the point that it is now conceivable for liposome encapsulation to become a routine process in the food industry. In liposome entrapment, liposomes consist of an aqueous phase that is completely surrounded by a phospholipid-based membrane. When phospholipids such as lecithin are dispersed in an aqueous phase, the liposomes form spontaneously. Liposomes are vesicles consisting of a lipid bilayer enclosing an aqueous compartment at the center and thus are nontoxic and acceptable for foods. The lipid bilayer used in the liposomes are usually made of phospholipids, that are arranged in such a way that the hydrophilic head is exposed outside and the lipophilic tails are aliened inside. This makes the liposomes water-soluble molecules. Permeability, stability, surface activity, and affinity can be varied through size and lipid composition variations. They can range from 25 nm to several microns in diameter, are easy to make, and can be stored by freeze drying. As shown in Figure 1.31, liposomes are structurally classified on the basis of lipid bilayers such as *small unilamellar vesicles* (*SUVs*: 20–200 nm), *multilamellar vesicles* (*MLVs*: >0.5 μm), *giant unilamellar vesicles* (*GUVs*: >1 μm) and *large unilamellar vesicles* (*LUVs*: 200 nm to 1 μm).

Liposome can be incorporated with drugs (antibiotics, antifungals), proteins/polypeptides (including antibiotics), and virus and bacteria can be incorporated in the liposomes. Hydrophilic drugs are incorporated within the central cavity which is hydrophilic, and lipophilic drug molecules are incorporated in the lipid bilayer. LUVs are the most appropriate liposomes for the food industry because of their high encapsulation efficiency, their simple production methods, and their good stability over time. The great advantage of liposomes over other microencapsulation technologies is the stability of liposomes to water-soluble material in high water activity application. Spray-dryers, extruders, and fluidized beds impart great stability to food ingredients in the dry state but release their content readily in high water activity application, giving up all protection properties. Another advantage of liposomes is the targeted delivery of their content in specific parts of the foodstuff. For example, it has been shown that liposome-encapsulated enzymes concentrate preferably in the curd during cheese formation, whereas nonencapsulated enzymes are usually distributed evenly in the whole milk mixture, which leads to very low (2–4%) retention of the flavor-producing enzymes in the curd.

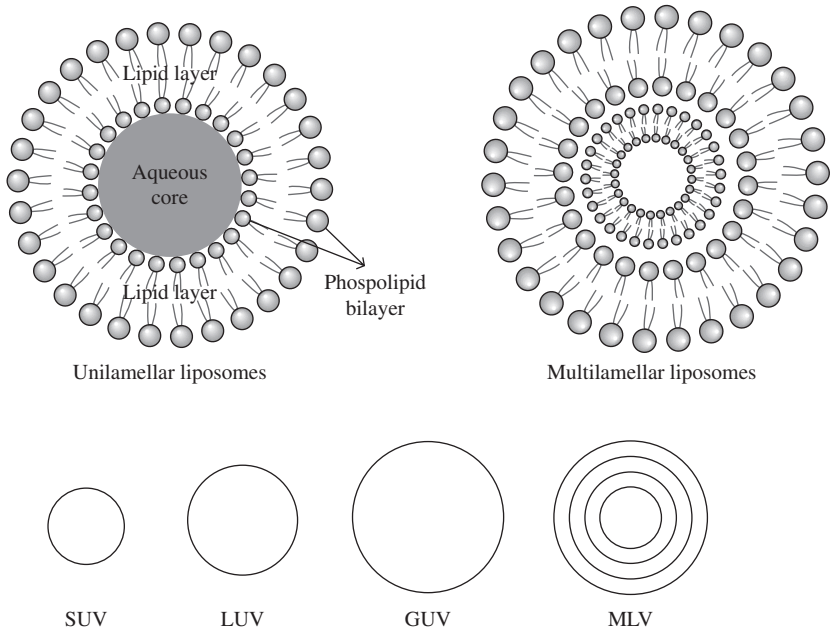

Figure 1.31 Schematic representation of basic structures and different types of liposomes. *Source*: Mishra GP, et al. 2011. Recent Applications of Liposomes in Ophthalmic Drug Delivery. Article ID 863734; doi:10.1155/2011/863734. Reproduced with permission from Journal of Drug Delivery.

Bromelain-loaded liposomes were also used as meat-tenderizer to improve stability of the enzyme during the food processing. Liposome-entrapped nisin retained higher activity against *Listeria*, which have improved stability in cheese production, proving a powerful tool to inhibit the growth of *Listeria* in cheese. Encapsulation of vitamin C also significantly improves the shelf life from a few days to up to 2 months, especially in the presence of common food components that would normally speed up decomposition, such as copper ions, ascorbate oxidase, and lysine. A cost-effective drying method of liposome microcapsules and a dry liposome formulation that readily reconstitutes upon rehydration would ensure a promising future to liposome encapsulation of food ingredients. Microfluidization has been shown to be an effective, cost-effective, and solvent-free continuous method for the production of liposomes with high encapsulation efficiency. The method can process a few hundred liters per hour of aqueous liposomes on a continuous basis. Liposome formulations are normally kept in relatively dilute aqueous suspensions that might be a serious drawback for the large-scale production, storage, and shipping of encapsulated food ingredients.

In an attempt to avoid the use of organic solvent in the production of liposomes, *supercritical fluids* like carbon dioxide, alkanes (C_2 to C_2) and nitrous oxide (N_2O) were used as the solvent for the phospholipids. In this process, supercritical fluid containing the active ingredient and the shell material are maintained at high pressure and then released at atmospheric pressure through a small nozzle. In this process called, *rapid expansion of supercritical solution* (*RESS*), the sudden drop in pressure causes desolvation of the shell material, which is deposited around the active ingredient (core) and forms a coating layer.

Although this method is scientifically interesting, the encapsulation efficiency is limited at 15% and also very few polymers with low cohesive energy densities are soluble in supercritical fluids.

1.13.1.2 Physicomechanical methods Centrifugal force processes were developed in the 1940s to encapsulate fish oils and vitamins, protecting them from oxidation. In this method, an oil and water emulsion is extruded through small holes in a cup rotating within an oil bath. The aqueous portion of the emulsion is rich in a water-soluble polymer, such as gelatin, that gels when cooled. The resulting droplets are cooled to form gelled polymer-matrix beads containing dispersed droplets of oil that are dried to isolate. Similar in concept to centrifugal force processes, submerged nozzle processes produce microcapsules when the oil core material is extruded with gelatin through a two-fluid nozzle. The oil droplets are enveloped in gelatin as they are extruded through the nozzle and then the capsules are cooled to gel the walls before being collected and dried. Centrifugal extrusion processes generally produce capsules of a larger size from 250 μm up to a few millimeters in diameter. The core and the shell materials which should be immiscible with one another are pushed through a spinning two-fluid nozzle. This movement forms an unbroken rope which naturally splits into round droplets directly after clearing the nozzle. The continuous walls of these droplets are solidified either by cooling or by a gelling bath, depending on the composition and properties of the coating material.

Spray drying, either hot or cold media, is a mechanical microencapsulation method developed in the 1930s and is a low-cost commercial process, which is mostly used for encapsulating fragrances, oils, and flavors. Core particles are dispersed in a polymer solution, usually an oil or active ingredient immiscible with water and sprayed into a hot chamber. The resultant emulsion is atomized into a spray of droplets by pumping the slurry through a rotating disk into the heated compartment of a spray drier. The fine droplets are encapsulated within the core materials in the drying medium. As the hot or cold medium evaporates, the powder entraps the encapsulated core within the material. Spray chilling is applied mainly to retard volatilization during thermal processing of sensitive components including vitamins, minerals, and flavors. The capsules are collected through continuous discharge from the spray drying chamber.

Fluidized-bed drying, another mechanical encapsulation method, is restricted to encapsulation of solid core materials, including liquids absorbed into porous solids. *Fluidized-bed processing* involves drying, cooling, agglomeration, granulation, and coating of particulate materials. It is ideal for a wide range of both heat sensitive and nonheat sensitive products. Uniform processing conditions are achieved by passing a gas (usually air) through a product layer under controlled velocity conditions to create a fluidized state. Solid particles to be encapsulated are suspended on a jet of air and then covered by a spray of liquid coating material. The capsules are then moved to an area where their shells are solidified by cooling or solvent vaporization. The process of suspending, spraying, and cooling is repeated until the capsules' walls are of the desired thickness. This process is known as the Wurster process when the spray nozzle is located at the bottom of the fluidized bed of particles. Both fluidized-bed coating and the *Wurster process* are variations of the pan coating method. In pan coating, solid particles are mixed with a dry coating material and the temperature is raised so that the coating material melts and encloses the core particles, and then is solidified by cooling; or, the coating material can be gradually applied to core particles tumbling in a vessel rather than being wholly mixed with the core particles from the start of encapsulation. This technology is one of the few advanced technologies capable of coating particles with any kind of shell material including starches, emulsifiers, fats, complex formulations, enteric or powder coatings, yeast extract, and so on. Fluidized-bed

drying is suited for powders, granules, agglomerates, and pellets with an average particle size normally between 50 and 5000 μm. Very fine, light powders or highly elongated particles may require vibration for successful fluid bed drying.

Applications include the manufacture of chemicals, pharmaceuticals and biochemicals, polymers and food and dairy products. This technology offers important advantages over other methods of drying particulate materials. Particle fluidization gives easy material transport, high rates of heat exchange at high thermal efficiency while preventing overheating of individual particles. In nontherapeutic applications, cell microencapsulation technology has successfully been applied in the food industry for the encapsulation of live probiotic bacteria cells to increase viability of the bacteria during processing of dairy products and for targeted delivery to the gastrointestinal tract. In addition to dairy products, microencapsulated probiotics have also been used in nondairy products, such as Theresweet™ which is a sweetener. The pH, DO, storage temperature, species and strain, and concentration of lactic and acetic acids are some of the factors that greatly affect the probiotic viability in the product. The probiotic product should contain at least 10^6–10^7 cfu of viable probiotic bacteria per gram. Oral administration of microcapsules containing live bacterial cells has potential as an alternative therapy for several diseases. The microcapsules should have adequate membrane strength (mechanical stability) to endure physical and osmotic stress such as during the exchange of nutrients and waste products. The microcapsules should not rupture on implantation as this could lead to an immune rejection of the encapsulated cells. Also, while investigating the potential of using *alginate-poly-L-lysine-alginate* (*APA*) *microcapsules* loaded with bile salt hydrolase (BSH) overproducing active *Lactobacillus plantarum* 80 cells in a simulated gastrointestinal tract (GIT) model for oral delivery applications, the mechanical integrity and shape of the microcapsules were good. However, the *genipin cross-linked alginate-chitosan* (*GCAC*) microcapsules possess a higher mechanical stability as compared to APA microcapsules for oral delivery applications. Extensive research into the mechanical properties of the biomaterial to be used for cell microencapsulation is necessary to determine the durability of the microcapsules during production and especially for *in vivo* applications where a sustained release of the therapeutic product over long durations is required.

Another mechanical encapsulation process is spinning disk and centrifugal coextrusion; they are both atomization methods used in modified spray cooling encapsulation. The internal phase is dispersed into the liquid wall material and the mixture is advanced onto a turning disk. Droplets of pure shell material are thrown off of the rim of the disk along with discrete particles of core material enclosed in a skin of shell material. After having been solidified by cooling, the microcapsules are collected separately from the particles of shell material. Delivery of lipid-based health-promoting components such as *omega-3 fatty acids* (*PUFA*) while preserving taste is an important, and thus for effective delivery, functional lipids must be protected and stabilized against oxidative degradation. Encapsulation is needed for the prevention of off-taste or strong flavor above certain concentrations of functional active ingredient; for instance, green tea extract has a naturally bitter taste, but in higher concentrations, the bitterness is more intense. Therefore, green tea is sometimes encapsulated to mask the flavor. Centrifugal coextrusion processes generally produce capsules of a larger size, from 250 μm up to a few millimeters in diameter. The core and the shell materials, which should be immiscible with one another, are pushed through a spinning two-fluid nozzle. This movement forms an unbroken rope which naturally splits into round droplets directly after clearing the nozzle. The continuous walls of these droplets are solidified either by cooling or by a gelling bath, depending on the composition and properties of the coating material.

Extrusion process is a physical entrapment method using mostly sugars and starch to encapsulate volatile and unstable flavors. The process uses shear force to blend two materials linking them physically and involves the preparation of low moisture (5–10%) melt at 100–130 °C and then the agglomerated starch entraps the flavor into encapsulated cavities. The carrier materials are added through the hopper, and a twin-screw extruder is used to grind the pellets into fine particles. The core material is then injected directly into the extruder to mix with the carrier materials that form complexes under shear and pressure.

As the mixture exits the extruder, the encapsulated mass is collected, dried, and milled to break up the aggregates into small particles. Two examples of extrusion for flavor encapsulation include the use of β-cyclodextrins and starch to entrap D-limone and retention of β-carotene (58–97%) in wheat flour matrix.

One disadvantage of this technology involves the formation of large particles (typically 500–1000 μm), which limits the use of extruded flavors in application where mouth feel is a crucial factor. Also shell materials are limited for extrusion encapsulation. Some low temperatures process using mixtures of corn starch and fat or corn starch and polyethyleneglycol for the encapsulation of enzymes.

Coacervation is a relatively simple and promising technique balancing the electrostatic interaction between two components of the encapsulation emulsion to create water- and heat-resistant microcapsules. Very high payloads can be achieved up to 99% based on mechanical stress, temperature, or sustained release. A complex coacervation process begins with the suspension or emulsification of core material in either gelatin or gum arabic solution. When a core solution is mixed with an oppositely charged encapsulating material, a complex is formed, resulting in phase segregation and associative complexation. The characteristic size of the capsules formed can be altered by changing the pH and temperature, also by the bioactive component properties or the type of encapsulating agent.

A core material such as hydrophobic citrus oil can be dispersed in hydrophilic gelatin, creating a two-phase system. The coalescence of the polymeric colloid normally occurs around the suspended core oil particles, creating small microcapsules. The final process involves adding a suitable cross-linking agent such as glutaraldehyde or formaldehyde, adjusting the pH, and subsequently collecting, washing and drying the encapsulated particles. Coacervation works by the aqueous-phase separation of immiscible solutions such as oil and water or protein and ionic polysaccharides. Complex coacervation is possible only at pH values below the isoelectric point of proteins. At the isoelectric point of proteins, the pH values of the protein become positively charged. Comatrix encapsulation or inclusion complexation is one form of encapsulation in which cyclic polymers such as β-cyclodextrins are used to encapsulate other molecules. *α-, β-, γ-cyclodextrins* have been shown to encapsulate and stabilize lycopene, flavors, colors, and vitamins. Inclusion complexation in porous carbohydrate structures possible with both spray drying and/or extrusion processing was also used to entrap unwanted odors (deodorization) or bitter molecules. Inclusion encapsulation or complexation generally refers to the supramolecular association of a ligand ("encapsulated" ingredient) into a cavity-bearing substrate ("shell" material). The encapsulated unit is kept within the cavity by hydrogen bonding, VDW forces or by the entropy-driven hydrophobic effect.

Despite coacervation's intrinsic advantage and unique properties compared to the other common encapsulation processes, a major problem is the high cost of the process, complex, and cross-linking agent, glutaraldehyde is a harmful toxicity, and so must be carefully used. Other cheaper processes such as spray drying can be used or a process in which a transglutaminase is used to crosslink the proteins in the shell material.

Table 1.33 Examples of microencapsulated meats, enzymes, and cells

Products	Technology	Purpose
Meats		
Pigment	Spray-rying	Color retention
Volatile flavor	Encapsulation	Flavor retention
Acidulate/antioxidant	Spray-drying/fluidized bed	Direct acidification
Antimicrobials	–	Shelf-life of dry sausages
Bacteriophage	–	Safety for animal pathogens
Enzymes		
Lactase	Liposome/Polyvinylalcohol hydrogel, and so on.	Lactose hydrolysis
Glucoamylase	Aginate beads, and so on.	Maltodextrin hydrolysis
Glucose isomerase	Alginate, and so on.	Fructose syrup
Invertase	Polyvinylalcohol hydrogel, and so on.	Invert sugar syrup
α-Amylase	Sodium alginate, and so on.	Starch hydrolysis
Inulinase	Alginate, and so on.	Sucrose hydrolysis
Chymosin	Liposome, and so on.	Continuos milk coagulation
Peptidases/Proteinases	Liposome, and so on.	Acclerated cheese ripening
Peptidase (recombinant)	Alginate + Chitosan	Acclerated cheese ripening
Flavorzyme	Liposome	Acclerated cheese ripening
Cells		
Lactobacillus casei	Alginate beads	Enhanced survival to heat
Lactobacilli	Spray-dried alginate	Enhanced survival to heat
Bifidobacterium longum	Encapsulated, and so on.	Enhanced survival to freezing
Lactococci	Alginate beads, and so on.	Protection against bacteriophage

Source: Author's compiled data; Champagne, C., L. Saucier, and B. H. Lee. 2009. Immobilization of cells and enzymes for fermented dairy or meat products: In: Encapsulation of Food Bioactive Ingredients and Food Processing (Eds: N.J. Zuidam and V. A. Nedovic), pp. 400, Springer, USA.

Some microencapsulated cells and enzymes have the potential to address many problems in food processing, among which some are already commercially used, particularly for flavor acceleration of cheeses and meats, stability of probiotics and protective cultures in foods (Table 1.33). Many works on encapsulation of probiotics have mainly focused on maintaining viability of probiotic bacterial cells at low pH and high bile concentrations, as well as during spray drying, freeze drying, and storage. Much research has focused on microencapsulation technologies and the manipulation of encapsulation parameters, such as coating material types and their concentrations and the use of multiple coating layers. By these efforts, few encapsulated probiotic cultures stable for up to one year or more at room temperature are on the markets.

1.13.2 Nanoencapsulation

While microencapsulation is similar to *nanoencapsulation* aside from it involving larger particles and having used for a longer period of time than nanoencapsulation, nanoencapsulation is a new technique of miniaturized microencapsulation using *nanocomposite*, *nanoemulsion*, and *nanoestruration*. Nanoencapsulation is the coating of various substances within another material at sizes on the nanoscale. Nanoparticles are colloidal

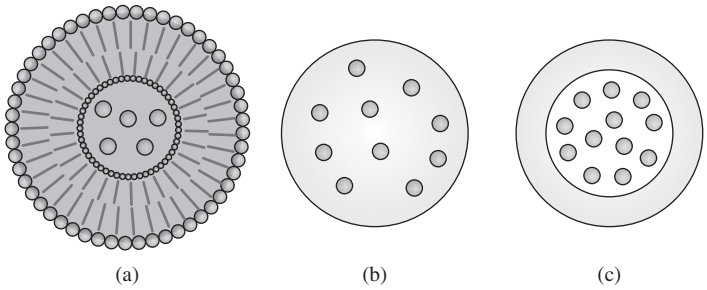

Figure 1.32 Schematic structure of (a) liposome, (b) nanosphere, and (c) nano-capsule. *Source*: Orive, G., et al. 2009. Reproduced with permission of Nature Publishing Group.

sized particles in ranging from 10 to 1000 nm diameter composed with nanocapsules and nanospheres including liposome (Figure 1.32). While nanocapsules are vesicular systems in which the bioactive compound is confined to a cavity surrounded by a polymer membrane, nanospheres are matrix systems where that compound is uniformly dispersed. Their small sizes are better suited for targeted delivery of drugs, nutrients, or bioactive compounds in small quantities to specific sites. As nanoencapsulation can improve the solubility and pharmacokinetic profiles of many insoluble drugs, targeted drug delivery is greatly enhanced, bioavailability to the target tissues and cells are significantly improved, while toxicity is reduced. Nanoencapsulation thus has the potential to enhance bioavailability, improve controlled release, and enable precision targeting of the bioactive compounds in a greater extent than microencapsulation.

A liposome already described in microencapsulation is an artificially prepared vesicle composed of a lipid bilayer, that can be used as a vehicle for administration of nutrients and pharmaceutical drugs. Liposomes are composed of natural phospholipids, and other mixed lipid chains with surfactant properties (e.g., egg phosphatidylethanolamine). Liposomes can be prepared by disrupting biological membranes such as by sonication. The major types of liposomes are the MLV, the SUV, the LUV, and the cochleate vesicle. Liposomes are different than micelles and reverse micelles composed of monolayers. Micelles are useful for encapsulating non-water soluble drugs to be administered intravenously. Nanospheres and nanocapsules are basically small vesicles used to transport materials. Nanospheres are typically solid polymers with drugs or active ingredients embedded in the polymer matrix. Nanocapsules are a shell with an inner space loaded with the ingredient of interest. Both systems are useful for controlling the release of a drug or bioactive molecule and/or protecting it from the surrounding environment. Dendrimers which are highly branched polymers with a controlled three-dimensional structure around a central core might be the most versatile of all nanocarriers.

In food nanoencapsulation, protection of bioactive compounds such as vitamins, antioxidants, proteins, lipids, and carbohydrates can better be achieved for the production of enhanced and stable functional foods. Flavors, oils, and other lipophilic materials are used in systems such as in salad dressings or mayonnaise where long-term stability is desirable. Nanoencapsulation is important for "*Nutraceutical*," a combined word of "nutrition" and "pharmaceutical." It is a food or food product that provides health and medical benefits, including the prevention and treatment of disease. While nutraceuticals are products isolated or purified from foods that are generally sold in medicinal forms capsules or tablets, *functional foods* are usually associated with food and consumed as a part of

foods. The effectiveness of nutraceuticals in preventing disease depends on preserving the bioavailability of bioactive ingredients until their release at targeted sites. Reducing the particle size may improve the bioavailability, delivery properties, and solubility of the nutraceuticals due to more surface area per unit volume and thus their biological activity. The bioavailabilities of these nutraceuticals are supposed to increase as a nanocarrier allows them to enter the bloodstream from the gut more easily.

Some of the nanoencapsulated hydrophilic nutraceuticals are ascorbic acid, polyphenols, and so on, and lipophilic compounds are insoluble in water but soluble in lipids and organic solvents. Nanoencapsulated lipophilic nutraceuticals include *lycopene*, *β-carotene*, *lutein*, *phytosterols*, and *DHA*. The solubility of the bioactive ingredients determines the release rate and release mechanism from a polymeric matrix system. Hydrophilic compounds show faster release rates and their release kinetics is determined by the appropriate combination of diffusion and erosion mechanisms. Lipophilic compounds often resulted in incomplete release due to poor solubility and low dissolution rates by an erosion mechanism. Although lipophilic compounds are highly permeable through the intestine via active transport and facilitated diffusion, hydrophilic compounds have low permeability that are absorbed only by active transport mechanism. Nanocarrier food systems such as lipid or natural biopolymer-based capsules are most often utilized for encapsulation, among which *nanoliposomes*, *archaeosomes*, and *nanocochleates* are three types of lipid-based nanocarrier systems that have applications in pharmaceutical, cosmetic, and food industries. Natural polymers such as albumin, gelatin, alginate, collagen, chitosan, α- and β-lactalbumin were used for the formulation of nano delivery systems. Whey protein has also been used as nanocarrier to improve the bioavailability of nutraceuticals, nanodrops mucosal delivery system of vitamins, and nano-based mineral delivery system. Nanoencapsulation of probiotics is desirable to develop designer probiotic bacterial preparations that could be delivered to certain parts of the gastrointestinal tract where they interact with specific receptors. These nanoencapsulated designer probiotic preparations may also act as *de novo* vaccines, with the capability of modulating immune responses. Biopolymer assemblies stabilized by various types of noncovalent forces have recently shown considerable progress. A starch-like nanoparticle can also help to stop lipids from oxidizing and therefore improve the stability of oil-in-water emulsions. The health benefits of anticancer compound *curcumin*, the natural pigment that gives the spice turmeric its yellow color, could be enhanced by encapsulation in nanoemulsions. Nanoemulsions could improve stability and oral bioavailability of *epigallocatechin gallate* and curcumin. A stearin-rich milk fraction was used, alone or in combination with α-tocopherol, for the preparation of oil-in-water sodium caseinate-stabilized nanoemulsions. Immobilization of α-tocopherol in fat droplets, composed by high melting temperature milk fat triglycerides, provided protection against degradation.

1.13.2.1 Nanoencapsulation techniques In general, the physicochemical properties such as particle size, size distribution, surface area, shape, solubility, encapsulation efficiency, and releasing mechanisms were reported to be altered by the encapsulation technique and delivery system. The appropriate encapsulation techniques must be based on the required size, physicochemical properties, nature of the core material, and wall material. Thus, the techniques used to achieve nanoencapsulation are more complex than microencapsulation, probably due to the difficulty in attaining a complex capsule and core material as well as the demands of releasing rates of nanoencapsulates. Various techniques developed and used for microencapsulation purpose such as emulsification, coacervation, inclusion complexation, emulsification–solvent evaporation, nanoprecipitation, and supercritical fluid

technique are all considered nanoencapsulation techniques since they can produce capsules in the nanometer range (10–1000 nm).

Nanoencapsulation techniques use either "top-down" or "bottom-up" approaches for the development of nanomaterials. "*Top down*" methodologies which consist in decreasing the size of macrostructures down to the nano-size scale or by "*Bottom-up*" techniques in which arrangements of atoms, molecules, or single particles are induced. A top-down approach such as emulsification and emulsification-solvent evaporation involves the application of precise tools that allow size reduction and structure shaping for desired application of the nanomaterials being developed. In the bottom-up approach such as the supercritical fluid technique, inclusion complexation, coacervation, and nanoprecipitation, materials are constructed by self-assembly and self-organization of molecules. These are influenced by many factors including pH, temperature, concentration, and ionic strength. These nanoencapsulation techniques can be used for encapsulation of various hydrophilic and lipophilic bioactive compounds. Emulsification, coacervation, and supercritical fluid technique are used for the encapsulation of both hydrophilic and lipophilic compounds, but inclusion complexation, emulsification-solvent evaporation, and nanoprecipitation techniques are mostly used for lipophilic compounds.

Nanoemulsions, which are nanoscale droplets of multiphase colloidal dispersions formed by dispersing of one liquid in another immiscible liquid by physically induced rupturing. Different size ranges of nanoemulsions less than 100, 100–500, and 100–600 nm have been reported, but the most appropriate ones are having the size ranges of less than 100 nm and processing different properties than ordinary emulsions. Nanoemulsions are liquid-in liquid dispersions with small droplets, typically in the range of 20–200 nm and are more thermodynamically stable. Nanoemulsions result from high kinetic energy input induced by shearing, which results in high energy emulsified small droplets making them stable against sedimentation or creaming. Stability of nanoemulsions can be enhanced by adding emulsifiers, which may be incorporated using high-shear homogenization. High-shear homogenization makes it possible for manufacturers to lower levels of surfactants in products.

Nanoemulsions are used in cosmetics, personal-care formulations and in some chemical industries and other nanoemulsions used for the delivery of micronutrients include the use of liposomes to deliver vitamins (A, D, E, and K) and caroteinoids. Nanoemulsions consisting of soybean-derived triglycerides and egg yolk phospholipids were used for parenteral high energy feeding. When dried, spray-dried emulsions of fish oil resulted in nanoparticles increased size in the range of 210–280 nm. Nanoemulsion can be prepared with mechanical (high-pressure homogenization, microfluidization, ultrasonication) and nonmechanical (solvent diffusion).

In high-pressure homogenization, the coarse dispersion of oil, aqueous phase, and emulsifier is passed through a small inlet orifice at pressures between 500 and 5000 psi. Microfludization uses a very high pressure of up to 2000 psi to force the liquid through the interaction chamber consisting of microchannels of a special configuration, where the emulsion feeds through configuration. The mechanism of nanoemulsion generation by ultrasonication is likely attributed to buddle cavitation and the collapse of the cavities provides sufficient energy to increase surface area of droplets. Although there is high potential of ultrasonication for research purpose, industrial applications do not appear to be practical and high-pressure homogenization or microfluidization is often preferred. Nanoemulsions formed by nonmechanical methods (solvent diffusion technique) have been used to prepare nanoemulsion (90–120 nm) of α-tocopherol first dissolved in an organic solvent. The resulting coarse dispersion is passed through a high-pressure homogenizer and then the solvent is removed from fine emulsion by evaporation. This method

has a limitation of using large amount of organic solvent to prepare and of removing organic solvent by expensive equipment before consumption.

Nanomaterials are divided in three categories: (i) nanoparticles, (ii) nanoplates (like silver, gold, ZnO, etc.), and (iii) nanocomponents. Nanoparticles are mostly used in the medical area to find disease in human body and many nanomaterials are commercially available. The subjects are out of this text; only related topics on food and agriculture are discussed. Nanocomposites are materials that are made based on different components like nanoclay or CNTs (to provide conductivity to other materials).

Nanoparticles (also known as *nanomaterials*) in Figure 1.33 are manufactured for use in an array of applications such as cosmetics, material coatings, biomedical, optical, electronic, toxicology, food, agriculture, and environmental remediation as well as a fuel additive. They are being increasingly investigated for use in medical applications such as drug delivery and release. Nanoparticles often posses unexpected optical properties as they are small enough to confine their electrons and produce quantum effects. This is the size scale where the so-called quantum effects rule the behavior and properties of particles. Although many of their effects have been well documented, some of their mechanisms of action are not fully understood. Nanoparticles, because of their small size, are better suited for targeted delivery of nutrients in small quantities to specific sites. Flavor and oils and other lipophilic materials are used in systems such as in salad dressings or mayonnaise where long-term stability is desirable.

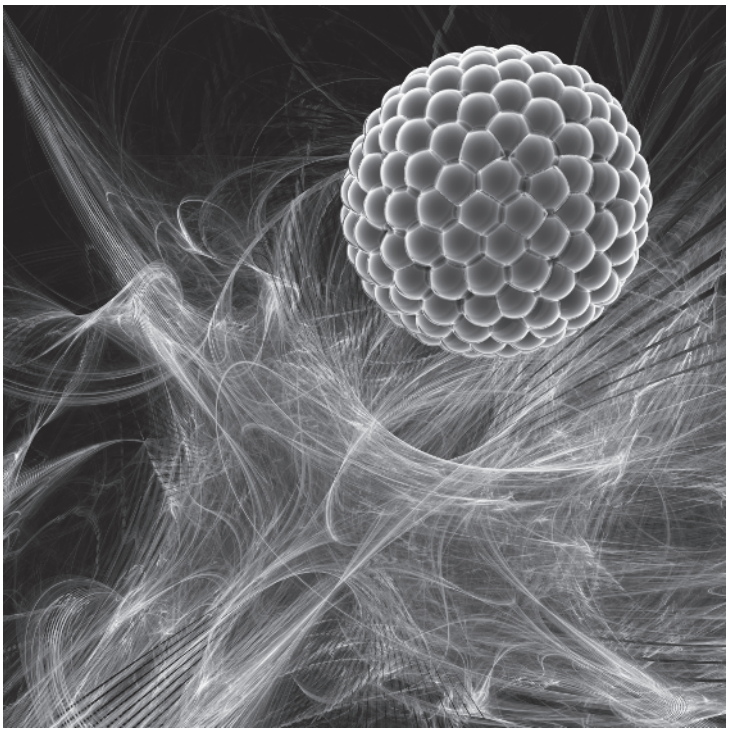

Figure 1.33 Representation of nanoparticles (millionths of a millimeter in size). *Source*: F005/0791 Nanoparticle, artwork LAGUNA DESIGN/SCIENCE PHOTO LIBRARY Nanoparticle, computer artwork.

Biocompatible and biodegradable biopolymers can be used to form delivery nanoparticles, which can maximize loading efficiency of nutrients in the particles, for site-specific delivery in the GIT. Nanoparticles emulsions and hydrogels can be made from egg white, soybean, and whey proteins. Mucosal delivery systems were also created from peptides derived from proteins and plasmids. Nanoparticles are easily dispersed in oil-based suspensions used in consumer products such as the delivery of omega-3 from fish oils. Nano-sized emulsions are kinetically stabilized monolayer of uniformly polydispersed spherical particles with a large surface area. The future application of nanoparticles technology in the areas of micronutrient and nutraceutical delivery will depend largely on the type of active molecule/ions and the product format, sprays or gels.

However, concern has arisen that widespread long-term nanoparticle use may "trickle down" into the environment, food, unforeseen effects on plant or animal or even human health.

Among two nanoparticles, *polymeric nanoparticles* (*PNs*), which include nanospheres and nanocapsules, are solid carriers ranging from 10 to 1000 nm in diameter made of natural or artificial polymers, which are generally biodegradable and in which therapeutic drugs can be adsorbed, dissolved, entrapped, encapsulated, or covalently linked to the particles. The synthetic materials used to prepare nanoparticles include *poly*(*lactic acid*) (*PLA*), *poly*(*glycolic acid*) (*PGA*), *poly*[*lactide-co-glycolide* (*PLGA*), *poly*(*alkylcyanoacrylate*) and *polyanhydride poly[bis(p-carboxyphenoxy)]propane-sebacic acid* (*PCPP-SA*); natural polymers such as chitosan, alginate, and gelatin have also been tested.

When systemically administered nanoparticles are generally more stable than liposomes but are limited by poor pharmacokinetic properties, and a poor ability to cross the blood–brain barrier. Similar to liposomes, the surface of nanoparticles can be coated with molecules to increase blood–brain barrier permeability and improve pharmacokinetics and even enable targeting for delivery and imaging purposes. More recently, other configurations of nanocarriers such as *solid lipid nanoparticles* (*SLNs*), micelles and dendrimers have been tested for brain drug delivery. SLNs consist of solid lipid matrices stabilized by surfactants to oral and parenteral drug delivery systems with low cytotoxicity and good physical stability. These combine the advantages of lipid emulsion and polymeric nanoparticle systems while overcoming the temporal and *in vivo* stability issues that trouble the conventional as well as PNs drug-delivery approaches. Other advantages are the avoidance of organic solvents, a potential wide application spectrum (dermal, per os, intravenous) and the high-pressure homogenization as an established production method.

1.13.2.2 Applications Nanomaterials and nanotechnologies are expected to yield numerous health and health care advances, such as more targeted methods of delivering drugs, new cancer therapies, methods of early detection of diseases, nanobiosensors as well as numerous benefits in agrifoods and environments (Table 1.34). Well over 100 nanoproducts are on the markets, but they also may have unwanted effects. Increased rate of absorption is the main concern associated with manufactured nanoparticles. In particular, nanoparticle research holds potential in areas such as diagnostics, cancer detection, and targeted drug delivery. However, their safety has been questioned, particularly through the discovery of toxic nanoparticles in sunscreens. The reactiveness of nanoparticles is difficult to assess, so properties are still relatively unknown.

Nanoparticles in biomedicine *Nano-biochip* is one more dimension of lab-on-a-chip technology in which magnetic nanoparticles bound to a suitable antibody are used to label specific molecules, structures, or microorganisms. GNPs tagged with short segments of

Table 1.34 Various applications of nanobiosensors and nanoparticles

Types	Applications
(a) Nanobiosensor/Biochips	— Pregnancy test (human chronionic gonadotrophin (hCG) in urine)* — Glucose test (diabetes patients)* — Infectious disease (from red blood cell)* — LAB-ON-A-CHIP (for genetic analysis, bacteria/viruses, cancers, blood DNA, etc.)* — Nanochemical sensor (for detecting harmful chemicals and biological weapons)* — Smart nanosensors for grain quality monitoring — Nano electronic nose (for recognizing smells, etc.)
(b) Nanocomposites/ Agrochemicals	— Nanoclay particle beer bottle* — Nanocleaning/nanoemulsion disinfectant* — Many paints/varnishes* — Nanoselenium feed for chicken* — NanoFungicide, NanoPlant Growth Regulator (PrimoMaxx, Syngenta, etc.)* — Nanofertilizers/pesticides — Nanofuel catalysts* — Nanopowered catalytic device (for deep flying oil)*
(c) Nanoencapsulation/Nanofoods	— Food ingredients and additives (AquaNova; Bioral omega-3 nanocochleates, Synthetic BSF lycopene, etc.)* — Beverage (oat chocolate/vanilla, etc.)* — Flavors/colors and enzymes* — Detection of food pathogens, virus, toxin (antibody fluorescence-based methods, etc.) — Structural control for texture, heterologous mixtures of emulsions and suspensions — Nanosilver antibacterial kitchenware (Nano Care Tech, etc.)/food contact material (A-Do Global)* — Food packaging (Durethan KU2-2601)/NanoZnO plastic wrap (SongSing Nanotech)* — Edible nano wrappers* — Chemical release food packaging — Nanomanipulation for seeds
(d) Nanocosmetics/Others	— Sunscreen cream with TiO_2, ZnO* — Nanorepair Q10 cream* — Nanocoatings (sunglass, fabrics, etc.)*
(e) Drug delivery/Environment/ Pathogens	— Delivery and directing of drugs to tumors and growing new organs — Detecting and filtering toxins out of water supplies — Cleaning up heavy metals and organic chemicals

*Commercialized products.

Source: From: (1) Authors compiled data; (2) Ljabadeniyi, O. A. 2012. *Afr J. Biotechnol*. 11:15258–15263; (3) Fathi, M. et al. 2012. *Trends Food Sci Technol*. 23: 13–27; (4) Rai, V. et al. 2012. J. *Biomater. Nanobiotech*. 3: 315–324.

DNA can be used for detection of genetic sequence in a sample. Multicolor optical coding for biological assays has been achieved by embedding different-sized quantum dots into polymeric microbeads. Nanopore technology for analysis of nucleic acids then converts strings of nucleotides directly into electronic signatures.

Nanoparticles have been a boon in *delivering drugs* to specific cells. The overall drug consumption and side-effects can be lowered significantly by depositing the active agent in the morbid region only and in no higher dose than needed. This highly selective approach such as dendrimers and nanoporous materials reduces costs and human suffering. Another example is to use block copolymers, which form micelles for drug encapsulation. They could hold small drug molecules transporting them to the desired location. Other small nanoelectromechanical systems are being investigated for the active release of drugs. Some potentially important applications include cancer treatment with iron nanoparticles or gold shells. To provide controllable release of bioactive compounds and local delivery of potentially therapeutic molecules, PLA, PGA, PLGA, poly(alkylcyanoacrylate) and PCPP-SA natural polymers such as chitosan, alginate, and gelatin have also been tested. When systemically administered, nanoparticles are generally more stable than liposomes but are limited by poor pharmacokinetic properties and a poor ability to cross the blood–brain barrier. PLGA nanoparticles and microparticles (particle size >1000 nm) have been successfully used to deliver drugs for the treatment of neurodegenerative disorders.

Nano electronic nose (*e-nose*) based on natural olfactory receptors using nanotechnologies could also help to develop the right smells for their foods, sniff out rotting ingredients, pick up the smells produced by bacteria and pathogens, and detect cancer infections, gastrointestinal disorder, liver diseases, and so on. As nano-nose applications can be applied to agrifoods, health, and environmental monitoring, this technology will revolutionize the electronic nose industry with its compact size and practical applicability. As compared with existing artificial e-nose devices and current e-nose devices based on metal oxide semiconductors or conducting polymers, nanowire chemiresistors are seen as critical elements in the future miniaturization of e-noses. Prototype nano e-nose was recently designed to detect harmful airborne agents such as pesticides, biological weapons, gas leaks, and other unwanted presences. The development has clear applications in military, industry, and agricultural area (www.neapplications.com/nanosensors-are-key-electronic-nose-prototype).

Nanoclays and nanofilms in packaging as barrier materials are used to prevent spoilage and prevent oxygen absorption. Plastic polymers containing or coating with nanomaterials are used to improve mechanical or functional properties. This is the largest share of the current short-term market for nanotechnology applications in food sector. Nanoparticles with silver and zinc oxide are used for antimicrobial/antifungal surface coatings. Nanocarrier systems for delivering of nutrients and supplements in the form of lyposomes or biopolymers-based nanoencapsulated substances are commercially available.

Nanofood and agriculture Commercially available nano-ingredients and nanomaterial additives include nanoparticles of iron or zinc, and nanocapsules containing ingredients like omega 3, or producing stronger flavors and colorings. In food packaging, nanoparticles are used to detect bacterial contamination, absorb oxygen or release preservatives to food, surface coating of bottles for ketchup or dressings, and so on. Untested nanotechnology is being used in more than 100 food products, food packaging, and contact materials currently on the shelf, without warning or new FDA testing. A list of some food products currently containing nanoproducts besides in Table 1.34 include: Canola Active Oil (Shemen, Haifa, Israel), Nanotea (Shenzhen Become Industry Trading Co. Guangdong,

China), Fortified Fruit Juice (High Vive.com, USA), Nanoceuticals Slim Shake (assorted flavors, RBC Lifesciences, Irving, USA), NanoSlim beverage(NanoSlim), Oat Nutritional Drink (assorted flavors, Toddler Health, Los Angeles, USA), and "Daily Vitamin Boost" fortified fruit juice (Jamba Juice Hawaii, USA) and nanocapsules containing tuna fish oil (a source of omega 3 fatty acids) in "Tip-Top" Up bread (Enfield, Australia). Polylysine, a food-grade polypeptide, can be added to the oil droplets to help protect from oxidation. Polylysine is much smaller than the phytoglycogen octenyl succinate nanoparticles, allowing it to fill in the gaps between phytoglycogen octenyl succinate nanoparticles.

The effects of two commonly used nanoparticles containing zinc oxide and cerium oxide were also for their effects on the growth of soybean. Zinc oxide is a common component of cosmetics and ultimately ends up as a contaminant of solid waste generated by sewage treatment. This waste is widely used as an organic fertilizer. Cerium oxide is used in some diesel fuels to improve combustion and reduce particulate emissions. The plants grown in the presence of zinc oxide nanoparticles actually grew slightly better than control plants grown in the absence of nanoparticles. However, zinc built up in the edible parts of the plants, which included the leaves and the beans. Zinc oxide nanoparticles have been shown to be toxic to mammalian cells grown in the laboratory, but effects in humans remain to be examined fully. After nanoparticles are found in foods, including bread, cornflakes, biscuits and caramels, their safety has been questioned, particularly through the discovery of toxic nanoparticles in sunscreens. As the reactiveness of nano-sized particles is difficult to assess, properties are still relatively unknown. Nanoparticles have also been known to pass through cell membranes and cause possible harm to otherwise healthy cells. The carbon nanoparticles were extracted from the food sources surrounded by polymerized sugar; thus some demonstrated that even at high concentrations, the nanoparticles had little or no toxicity.

1.13.2.3 Safety issues of nanoparticles

Health aspects on nanotechnology Many applications of nanomaterials are expected to food and additive preparations such as in (i) encapsulation of additives/nutrient, flavors and colors, and enzymes, (ii) structure control for texture, emulsions and suspensions, and (iii) detection of food pathogens, virus, and toxins. Consumers are increasingly concerned about pesticide and antibiotic residues, food allergies, GMF, irradiation, and food adulteration, and so on, and now more concern about health implications of unintended consequences of nanoparticles.

Very little is known about how the particles interact with the environment and human body, but there is evidence that nanoparticles might ferry toxins right past the body's normal defense. Nanoparticles are readily inhaled and injested and at least some will cross the skin, and may gain access to tissues and cells. The Royal Society in the United Kingdom mentioned that nanotechnology poses health and environmental risks great enough to justify banning. Also there is a lack of analytical methods and predictive model to evaluate the safety of nanofood and food contact materials incorporated with nanomaterials. Currently, a high degree of uncertainty exists as to whether regulatory system and statute would give attention to the "nano" scale. According to WHO, new data and measurement approaches needed to ensure safety of products using nanotech can be properly assessed, in which nanobiotechnology can easily fall into the premade trap of GMO.

Although possible dangers of nanoparticles have been discussed and concerns are now arising that nanomaterial may prove toxic to humans or the environment, it is not simply uncertain on what effects, if any, nanoparticles will have on the *environment*, *health*, *and safety* (*EHS*). This nanotechnology should not completely halt on EHS grounds, since

nanotechnologies are extremely beneficial to both the environment and human health in the long term.

When materials are made into nanoparticles, their surface area to volume ratio increases. The greater specific surface area (surface area per unit weight) which can make the particles very reactive or catalytic, so that they can easily pass through cell membranes in organisms that may cause unwanted effects to the lungs as well as other organs. Their interactions with biological systems are relatively unknown. However, the particles must be absorbed in sufficient quantities in order to pose health risks. Although the results in 2008 showed that iron oxide nanoparticles caused little DNA damage and were nontoxic, but zinc oxide nanoparticles were slightly worse and titanium dioxide caused only DNA damage. While CNTs caused DNA damage at low levels, copper oxide was found to be the worst offender.

Whether cosmetics and sunscreens containing nanomaterials pose health risks also remains largely unknown at this stage. However considerable research has demonstrated that zinc nanoparticles are not absorbed into the bloodstream *in vivo*. *Diesel nanoparticles* have been found to damage the cardiovascular system in a mouse model. Concern has also been raised over the negative health effects of respirable nanoparticles from certain combustion processes. However, other research showed that most nanoparticles do not pose a serious threat to human and environmental safety, and thus in theory nanoparticles should be benign.

Some nanoparticles have the unique ability to easily pass through cell walls and can permeate the blood–brain barrier and may also be bactericidal, that can highly damage into ecosystems where bacteria are at the bottom of the food chain. Thus, this safety issue is currently done on a case-by-case basis, but risk assessment methods need to be kept up to date as the use of nanomaterials expands, especially as they find their way into consumer products.

Summary

Microencapsulation system offers potential advantages over conventional drug delivery systems and also established as unique carrier systems for many pharmaceuticals (targeted drug delivery systems). Although a wide range of encapsulated products have been developed and successfully marketed in the pharmaceutical and cosmetic industries, microencapsulation has a comparatively much smaller market in the food industry. Also the development time is rather long involving multidisciplinary cooperation and the low margins typically achieved in food ingredients are a deterrent factor. Despite the significant advances in the field of microencapsulation, still many challenges need to be rectified during the appropriate selection of core materials, coating materials, and process techniques. A wide variety of different types of delivery systems are available for functional bioactives, but each type of delivery system has its own specific advantages and disadvantages. Techniques such as microencapsulation, microemulsions, colloidal particulation, and nanostructuring may be adequate for the delivery of bioactive components at health-optimizing doses, at appropriate site, using the proper natural triggers. Future work will improve encapsulation and coencapsulation delivery of functional foods like live probiotic bacterial cells. Simulated human gastrointestinal models are making the evaluation of functional bioactives much robust allowing for *in vitro* simulation of harsh conditions in the GIT.

Colloidal dispersions of soft matter systems are used to create atomic systems. Nanoparticles are easily dispersed in oil-based suspensions used in consumer products such as the delivery of omega-3 from fish oils. Nano-sized emulsions are kinetically stabilized

monolayer of uniformly polydispersed spherical particles with large surface area. Colloidal particles and nanoemulsions could be used as delivery systems for micronutrients and nutraceuticals. Future application of nanoparticles technology in the areas of micronutrient and nutraceutical delivery will depend largely on the type of active molecule/ions and the product format, sprays or gels.

Nanotechnology may offer many benefits for human health and the environment. The properties of nanoparticles make them excellent candidates for advanced medication and new drug delivery methods, as well as for curing diseases like cancer and AIDS. Nanotechnologies can also help monitor pollution, lower energy requirements, and reduce the use of harmful cleansing chemicals. This technology can also be used to enhance food flavor and texture, to reduce fat content, or to encapsulate nutrients to ensure they do not degrade during a product's shelf life.

Nanoparticles emulsions and hydrogels can be made from egg white, soybean, and whey proteins. Nanoparticle-sized colloidal dispersions are used in a broad range of products including foods, cosmetics, paints, and drugs. Nanoparticle structures are created with technologies and processes that can accommodate soft materials. Nanomaterials can be used to make packaging that keeps the product fresher for longer. Intelligent food packaging, incorporating nanosensors, could even provide consumers with information on the state of the food inside and alert consumers when a product is no longer safe to eat. Sensors can warn before the food goes rotten or can inform us the exact nutritional status contained in the contents. Food nanotechnology advances offers important challenges for both government and industry. The food processing industry must ensure consumer confidence and acceptance of nanofoods.

Currently available information of risks associated to manipulation of nano-sized products is limited, and there are not internationally agreed regulatory parameters related to manipulation of nano-sized food materials. Thus, many products reach markets without prior evaluation on safety aspects.

Very little information is available on the development of foodstuffs which contain nanoparticles and on their physicochemical properties used as additives during their transit through the gastrointestinal tract. Until now, consumers lean to be more unwilling to nanofood applications than other uses. Regulatory aspects led by the United States of America and The National Nanotechnology Initiative (NNI) and other countries are centered on the uses and the applications of nanotechnology in three main areas: (i) Research and technology on the development of products at the atomic, molecular or macromolecular scales in the length of 1–100 nm, (ii) creating and using structures that have novel properties and functions because of their small sizes, and (iii) ability to control or manipulate on the atomic scale. The EU organization Strategy for Nanotechnology asserts that nanotechnology has the potential to enhance quality of life and industrial competitiveness, and thus lobbies aggressively for minimal legislation on nanotechnology. Current laws state that anyone producing or importing nanomaterials into Europe is required to provide written notification to public authorities; that requires the manufacturer to conduct research illustrating the properties and dangers of the product.

As the long-term impact of nanomaterials on the natural environment and human health is unknown, it is difficult to comprehensively regulate this technology in a single piece of legislation that would capture its risks. Nanotechnology should rather be regulated by a series of laws which govern the exposure of nanotechnology on specific areas: food, environment, medicine, and agriculture. Mishandling of previous GM food debates will put nonfoods at a disadvantage and nanofoods might be the next "next GMO." The possible solution to current problem will be the proper labeling on nanoproducts and legislation.

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