3

Genetics and biotechnology

3.1 Introduction

In essence, all properties of organisms depend on the sum of their genes. There are two broad categories of genes: structural and regulatory. *Structural genes* encode for amino acid sequences of proteins which, as enzymes, determine the biochemical capabilities of the organism by catalysing particular synthetic or catabolic reactions or, alternatively, play more static roles as components of cellular structures. In contrast, the *regulatory genes* control the expression of the structural genes by determining the rate of production of their protein products in response to intra- or extracellular signals. The derivation of these principles has been achieved using well- known genetic techniques which will not be considered further here.

The seminal studies of Watson and Crick and others in the early 1950s led to the construction of the double-helix model depicting the molecular structure of DNA and subsequent hypotheses on its implications for the understanding of gene replication. Since then there has been a spectacular unravelling of the complex interactions required to express the coded chemical information of the DNA molecule into cellular and organismal expression. Changes in the DNA molecule making up the genetic complement of an organism is the means by which organisms evolve and adapt themselves to new environments. In nature, changes in the DNA of an organism can occur in two ways:

(1) By *mutation*, which is a chemical deletion or addition of one or more of the chemical parts of the DNA molecule.

(2) By the interchange of genetic information or DNA between like organisms normally by *sexual reproduction* and by *horizontal transfer* in bacteria. In eukaryotes, sexual reproduction is achieved by a process of conjugation in which there is a donor, called 'male', and a recipient, called 'female'. Often, these are determined physiologically and not morphologically. Bacterial conjugation involves the transfer of DNA from a donor to a recipient cell. The transferred DNA (normally plasmid DNA) is always in a single-stranded form and the complementary strand is synthesised in the recipient. *Transduction* is the transfer of DNA mediated by a bacterial virus (*bacteriophage* or *phage*), and cells that have received transducing DNA are referred to as '*transductants*'. *Transformation* involves the uptake of isolated DNA, or DNA present in the organism's environment, into a recipient cell which is then referred to as a '*transformant*'. Genetic transfer by this way in bacteria is a natural characteristic of a wide variety of bacterial genera such a *Campylobacter, Neisseria* and *Streptomyces*. Strains of bacteria that are not naturally transformable can be induced to take up isolated DNA by chemical treatment or by *electroporation*.

Classical genetics was, until recently, the only way in which heredity could be studied and manipulated. However, in recent years, new techniques have permitted unprecedented alterations in the genetic make-up of organisms, even allowing exchange in the laboratory of DNA between unlike organisms.

The manipulation of the genetic material in organisms can now be achieved in three clearly definable ways: organismal, cellular and molecular.

Organismal manipulation

Genetic manipulation of whole organisms has been happening naturally by sexual reproduction since the beginning of time. The evolutionary progress of almost all living creatures has involved active interaction between their genomes and the environment. Active control of sexual reproduction has been practised in agriculture for decades – even centuries. In more recent times it has been used with several industrial microorganisms, e.g. yeasts. It involves selection, mutation, sexual crosses, hybridisation, etc. However, it is a very random process and can take a long time to achieve desired results – if at all in some cases. In agriculture, the benefits have been immense with much improved plants and animals, while in the biotechnological industries there has been greatly improved productivity, e.g. antibiotics and enzymes.

Cellular manipulation

Cellular manipulations of DNA have been used for over two decades, and involve either cell fusion or the culture of cells and the regeneration of whole plants from these cells (Chapter 10). This is a semi-random or directed process in contrast to organismal manipulations, and the changes can be more readily identified. Successful biotechnological examples of these methods include monoclonal antibodies and the cloning of many important plant species.

Molecular manipulation

Molecular manipulations of DNA and RNA first occurred over two decades ago and heralded a new era of genetic manipulations enabling – for the first time in biological history – a directed control of the changes. This is the much publicised area of *genetic engineering* or *recombinant DNA technology*, which is now bringing dramatic changes to biotechnology. In these techniques the experimenter is able to know much more about the genetic changes being made. It is now possible to add or delete parts of the DNA molecule with a high degree of precision, and the product can be easily identified. Current industrial ventures are concerned with the production of new types of organism and numerous compounds ranging from pharmaceuticals to commodity chemicals, and are discussed in more detail in later chapters.

3.2 Industrial genetics

Biotechnology has so far been considered as an interplay between two components, one of which is the selection of the best biocatalyst for a particular process, while the other is the construction and operation of the best environment for the catalyst to achieve optimum operation.

The most effective, stable and convenient form for the biocatalyst is a whole organism; in most cases it is some type of microbe, e.g. a bacterium, yeast or mould, although mammalian cell cultures and (to a lesser extent) plant cell cultures are finding ever-increasing uses in biotechnology.

Most microorganisms used in current biotechnological processes were originally isolated from the natural environment, and have subsequently been modified by the industrial geneticist into superior organisms for specific productivity. The success of strain selection and improvement programmes practised by all biologically based industries (e.g. brewing, antibiotics, etc.) is a

direct result of the close cooperation between the technologist and the geneticist. In the future, this relationship will be even more necessary in formulating the specific physiological and biochemical characteristics that are sought in new organisms in order to give the fullest range of biological activities to biotechnology.

In biotechnological processes, the aim is primarily to optimise the particular characteristics sought in an organism, e.g. specific enzyme production or by-product formation. Genetic modification to improve productivity has been widely practised. The task of improving yields of some primary metabolites and macromolecules (e.g. enzymes) is simpler than trying to improve the yields of complex products such as antibiotics. Advances have been achieved in this area by using *screening* and *selection* techniques to obtain better organisms. In a selection system, all rare or novel strains grow while the rest do not; in a screening system, all strains grow but certain strains or cultures are chosen because they show the desired qualities required by the industry in question.

In most industrial genetics the basis for changing the organism's genome has been by mutation using X-rays and mutagenic chemicals. However, such methods normally lead only to the loss of undesired characters or increased production due to loss of control functions. It has rarely led to the appearance of a new function or property. Thus, an organism with a desired feature will be selected from the natural environment, propagated and subjected to a mutational programme, then screened to select the best progeny.

Unfortunately, many of the microorganisms that have gained industrial importance do not have a clearly defined sexual cycle. In particular, this has been the case in antibiotic-producing microorganisms; this has meant that the only way to change the genome, with a view to enhancing productivity, has been to indulge in massive mutational programmes followed by screening and selection to detect the new variants that might arise.

Once a high-producing strain has been found, great care is required in maintaining the strain. Undesired spontaneous mutations can sometimes occur at a high rate, giving rise to degeneration of the strain's industrial importance. Strain instability is a constant problem in industrial utilisation of microorganisms. Industry has always placed great emphasis on strain viability and productivity potential of the preserved biological material. Most industrially important microorganisms can be stored for long periods, for example in liquid nitrogen, by lyophilisation (freeze-drying) or under oil, and still retain their desired biological properties.

However, despite elaborate preservation and propagation methods, a strain has generally to be grown in a large-production bioreactor in which the chances of genetic changes through spontaneous mutation and selection are very high.

The chance of a high rate of spontaneous mutation is probably greater when the industrial strains in use have resulted from many years of mutagen treatment. Great secrecy surrounds the use of industrial microorganisms and immense care is taken to ensure that they do not unwittingly pass to outside agencies (Section 12.2).

There is now a growing movement away from the extreme empiricism that characterised the early days of the fermentation industries. Fundamental studies of the genetics of microorganisms now provide a background of knowledge for the experimental solution of industrial problems and increasingly contribute to progress in industrial strain selection.

In recent years, industrial genetics has come to depend increasingly on two new ways of manipulating DNA: – (1) protoplast and cell fusion, and (2) recombinant DNA technology (genetic engineering). These are now important additions to the technical repertoire of the geneticists involved with biotechnological industries. A brief examination of these techniques will attempt to show their increasingly indispensable relevance to modern biotechnology.

3.3 Protoplast and cell-fusion technologies

Plants and most microbial cells are characterised by a distinct outer wall or exoskeleton which gives the shape characteristic to the cell or organism. Immediately within the cell wall is the living membrane, or plasma membrane, retaining all the cellular components such as nuclei, mitochondria, vesicles, etc. For some years now it has been possible, using special techniques (in particular, hydrolytic enzymes), to remove the cell wall, releasing spherical membrane-bound structures known as *protoplasts*. These protoplasts are extremely fragile but can be maintained in isolation for variable periods of time. Isolated protoplasts cannot propagate themselves as such, requiring first the regeneration of a cell wall before regaining reproductive capacity.

In practice, it is the cell wall which largely hinders the sexual conjugation of unlike organisms. Only with completely sexually compatible strains does the wall degenerate, allowing protoplasmic interchange. Thus natural sexual mating barriers in microorganisms may, in part, be due to cell-wall limitations, and by removing this cell wall, the likelihood of cellular fusions may increase.

Protoplasts can be obtained routinely from many plant species, bacteria, yeasts and filamentous fungi. Protoplasts from different strains can sometimes be persuaded to fuse and so overcome the natural sexual mating barriers. However, the range of protoplast fusions is severely limited by the need for

DNA compatibility between the strains concerned. Fusion of protoplasts can be enhanced by treatment with the chemical polyethylene glycol, which, under optimum conditions, can lead to extremely high frequencies of recombinant formation which can be increased still further by ultraviolet irradiation of the parental protoplast preparations. Protoplast fusion can also occur with human or animal cell types.

Protoplast fusion has obvious empirical applications in yield improvement of antibiotics by combining yield-enhancing mutations from different strains or even species. Protoplasts will also be an important part of genetic engineering, in facilitating recombinant DNA transfer. Fusion may provide a method of re-assorting whole groups of genes between different strains of macro- and microorganisms.

One of the most exciting and commercially rewarding areas of biotechnology involves a form of mammalian cell fusion leading to the formation of monoclonal antibodies. It has long been recognised that certain cells (β-lymphocytes) within the body of vertebrates have the ability to secrete antibodies which can inactivate contaminating or foreign molecules (the antigen) within the animal system. The antibody has a Y-shaped molecular structure and uses one part of this structure to bind the invading antigen and the other part to trigger the body's response to eliminate the antigen/antibody complex. It has been calculated that a mammalian species can generate up to 100 million different antibodies, thereby ensuring that most invading foreign antigens will be bound by some antibody. Antibodies have high binding affinities and specificity against the chosen antigen. For the mammalian system it is the major defence against disease-causing organisms and other toxic molecules.

Attempts to cultivate the antibody-producing cells in artificial media have generally proved unsuccessful, with the cells either dying or ceasing to produce the antibodies. It is now known that individual β-lymphocyte cells produce single-antibody types. However, in 1975, Georges Köhler and Cesar Milstein successfully demonstrated the production of pure or *monoclonal antibodies* from the fusion product (*hybridoma*) of β-lymphocytes (antibody-producing cells) and myeloma tumour cells. In 1984, they were awarded the Nobel Prize for this outstanding scientific achievement. The commercial importance of their scientific findings can be judged from the estimate that, in the late 1990s, the value of therapeutic antibodies alone was $6 billion.

The monoclonal-antibody technique changes antibody-secreting cells (with limited life span) into cells that are capable of continuous growth (immortalisation) while maintaining their specific antibody-secreting potential. This immortalisation is achieved by a fusion technique, whereby β-lymphocyte cells are fused to 'immortal' cancer or myeloma cells in a one-to-one ratio, forming hybrids or hybridomas that are capable of continuous growth and

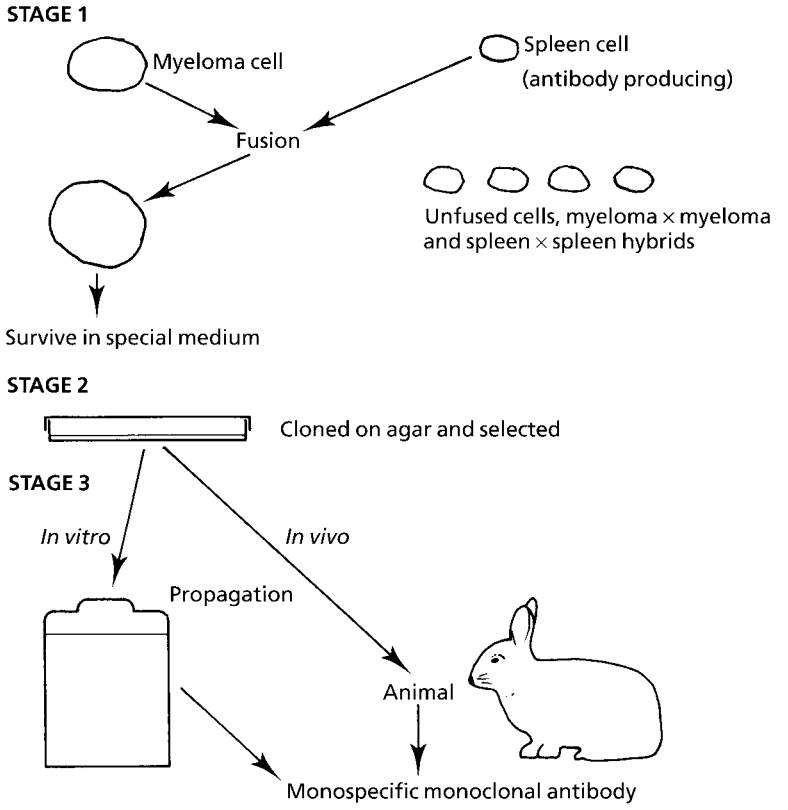

Fig. 3.1 The formation of antibody-producing hybridomas by fusion techniques. Stage 1: myeloma cells and antibody-producing cells (derived from immunised animal or man) are incubated in a special medium containing polyethylene glycol, which enhances fusion. Stage 2: the myeloma spleen hybridoma cells are selected out and cultured in closed agar dishes. Stage 3: the specific antibody-producing hybridoma is selected and propagated in culture vessels (*in vitro*) or in animal (*in vivo*) and monoclonal antibodies are harvested.

antibody secretion in culture. Single hybrid cells can then be selected and grown as clones or pure cultures of the hybridomas. Such cells continue to secrete antibody, and the antibody is of one particular specificity, as opposed to the mixture of antibodies that occurs in an animal's bloodstream after conventional methods of immunisation.

Monoclonal antibody formation is performed by injecting a mouse or rabbit with the antigen, later removing the spleen, and then allowing fusion of individual spleen cells with individual myeloma cells. Approximately l% of the spleen cells are antibody-secreting cells and l0% of the final hybridomas consist of antibody-secreting cells (Fig. 3.1). Techniques are available to identify the

correct antibody-secreting hybridoma cell, cloning or propagating that cell into large populations with subsequent large formation of the desired antibody. These cells may be frozen and later re-used.

Monoclonal antibodies have now gained wide application in many diagnostic techniques which require a high degree of specificity. Specific monoclonal antibodies have been combined into test kits for diagnostic purposes in health care, in plant and animal agriculture, and in food manufacture. Monoclonal antibodies may also be used in the future as antibody therapy to carry cytotoxic drugs to the site of cancer cells. In the fermentation industry they are already widely used as affinity ligands to bind and purify expensive products.

Since the development of the first monoclonal antibody the methodology has developed from a purely scientific tool into one of the fastest expanding fields of biotechnology, which has revolutionised, expanded and diversified the diagnostic industry. The monoclonal-antibody market is expected to continue to grow at a very high rate and, in health care alone, the anticipated annual world market could be several billion US dollars over the next decade. It is undoubtedly one of the most commercially successful and useful areas of modern biotechnology and will be expanded on in several chapters.

3.4 Genetic engineering

Genes are the fundamental basis of all life, determine the properties of all living forms of life, and are defined segments of DNA. Because the DNA structure and composition of all living forms is essentially the same, any technology that can isolate, change or reproduce a gene is likely to have an impact on almost every aspect of society.

Genetic recombination, as occurs during normal sexual reproduction, consists of the breakage and rejoining of DNA molecules of the chromosomes, and is of fundamental importance to living organisms for the reassortment of genetic material. Genetic manipulation has been performed for centuries by selective breeding of plants and animals superimposed on natural variation. The potential for genetic variation has, thus, been limited to close taxonomic relatives.

In contrast, recombinant DNA techniques, popularly termed 'gene cloning' or 'genetic engineering', offer potentially unlimited opportunities for creating new combinations of genes which, at the moment, do not exist under natural conditions.

Genetic engineering has been defined as the formation of new combinations of heritable material by the insertion of nucleic acid molecules – produced

by whatever means outside the cell – into any virus, bacterial plasmid or other vector system so as to allow their incorporation into a host organism in which they do not naturally occur but in which they are capable of continued propagation. In essence, gene technology is the modification of the genetic properties of an organism by the use of recombinant DNA technology. Genes may be viewed as the biological software and are the programs which drive the growth, development and functioning of an organism. By changing the software in a precise and controlled manner, it becomes possible to produce desired changes in the characteristics of the organism.

These techniques allow the splicing of DNA molecules of quite diverse origin and, when combined with techniques of genetic transformation, etc., facilitate the introduction of foreign DNA into other organisms. The foreign DNA or gene construct is introduced into the genome of the recipient organism host in such a way that the total genome of the host is unchanged except for the manipulated gene(s).

Thus DNA can be isolated from cells of plants, animals or microorganisms (the donors) and can be fragmented into groups of one or more genes. Such fragments can then be coupled to another piece of DNA (the *vector*) and then passed into the host or recipient cell, becoming part of the genetic complement of the new host. The host cell can then be propagated in mass to form novel genetic properties and chemical abilities that were unattainable by conventional ways of selective breeding or mutation. While traditional plant and animal genetical breeding techniques also change the genetic code, it is achieved in a less direct and controlled manner. Genetic engineering will now enable the breeder to select the particular gene required for a desired characteristic and modify only that gene.

Although much work to date has involved bacteria, the techniques are evolving at an astonishing rate and ways have been developed for introducing DNA into other organisms such as yeasts and plant and animal cell cultures. Provided that the genetic material transferred in this manner can replicate and be expressed in the new cell type, there are virtually no limits to the range of organisms with new properties which could be produced by genetic engineering. Life forms containing 'foreign' DNA are termed '*transgenic*' and will be discussed in more detail in chapter 10.

These methods potentially allow totally new functions to be added to the capabilities of organisms, and open up vistas for the genetic engineering of industrial microorganisms and agricultural plants and animals which are quite breathtaking in their scope. This is undoubtedly the most significant new technology in modern bioscience and biotechnology. In industrial microbiology it will permit the production in microorganisms of a wide range of hitherto unachievable products such as human and animal proteins and enzymes such

as insulin and chymosin (rennet); in medicine better vaccines, hormones and improved therapy of diseases; in agriculture improved plants and animals for productivity, quality of products, disease resistance, etc; in food production improved quality, flavour, taste and safety; and in environmental aspects a wide range of benefits such as pollution control can be expected. It should be noted that genetic engineering is a way of doing things rather than an end in itself. Genetic engineering will add to, rather than displace, traditional ways of developing products. However, there are many who view genetic engineering as a transgression of normal life processes that goes well beyond normal evolution. These concerns will be discussed in chapter 14.

Genetic engineering holds the potential to extend the range and power of almost every aspect of biotechnology. In microbial technology these techniques will be widely used to improve existing microbial processes by improving the stability of existing cultures and eliminating unwanted side-products. It is confidently anticipated that, within this decade, recombinant DNA techniques will form the basis of new strains of microorganisms with new and unusual metabolic properties. In this way fermentations based on these technical advances could become competitive with petrochemicals for producing a whole range of chemical compounds, for example ethylene glycol (used in the plastics industry). In the food industry, improved strains of bacteria and fungi are now influencing such traditional processes as baking and cheese-making and bringing greater control and reproducibility of flavour and texture.

A full understanding of the working concepts of recombinant DNA technology requires a good knowledge of molecular biology. A brief explanation will be attempted here but readers are advised to consult some of the many excellent texts that are available in this field.

The basic molecular techniques for the *in vitro* transfer and expression of foreign DNA in a host cell (*gene transfer technology*) include isolating, cutting and joining molecules of DNA, inserting into a vector (carrying) molecule that can be stably retained in the host cell.

These techniques may be defined thus:

Isolation and purification of nucleic acids. Nucleic acids from most organisms can now be routinely extracted and purified by means of a range of biochemical techniques (Fig. 3.2).

Cutting and splicing DNA. The most significant advances towards the construction of hybrid DNA molecules *in vitro* have come from the discovery that site-specific *restriction endonuclease enzymes* produce specific DNA fragments that can be joined to any similarly treated DNA molecule using another enzyme, *DNA ligase*. Restriction enzymes are present in a wide

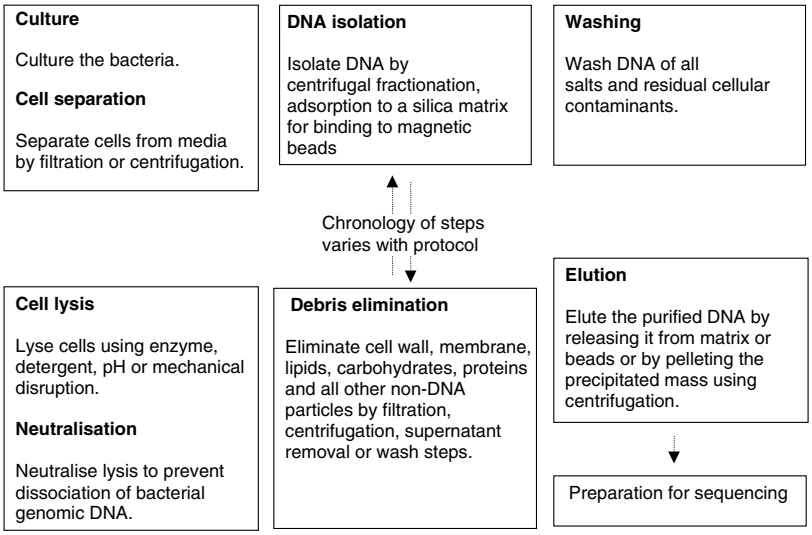

Fig. 3.2 Diagram of a typical series of sample preparation steps required for DNA purification from bacterial cells (from Wells and Herron, 2002).

range of bacteria and can distinguish between DNA from their own cells and foreign DNA by recognising certain sequence of nucleotides. There are techniques available for breaking open a length of DNA into shorter fragments which contain a number of genes determined by the enzyme used. Such DNA fragments can then be separated from each other on the basis of differing molecular weights, and can subsequently be joined together in a number of ways, provided that the ends are complementary. The sources of DNA can be quite different, giving an opportunity to replicate the DNA biologically by inserting it into other cells.

The composite molecules into which DNA has been inserted have also been termed 'DNA chimeras' because of the analogy with the Chimera of mythology – a creature with the head of a lion, the body of a goat and the tail of a serpent.

The vector or carrier system. Two broad categories of vector molecules have been developed as vehicles for gene transfer, namely *plasmids* (small units of DNA distinct from chromosomes) and *bacteriophages* (or bacterial viruses). Vector molecules will normally exist within a cell in an independent or extra-chromosomal form, not becoming part of the chromosomal system of the organism. Vector molecules should be capable of entering the host cell and replicating within it. Ideally, the vector

should be small, easily prepared and must contain at least one site where integration of foreign DNA will not destroy an essential function. Plasmids will undoubtedly offer the greatest potential in biotechnology and have been found in an increasingly wide range of organisms, e.g. bacteria, yeasts and mould fungi; they have been mostly studied in gram-negative bacteria.

Introduction of vector DNA recombinants. The new recombinant DNA can now be introduced into the host cell by transformations (the direct uptake of DNA by a cell from its environment) or transductions (DNA transferred from one organism to another by way of a carrier or vector system) and, if acceptable, the new DNA will be cloned with the propagation of the host cell.

Novel methods of ensuring DNA uptake into cells include *electroporation* and *mechanical particle delivery* or *biolistics*. Electroporation is a process of creating transient pores in the cell membrane by application of a pulsed electric field. Creation of such pores in a membrane allows the introduction of foreign molecules such as DNA, RNA, antibodies, drugs, etc., into the cell cytoplasm. Development of this technology has arisen from synergy of biophysics, bioengineering and cell and molecular biology. While the technique is now widely used to create transgenic microorganisms, plants and animals, it is also being increasingly used for the application of therapeutics and gene therapy. The mechanical particle delivery or 'gene gun' methods deliver DNA on microscopic particles into target tissue or cells. This process is increasingly used to introduce new genes into a range of bacterial, fungal, plant and mammalian species and has become a main method of choice for genetic engineering of many plant species including rice, corn, wheat, cotton and soybean.

The strategies involved in genetic engineering are summarised in Table 3.1 and Fig. 3.3.

Although the theory underlying the exchange of genetic information between unrelated organisms and their propagation is becoming better understood, difficulties still persist at the level of some applications. Further research is required before such exchanges become commonplace and the host organisms are propagated in large quantities.

Early studies on genetic engineering were mainly carried out with the bacterium *Escherichia coli* but, increasingly, other bacteria, yeast and filamentous fungi have been used. Mammalian systems have been increasingly developed using the simian virus (SV40) and oncogenes (genes that cause cancer), while several successful methods are available for plant cells, in particular the

Table 3.1. Strategies involved in genetic engineering

Strategy	Method
Formation of DNA fragments	Extracted DNA can be cut into small sequences by specific enzymes – restriction endonucleases found in many species of bacteria.
Splicing of DNA into vectors	The small sequences of DNA can be joined or spliced into the vector DNA molecules by an enzyme DNA ligase, creating an artificial DNA molecule.
Introduction of vectors into host cells	The vectors are either viruses or plasmids, and are replicons and can exist in an extra-chromosomal state; they can be transferred normally by transduction or transformation.
Selection of newly acquired DNA	Selection and ultimate characterisation of the recombinant clone.

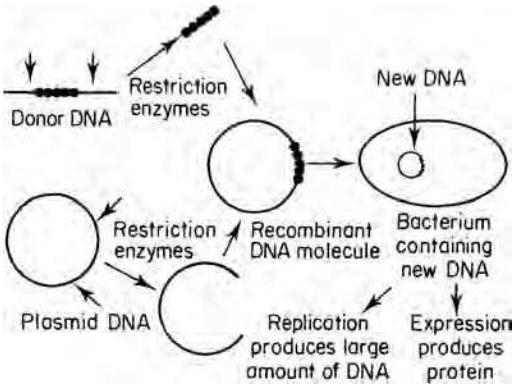

Fig. 3.3 Recombinant DNA: the technique of recombining genes from one species with those of another.

Agrobacterium system (Chapter 10). Thus, in the last four decades, molecular biology has formulated evidence for the unity of genetic systems together with the basic mechanisms that regulate cell function. Genetic engineering has confirmed the unity of the living world, demonstrating that all living creatures are built of molecules that are more or less identical. Thus, the diversity of life forms on this planet derives from small changes in the regulatory systems that control the expression of genes.

3.5 The polymerase chain reaction and DNA sequencing

Two molecular biology techniques in recent years have revolutionised the availability of DNA data, namely the polymerase chain reaction (PCR) and the development of automated DNA sequencing. A PCR is basically a technique which allows the selective amplification of any fragment of DNA provided that the DNA sequences flanking the fragment are known – described as a technique which finds a needle in a haystack and then produces a haystack of needles by specific amplification! The inventor of PCR, Kary Mullis, shared the Nobel Prize in Chemistry in 1993.

The PCR process relies on the sequence of 'base pairs' along the length of the two strands that make the complete DNA molecule. In DNA there are four deoxynucleotides derived from the four bases, adenine (A), thymine (T), guanine (G) and cytosine (C). The strands or polymers that comprise the DNA molecule are held to each other by hydrogen bonds between the base pairs. In this arrangement, A only binds to T while G only binds to C, and this unique system folds the entire molecule into the now well-recognised double-helix structure.

PCR involves three processing steps: *denaturation, annealing* and then *extension* by DNA polymerase (Fig. 3.4a, b). In Step 1, the double-stranded DNA is heated (95–98°C) and separates into two complementary single strands. In Step 2 (60°C), the synthetic oligonucleotide primers (chemically synthesised short-chain nucleotides) – short sequences of nucleotides (usually about 20 nucleotide base pairs long) – are added and bind to the single strands in places where the strand's DNA complements their own. In Step 3 (37°C), the primers are extended by DNA polymerase in the presence of all four deoxynucleoside triphosphates, resulting in the synthesis of new DNA strands that are complementary to the template strands. The completion of the three steps comprises a cycle and the real power of PCR is that, with 25–30 cycles, this experimental synthesis leads to massive amplification of DNA which can then be used for analytical purposes. A major recent advance has been the development of automated thermal cyclers (PCR machines), which allow the entire PCR to be performed automatically in several hours.

PCR was first patented in l987 and then commercialised by the American Cetus Corporation in 1988. However, in 1991, Hoffman La Roche and Perkin Elmer purchased the full operating rights of PCR for $300 million. The applications of PCR increase almost daily and include: molecular biology/genetic engineering, infectious and parasitic disease diagnosis, human genetic disease diagnosis, forensic validation, plant and animal breeding, and environmental

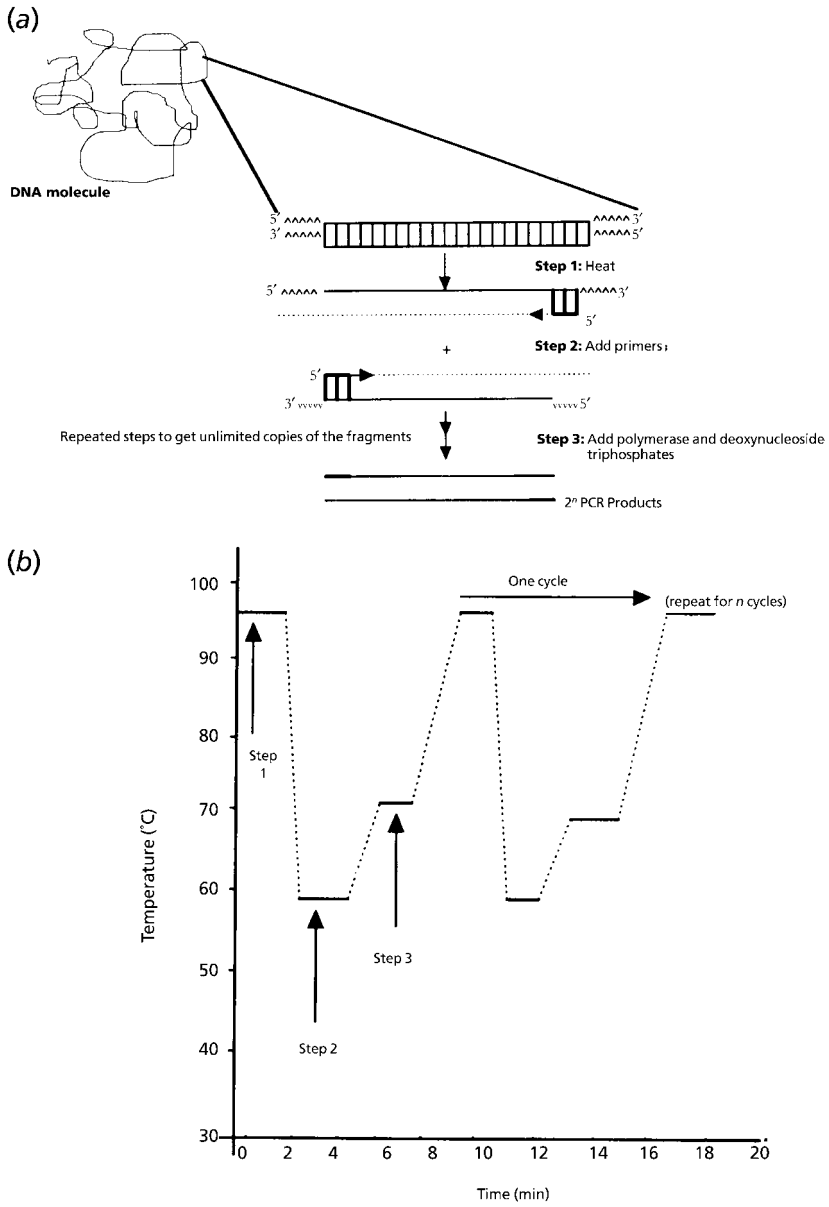

Fig. 3.4 (a) The polymerase chain reaction. The double-stranded DNA is heated and separates into two single strands. The synthetic oligonucleotide primers then bind to their complementary sequence and are extended in the direction of the arrows, giving a new strand of DNA identical to the template's original partner;
(b) PCR temperature cycling profile (see Graham, 1994).

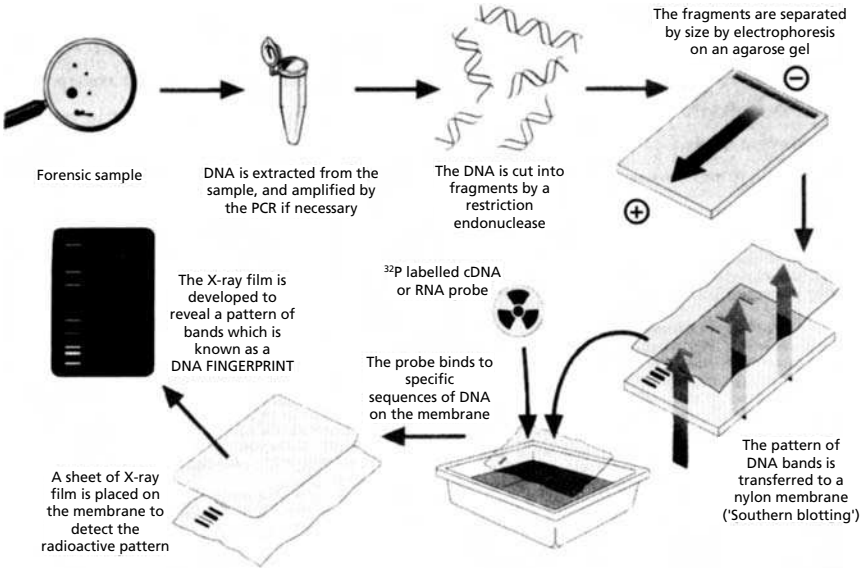

Fig. 3.5 DNA fingerprinting (from Grainger and Madden, 1993).

monitoring. PCR has been extensively used in the well-known procedure of genetic or DNA fingerprinting, the fallibility of which is now being challenged in courts of law (Fig. 3.5).

While PCR is finding considerable and unique use in archaeology, it is doubtful whether we will ever be able to resurrect woolly mammoths and dinosaurs from ancient animal remains, as recently epitomised in Michael Crighton's *Jurassic Park*.

Genomes of all organisms consist of millions of repetitions of the four nucleotides – C, G, A and T. In humans, there are over 3000 million nucleotides. Analysing the sequence of the nucleotides (*DNA sequencing*) has become a critically useful technique for the identification, analysis and directed manipulation of genomic DNA. Originally, methods of separation and identification relied upon gel electrophoresis and autoradiography. However, recent developments in sequencing technology have allowed the process to be automated and greatly speeded up. Fluorescent dye-labelled substrates are used, which allow the use of a laser-induced fluorescent detection system. In many applications automated sequencers can produce over 1000 base pairs of sequences from overnight operations. There are now publicly available databases such as GenBank, which provide numerous online services for identifying, aligning and comparing sequences. Individual chromosomes contain

many thousands of sequences, some of which are organised into genes while others appear to be merely flanking or spacer regions.

3.6 Genomics and proteomics

The genetic heritable material of living cells resides with the nucleic acids of the chromosomes and is termed the '*genome*'. Arising from the previously described techniques, it was possible in 1995 to determine the first complete genome or DNA sequence of a free-living organism, the bacterium *Haemophilus influenzae*. Since then, a considerable number of prokaryotes, the yeast *Saccharomyces cerevisiae*, the fruit-fly *Drosophila melanogaster* and the plant *Arabidopsis thaliana* have been sequenced. However, the major event in molecular genetics was the elucidation of the human genome sequence in 2001. The academic and commercial drive to decipher the human genome was largely driven by a belief that major medical developments would unfold. Consequently, many billions of dollars have been spent to achieve this momentous level of genomic knowledge. While there has been much hype concerning the ethical and commercial implications of these discoveries, this is only the beginning of the understanding of the real functional activity within cells, in tissues and in whole organisms. Throughout this last decade of genomic research there has been insufficient emphasis on other aspects of cellular organisation and much ill-judged scientific belief that the enigma of cell function in health and disease could be understood solely through knowledge of genes alone.

Biochemical studies over many decades have shown that cellular activity is achieved through a vast array of signalling and regulatory and metabolic pathways, each involving many specific molecules. There still exists a vast gulf between our understanding of individual molecular mechanisms and pathways and how they are integrated into an orderly *homeostatic* system.

Major molecular biology attention has now moved dramatically to the study of the *proteome* – the collective body of proteins made within an organism's cells and tissues. While the genome supplies the recipes for making the cell's proteins, it is the proteome that represents the bricks and mortar of the cells and carries out the cellular functions. The proteome is infinitely more complicated than the genome. While a cell will have only one genome, it can have many proteomes. The DNA alphabet is composed of four chained bases, while proteins, in contrast, are constructed from approximately 20 amino acids. While the genes through transcription determine the sequence of amino acids in a protein, it is not totally clear what the protein does and how

it interacts with other proteins. Unlike genes which are linear, proteins fold into three-dimensional structures which are difficult to predict. The proteome is extremely dynamic, and minor alterations in the external or internal environment can modify proteome function. Understanding proteomics should give a better holistic view of cellular metabolism.

The dominant biochemical approach to proteomics combines two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), which separates, maps and quanitifies proteins, with mass spectrometry (MS)-based sequencing techniques which identify both the amino acid sequences of proteins and the post-translationary molecular additions. Proteomics will relate to genomic databases to assist protein identification and consequently will indicate which genes within the database are important in specific conditions. The two areas of genomics and proteomics must have a strong synergistic relationship. The potential of proteomics to identify and compare complex protein profiles is now generating highly accurate but sensitive molecular fingerprints of proteins present in human body fluids at a given time. These may well offer early markers of diseased status in the human system. Such molecular medicine could well be one of the most remarkable achievements of biotechnology of this century.

The ability to clone DNA or manipulate genes and to obtain successful expression in an organism is nowadays a core technology of quite unparalleled importance in modern bioscience and biotechnology. The expression and acceptance of genetic engineering in the context of biotechnology, where novel gene pools can be created and expressed in large quantities, will offer outstanding opportunities for the well-being of humanity.

3.7 Potential laboratory biohazards of genetic engineering

The early studies on gene manipulation provoked wide discussion and considerable concern at the possible risks that could arise with certain types of experiment. Thus it was believed by some that the construction of recombinant DNA molecules and their insertion into microorganisms could create novel organisms which might inadvertently be released from the laboratory and become a biohazard to humans or the environment. In contrast, others considered that newly synthesised organisms with their additional genetic material would not be able to compete with the normal strains present in nature. The present views of gene manipulation studies are becoming more moderate as experiments have shown that this work can proceed within a strict

safety code when required, involving physical and biological containment of the organism.

The standards of containment enforced in the early years of recombinant DNA studies were unnecessarily restrictive and there has been a steady relaxation of the regulations governing much of the routine genetic engineering activities. However, for many types of study – particularly with pathogenic microorganisms – the standards will remain stringent. Thus, for strict physical containment, laboratories involved in this type of study must have highly skilled personnel and correct physical containment equipment, e.g. negative pressure laboratories, autoclaves and safety cabinets.

Biological containment can be achieved or enhanced by selecting non-pathogenic organisms as the cloning agents of foreign DNA or by the deliberate genetic manipulation of a microorganism to reduce the probability of survival and propagation in the environment. *Escherichia coli*, a bacterium which is extremely prevalent in the intestinal tracts of warm-blooded and cold-blooded animals as well as in humans, is the most widely used cloning agent. To offset the risk of this cloning agent becoming a danger in the environment, a special strain of *E. coli* has been constructed by genetic manipulation which incorporates many fail-safe features. This strain can only grow under special laboratory conditions and there is no possibility that it can constitute a biohazard if it escapes out of the laboratory.

The government-controlled Health and Safety Executive controls and monitors recombinant DNA work within the UK. This committee seeks advice from the Genetic Manipulation Advisory Group (GMAG), who formulate realistic procedural guidelines which, in general, have proved widely acceptable to the experimenting scientific community. Most other advanced scientific nations involved in recombinant DNA studies have set up similar advisory committees. The deliberate releasing of genetically manipulated organisms to the environment is discussed in Chapter 14.

4

Bioprocess/fermentation technology

4.1 Introduction

Bioprocess or fermentation technology is an important component of most 'old' and 'new' biotechnology processes and will normally involve complete living cells (microbe, mammalian or plant), organelles or enzymes as the biocatalyst and will aim to bring about specific chemical and/or physical changes in organic materials (the medium). In order to be viable in any specific industrial context, bioprocessing must possess advantages over competing methods of production such as chemical technology. In practice, many bioprocessing techniques will be used industrially because they are the only practical way in which a specific product can be made (e.g. vaccines, antibiotics).

The very beginnings of fermentation technology, or as it is now better recognised, 'bioprocess technology', were derived in part from the use of microorganisms for the production of foods such as cheeses, yoghurts, sauerkraut, fermented pickles and sausages, soy sauce, and other Oriental products, and beverages such as beers, wines and derived spirits (Table 4.1). In many cases, the present-day production processes for such products are still remarkably similar. These forms of bioprocessing were long viewed as arts or crafts but are now increasingly subjected to the full array of modern science and technology. Paralleling these useful product formations was the identification of the roles that microorganisms could play in removing obnoxious and unhealthful wastes, which has resulted in worldwide service industries involved in water purification, effluent treatment and solid waste management (Chapter 9).

Bioprocessing in its many forms involves a multitude of complex enzyme-catalysed reactions within specific cellular systems, and these reactions are

Table 4.1. Fermentation products according to industrial sectors

Sector	Products/activities
Chemicals	
Organic (bulk)	Ethanol, acetone, butanol
	Organic acids (citric, itaconic)
Organic (fine)	Enzymes
	Perfumeries
	Polymers (mainly polysaccharides)
Inorganic	Metal beneficiation, bioaccumulation and leaching (Cu, U)
Pharmaceuticals	Antibiotics
	Diagnostic agents (enzymes, monoclonal antibodies)
	Enzyme inhibitors
	Steroids
	Vaccines
Energy	Ethanol (gasohol)
	Methane (biogas)
	Biomass
Food	Dairy products (cheeses, yoghurts, fish and meat products)
	Beverages (alcoholic, tea and coffee)
	Baker's yeast
	Food additives (antioxidants, colours, flavours, stabilisers)
	Novel foods (soy sauce, tempeh, miso)
	Mushroom products
	Amino acids, vitamins
	Starch products
	Glucose and high-fructose syrups
	Functional modifications of proteins, pectins
Agriculture	Animal feedstuffs (SCP)
	Veterinary vaccines
	Ensilage and composting processes
	Microbial pesticides
	Rhizobium and other N-fixing bacterial inoculants
	Mycorrhizal inoculants
	Plant cell and tissue culture (vegetative propagation, embryo production, genetic improvement)

Adapted from Bull *et al*. (1982).

critically dependent on the physical and chemical conditions that exist in their immediate environment. Successful bioprocessing will only occur when all the essential factors are brought together.

Although the traditional forms of bioprocess technology related to foods and beverages still represent the major commercial bioproducts, new products are increasingly being derived from microbial and mammalian fermentations, namely:

(1) in the overproduction of essential primary metabolites, e.g. acetic and lactic acids, glycerol, acetone, butyl alcohol, organic acids, amino acids, vitamins and polysaccharides;
(2) in the production of secondary metabolites (metabolites that do not appear to have an obvious role in the metabolism of the producer organism), e.g. penicillin, streptomycin, cephalosporin, gibberellins;
(3) in the production of many forms of industrially useful enzymes, e.g. exocellular enzymes such as amylases, pectinases and proteases and intracellular enzymes such as invertase, asparaginase and restriction endonucleases;
(4) in the production of monoclonal antibodies, vaccines and novel recombinant products, e.g. therapeutic proteins.

All of these products now command large industrial markets and are essential to modern society (Table 4.1).

More recently, bioprocess technology is increasingly using cells derived from higher plants and animals to produce many important products. Plant cell culture is largely aimed at secondary product formations such as flavours, perfumes and drugs, while mammalian cell culture has been concerned with vaccine and antibody formation and the recombinant production of protein molecules such as interferon, interleukins and erythropoietin.

The future market growth of these bioproducts is largely assured because, with limited exceptions, most cannot be produced economically by other chemical processes. It will also be possible to make further economies in production by genetically engineering organisms to higher or unique productivities and utilising new technological advances in processing. The advantages of producing organic products by biological, as opposed to purely chemical, methods are listed in Table 4.2.

The product formation stages in bioprocess technology are essentially very similar regardless of the organism selected, the medium used and the product formed. In all examples, large numbers of cells are grown under defined controlled conditions. The organisms must be cultivated *and* motivated to form the desired products by means of a physical/technical containment system (*bioreactor*) and the correct medium composition and environmental

Table 4.2. Advantages and disadvantages of producing organic compounds by biological rather than chemical means

Advantages	Disadvantages
Complex molecules such as proteins and antibodies cannot be produced by chemical means.	The product can be easily contaminated with foreign unwanted microorganisms, etc.
Bioconversions give higher yields.	The desired product will usually be present in a complex product mixture requiring separation.
Biological systems operate at lower temperatures, near neutral pH, etc.	There is a need to provide, handle and dispose of large volumes of water.
There is much greater specificity of catalytic reaction.	Bioprocesses are usually extremely slow when compared with conventional chemical processes.
Exclusive production of an isomeric compound can be achieved.	

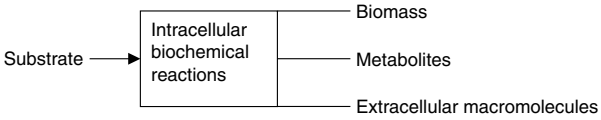

Fig. 4.1 The biotechnology process.

growth-regulating parameters such as temperature and aeration. Optimisation of the bioprocess spans both the bio- and the technical systems. The proper exploitation of an organism's potential to form distinct products of defined quality *and* in large amounts requires a detailed knowledge of the biochemical mechanisms of product formation.

Bioprocessing in its many forms is catalysed with each respective cellular system by a large number of intracellular biochemical reactions. Substrates derived from the medium are converted into primary and secondary products, intra- and extracellular macromolecules, and biomass components such as DNA, RNA, proteins and carbohydrates (Fig. 4.1).

These reactions will be dependent on the physical and chemical parameters that exist in their immediate environments.

The same apparatus with modifications can be used to produce an enzyme, an antibiotic, an amino acid or a single cell protein. In its simplest form, the

Table 4.3. Examples of products in different categories in biotechnological industries

Category	Example
Cell mass[a]	Baker's yeast, SCP
Cell components[b]	Intracellular proteins
Biosynthetic products[b]	Antibiotics, vitamins, amino and organic acids
Catabolic products[a]	Ethanol, methane, lactic acid
Bioconversion[a]	High-fructose corn syrup, 6-aminopenicillanic acid
Waste treatment	Activated sludge, anaerobic digestion

[a] Typically, conversion of feedstock cost-intensive processes.
[b] Typically, recovery cost-intensive process.

bioprocess can be viewed merely by mixing the microorganisms with a nutrient broth and allowing the components to react, e.g. mixing yeast cells with a sugar solution to give alcohol. More advanced and sophisticated processes operating on a large scale need to control the entire system so that the bioprocess can proceed efficiently and be readily and exactly repeated with the same amounts of raw materials and inoculum (the particular organism) to produce precisely the same amount of product.

All biotechnological processes are essentially performed within containment systems or bioreactors. Large numbers of cells are invariably involved in these processes and the bioreactor ensures their close involvement with the correct medium and conditions for growth and product formation. It also should restrict the release of the cells into the environment. A main function of a bioreactor is to minimise the cost of producing a product or service. Examples of the diverse product categories produced industrially in bioreactors are given in Table 4.3.

4.2 Principles of microbial growth

The growth of organisms may be seen as the increase of cell material expressed in terms of mass or cell number and results from a highly complicated and coordinated series of enzymatically catalysed biological steps. Growth will be dependent both on the availability and transport of necessary nutrients to the cell and subsequent uptake and on environmental parameters such as temperature, pH and aeration being optimally maintained.

The quantity of biomass or specific cellular component in a bioreactor can be determined gravimetrically (by dry weight, wet weight, DNA or protein)

Table 4.4. Approximate size of cells used in biotechnology processes

Cell type	Size (μm)
Bacterial cells	1 × 2
Yeast cells	7 × 10
Mammalian cells	40 × 40
Plant cells	100 × 100

or numerically for unicellular systems (by number of cells). Doubling time refers to the period of time required for the doubling in the weight of biomass, while generation time relates to the period necessary for the doubling of cell numbers. Average doubling times increase with increasing cell size (Table 4.4) and complexity, e.g. doubling time for bacteria is 0.25–1 h; yeast 1–2 h; mould fungi 2–6.5 h; plant cells 20–70 h; and mammalian cells 20–48 h.

In normal practice an organism will seldom have totally ideal conditions for unlimited growth; rather, growth will be dependent on a limiting factor, for example an essential nutrient. As the concentration of this factor drops, so also will the growth potential of the organism decrease.

In biotechnological processes there are three main ways of growing microorganisms in the bioreactor: batch, semi-continuous or continuous. Within the bioreactor, reactions can occur with static or agitated cultures, in the presence or absence of oxygen, and in liquid or low-moisture conditions (e.g. on solid substrates). The microorganisms can be free or can be attached to surfaces by immobilisation or by natural adherence.

In a *batch culture*, the microorganisms are inoculated into a fixed volume of medium and, as growth takes place, nutrients are consumed and products of growth (biomass, metabolites) accumulate. The nutrient environment within the bioreactor is continuously changing and, thus, in turn, enforcing changes to cell metabolism. Eventually, cell multiplication ceases because of exhaustion or limitation of nutrient(s) and accumulation of toxic excreted waste products.

The complex nature of batch growth of microorganisms is shown in Fig. 4.2. The initial *lag phase* is a time of no apparent growth but actual biochemical analyses show metabolic turnover, indicating that the cells are in the process of adapting to the environmental conditions and that new growth will eventually begin. There is then a *transient acceleration* phase as the inoculum begins to grow, which is quickly followed by the *exponential phase*. In the exponential phase microbial growth proceeds at the maximum possible rate for that organism with nutrients in excess, ideal environmental parameters

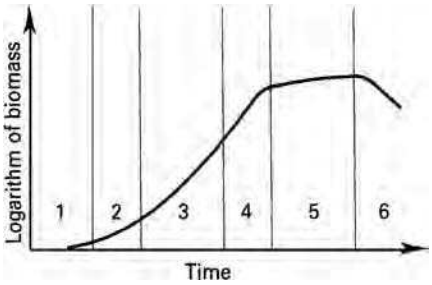

Fig. 4.2 Growth characteristics in a batch culture of a microorganism. 1, lag phase; 2, transient acceleration; 3, exponential phase; 4, deceleration phase; 5, stationary phase; 6, death phase.

and growth inhibitors absent. However, in batch cultivations exponential growth is of limited duration and, as nutrient conditions change, growth rate decreases, entering the *deceleration phase*, to be followed by the *stationary phase*, when overall growth can no longer be obtained owing to nutrient exhaustion. The final phase of the cycle is the *death phase*, when growth rate has ceased. Most biotechnological batch processes are stopped before this stage because of decreasing metabolism and cell lysis.

In industrial usage, batch cultivation has been carried out to optimise organism or biomass production and then to allow the organism to perform specific biochemical transformations such as end-product formation (e.g. amino acids, enzymes) or decomposition of substances (sewage treatment, bioremediation). Many important products such as antibiotics are optimally formed during the stationary phase of the growth cycle in batch cultivation.

However, there are means of prolonging the life of a batch culture and thus increasing the yield by various substrate feed methods:

(1) by the gradual addition of concentrated components of the nutrient, e.g. carbohydrates, so increasing the volume of the culture (*fed batch*) – used for the industrial production of baker's yeast;
(2) by the addition of medium to the culture (*perfusion*) and withdrawal of an equal volume of used cell-free medium – used in mammalian cell cultivations.

In contrast to batch conditions, the practice of *continuous cultivation* gives near balanced growth with little fluctuation of nutrients, metabolites or cell numbers or biomass. This practice depends on fresh medium entering a batch system at the exponential phase of growth with a corresponding withdrawal

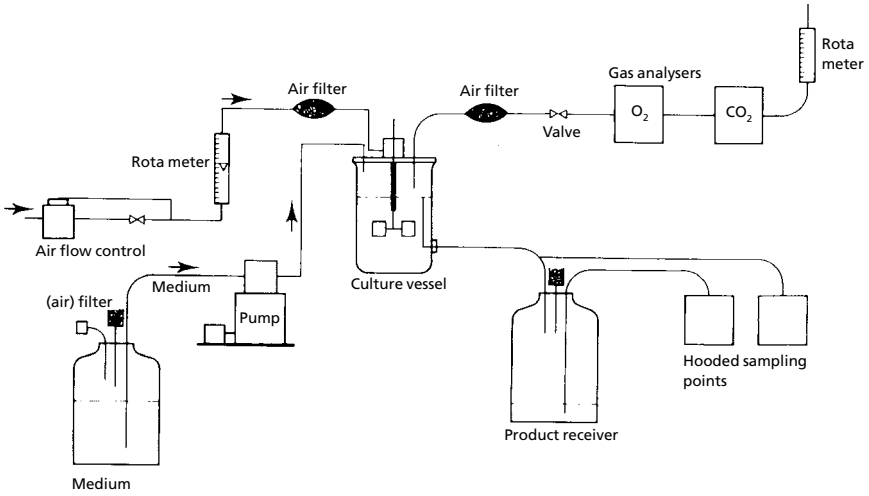

Fig. 4.3 A simple laboratory fermenter operating on a continuous-cultivation basis.

of medium *plus* cells. Continuous methods of cultivation will permit organisms to grow under steady state (unchanging) conditions in which growth occurs at a constant rate and in a constant environment. In a completely mixed continuous-culture system, sterile medium is passed into the bioreactor (Fig. 4.3) at a steady flow rate and culture broth (medium, waste products and organisms) emerges from it at the same rate, keeping the volume of the total culture in the bioreactor constant. Factors such as pH and the concentrations of nutrients and metabolic products, which inevitably change during batch cultivation, can be held near constant in continuous cultivations. In industrial practice continuously operated systems are of limited use and include only single cell protein (SCP) and ethanol productions and some forms of waste water treatment processes. However, for many reasons (Table 4.5) batch cultivation systems represent the dominant form of industrial usage. The full range of cultivation methods for microorganisms is shown in Table 4.6.

Microorganisms utilised in industrial biotechnology processes are normally held in great secrecy by the commercial companies. They have been derived from extensive selection processes and optimised by culture development for optimum productivity. Methods have been developed for long-term storage to maintain culture stability and productivity. National and International Culture Collection Centres conserve a wide range of microbial cultures, which provide an organism base for biosystematics and support bioscience and biotechnology research and development.

Table 4.5. Advantages of batch and fed-batch culture techniques in industry

(1) The products may be required only in relatively small quantities at any given time.
(2) Market needs may be intermittent.
(3) The shelf-life of certain products is short.
(4) High product concentration is required in broth to optimise downstream processing operations.
(5) Some metabolic products are produced only during the stationary phase of the growth cycle.
(6) The instability of some production strains requires their regular renewal.
(7) Continuous processes can offer many technical difficulties.

4.3 The bioreactor

Bioreactors are the containment vehicles of any biotechnology-based production process, be it for brewing, organic or amino acids, antibiotics, enzymes or vaccines or for bioremediation. For each biotechnology process the most suitable containment system must be designed to give the correct environment for optimising the growth and metabolic activity of the biocatalyst. Bioreactors range from simple stirred or non-stirred open containers to complex aseptic integrated systems involving varying levels of advanced computer control (Fig. 4.4).

Bioreactors occur in two distinct types (Fig. 4.4). In the first instance they are primarily non-aseptic systems where it is not absolutely essential to operate with entirely pure cultures, e.g. brewing, effluent disposal systems, while in the second type aseptic conditions are a prerequisite for successful product formation, e.g. antibiotics, vitamins, polysaccharides. This type of process involves considerable challenges on the part of engineering construction and operation.

The physical form of many of the most widely used bioreactors has not altered much over the past 40 years; however, in recent years, novel forms of bioreactors have been developed to suit the needs of specific bioprocesses, and such innovations are finding increasingly specialised roles in bioprocess technology (Fig. 4.4).

In all forms of fermentation the ultimate aim is to ensure that all parts of the system are subject to the same conditions. Within the bioreactor the microorganisms are suspended in the aqueous nutrient medium containing the necessary substrates for growth of the organism and required product

Table 4.6. Characteristics of cultivation methods

Type of culture	Operational characteristics	Application
Solid	Simple, cheap selection of colonies from single cell possible; process control limited	Maintenance of strains, genetic studies; production of enzymes; composting
Film	Various types of bioreactors; trickling filter, rotating disc, packed bed, sponge reactor, rotating tube	Waste-water treatment, monolayer culture (animal cells); bacterial leaching; vinegar production
Submerged homogeneous distribution of cells; batch	'Spontaneous' reaction, various types of reactor: stirred tank bioreactor, air lift, loop, deep shaft, etc; agitation by stirrers, air, liquid process control for physical parameters possible; less for chemical and biological parameters	Standard type of cultivation: antibiotics, solvents, acids, etc.
Fed-batch	Simple method for control of regulatory effects, e.g. glucose repression	Production of baker's yeast
Continuous one-stage homogeneous	Proper control of reaction; excellent role for kinetic and regulatory studies; higher costs for experiment; problem of aseptic operation, the need for highly trained operators	Few cases of application in industrial scale; production of SCP; waste water treatment

formation. All nutrients, including oxygen, must be provided to diffuse into each cell and waste products such as heat, CO_2 and waste metabolites removed.

The concentration of the nutrients in the vicinity of the organism must be held within a definite range since low values will limit the rate of organism metabolism while excessive concentrations can be toxic. Biological reactions run most efficiently within optimum ranges of environmental parameters, and in biotechnological processes these conditions must be provided on a micro-scale so that each cell is equally provided for. When the large scale of many bioreactor systems is considered, it will be realised how difficult it is to achieve

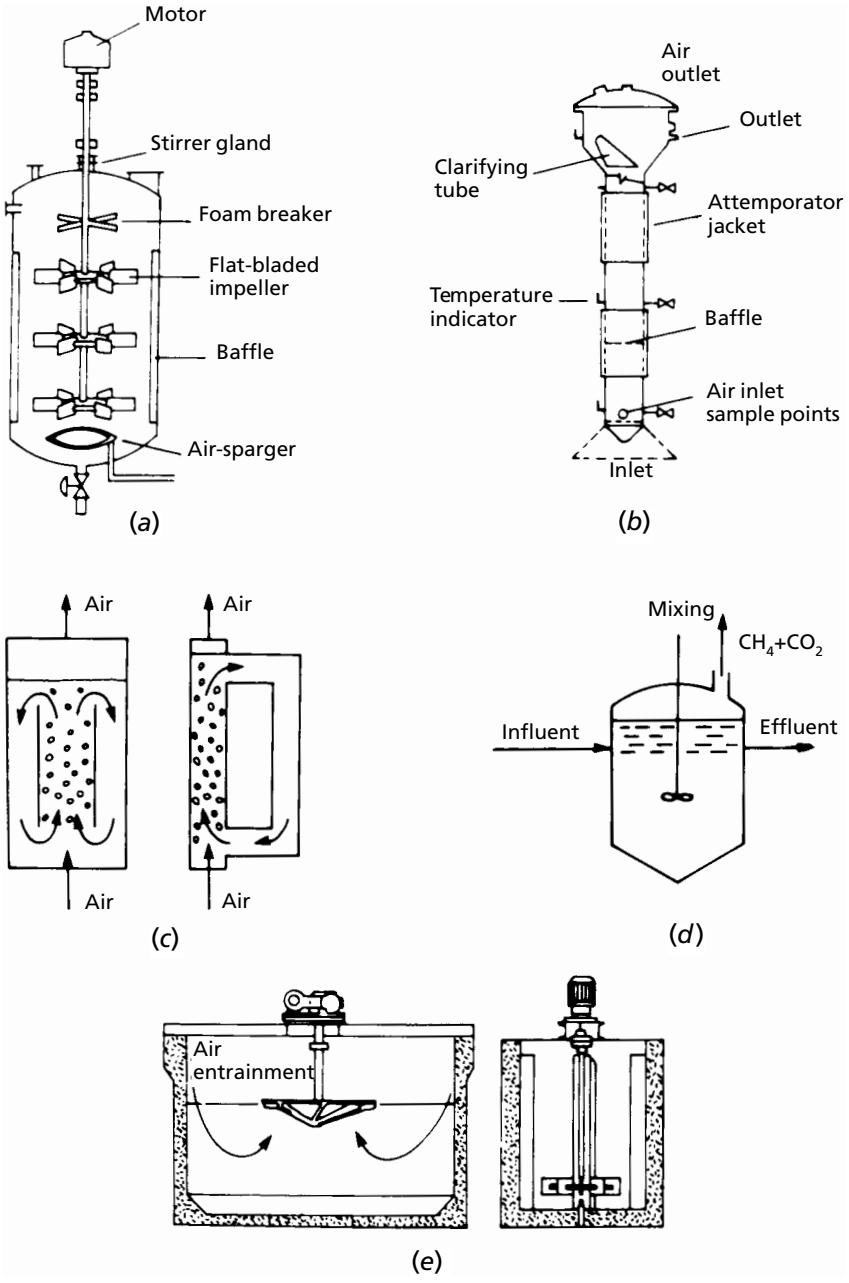

Fig. 4.4 Various forms of bioreactor. (a) Stirred tank bioreactor; (b) tower reactor; (c) loop (recycle) bioreactor; (d) anaerobic digester or bioreactor; (e) activated sludge bioreactor. (a) and (b) reproduced by permission from Kristiansen and Chamberlain (1983).

Table 4.7. Standards of materials used in sophisticated fermenter design

(1) All materials coming into contact with the solutions entering the bioreactor or the actual organism culture must be corrosion resistant to prevent trace-metal contamination of the process.
(2) The materials must be non-toxic so that slight dissolution of the material or components does not inhibit culture growth.
(3) The materials of the bioreactor must withstand repeated sterilisation with high-pressure steam.
(4) The bioreactor stirrer system, entry ports and end plates must be easily machinable and sufficiently rigid so as not to be deformed or broken under mechanical stress.
(5) Visual inspection of the medium and culture is advantageous; transparent materials should be used wherever possible.

these conditions in a whole population. It is here that the skills of the process or biochemical engineer and the microbiologist must come together.

Fermentation reactions are multiphase, involving a gas phase (containing N_2, O_2 and CO_2), one or more liquid phases (aqueous medium and liquid substrate) and a solid microphase (the microorganisms and, possibly, solid substrates). All phases must be kept in close contact to achieve rapid mass and heat transfer. In a perfectly mixed bioreactor, all reactants entering the system must be immediately mixed and uniformly distributed to ensure homogeneity inside the reactor.

To achieve optimisation of the bioreactor system, the following operating guidelines must be closely adhered to:

(1) The bioreactor should be designed to exclude entrance of contaminating organisms as well as containing the desired organisms;
(2) The culture volume should remain constant, i.e. no leakage or evaporation;
(3) The dissolved oxygen level must be maintained above critical levels of aeration and culture agitation for aerobic organisms;
(4) Environmental parameters such as temperature, pH, etc., must be controlled, and the culture volume must be well mixed.

The standards of materials used in the construction of sophisticated fermenters is important (Table 4.7).

Fermentation technologists seek to achieve a maximisation of culture potential by accurate control of the bioreactor environment. But still there is a great lack of true understanding of just what environmental conditions will produce an optimal yield of organism or product.

Successful bioprocessing will only occur when all the specific growth-related parameters are brought together and the information used to improve and optimise the process. For successful commercial operation of these bioprocesses, quantitative description of the cellular processes is an essential prerequisite. The two most relevant aspects, *yield* and *productivity,* are quantitative measures that will indicate how the cells convert the substrate into the product. The yield represents the amount of product obtained from the substrate while the productivity specifies the rate of product formation.

To understand and control a fermentation process it is necessary to know the state of the process over a small time increment and, further, to know how the organism responds to a set of measurable environmental conditions. Process optimisation requires accurate and rapid feedback control. In the future, the computer will be an integral part of most bioreactor systems. However, there is a lack of good sensor probes that will allow on-line analysis to be made on the chemical components of the fermentation process.

A large worldwide market exists for the development of new rapid methods for monitoring the many reactions within a bioreactor. In particular, the greatest need is for innovatory micro-electronic designs.

When endeavouring to improve existing process operations or designing, it is often advisable to set up mathematical models of the overall system. A model is a set of relationships between the variables in the system being studied. Such relationships are usually expressed in the form of mathematical equations but can also be specific as cause/effect relationships which can be used in the operation of the specific processes. The actual variables involved can be extensive but will include any parameter that is of importance for the process, and can include pH, temperature, substrate concentration, agitation, feed rate, etc.

Bioreactor configurations have changed considerably over the last few decades. The original fermentation system was a shallow tank that was agitated or stirred by manpower. From this has developed the basic aeration tower system which now dominates industrial usage. As fermentation systems were further developed, two design solutions to the problems of aeration and agitation have been implemented. The first approach uses mechanical aeration and agitation devices, with relatively high power requirements; the standard example is the stirred tank bioreactor, which is widely used throughout conventional laboratory and industrial fermentations. Such bioreactors ensure good gas/liquid mass transfer, have reasonable heat transfer, and ensure good mixing of the bioreactor contents.

The second main approach to aerobic bioreactor design uses air distribution (with low power consumption) to create forced and controlled liquid flow in a

recycle or loop bioreactor. In this way the contents are subjected to a controlled recycle flow, either within the bioreactor or involving an external recycle loop. Thus stirring has been replaced by pumping, which may be mechanical or pneumatic, as in the case of the airlift bioreactor.

The CSTR consists of a cylindrical vessel with a motor-driven central shaft that supports one or several agitators, with the shaft entering either through the top or the bottom of the vessels. The aspect ratio (i.e. height-to-diameter ratio) of the vessel is 3 : 5 for microbial systems, while for mammalian cell culture the aspect ratios do not normally exceed 2. Sterile air is sparged into the bioreactor liquid below the bottom impeller by way of a perforated ring sparger. The speed of the impellors will be related to the degree of fragility of the cells. Mammalian cells are extremely fragile when compared with most microorganisms. In a great many of the high-value processes, the bioreactors will be operated in a batch manner under aseptic monoculture. The bioreactors can range from about 20 litres to in excess of 250 m^3 for particular processes. The initial culture expansion of the microorganisms will commence in the smallest bioreactor, and when growth is optimised, will then be transferred to a larger bioreactor, and so forth, until the final-operation bioreactor. Throughout such operations it is imperative to maintain aseptic conditions to ensure the success of the process. Bioreactors are normally sterilised prior to inoculation, and contamination must be avoided during all subsequent operations. If contamination occurs during the cultivation this will invariably lead to process failure since, more often, the contaminant can outgrow the participating monoculture.

Large amounts of organic waste waters from domestic and industrial sources are routinely treated in aerobic and anaerobic systems. Activated sludge processes are widely used for the oxidative treatment of sewage and other liquid wastes (Fig. 4.4d). Such processes use batch or continuously agitated bioreactor systems to increase the entrainment of air to optimise oxidative breakdown of the organic material. These bioreactors are large and, for optimum functioning, will have several or many agitator units to facilitate mixing and oxygen uptake. They are widely used in most municipal sewage treatment plants.

Anaerobic bioreactors or digestors have long been used to treat sewage matter. In the absence of free oxygen, certain microbial consortia are able to convert biodegradable organic material to methane, carbon dioxide and new microbial biomass. Most common anaerobic digesters work on a continuous or semi-continuous manner.

An outstanding example of methane generation is the Chinese biogas programme, where millions of family-size anaerobic bioreactors are in operation. Such bioreactors are used for the treatment of manure, human excreta, etc.,

producing biogas for cooking and lighting and the sanitisation of the waste, which then becomes an excellent fertiliser.

In almost all fermentation processes performed in a bioreactor there is generally a need to measure specific growth-related and environmental parameters, record them and then use the information to improve and optimise the process. Bioreactor control measurements are made in either an on-line or an off-line manner. With an on-line measurement, the sensor is placed directly with the process stream, whereas for off-line measurement a sample is removed aseptically from the process stream and analysed. Bioreactor processing is still severely limited by a shortage of reliable instruments capable of on-line measurement of important variables such as DNA, RNA, enzymes and biomass. Off-line analysis is still essential for these compounds, and since the results of these analyses are usually not available until several hours after sampling, they cannot be used for immediate control purposes. However, on-line measurement is readily available for temperature, pH, dissolved oxygen and CO_2 analyses.

The continued discovery of new products such as therapeutic drugs from microorganisms and mammalian cells will continue to depend on the development of innovative exploratory culture systems which encourage the biosynthesis of novel compounds. New miniaturised, computer-controlled incubator systems with automated analysis units are now available as single units which can perform hundreds of experiments simultaneously, thus producing a wealth of data in a short time to facilitate optimum fermentation conditions for product formation.

A new and quite novel approach involving combinatorial biology generates new products from genetically engineered microorganisms. DNA fragments or genes derived from unusual microorganisms that are not easily cultivated (recalcitrant microorganisms) can be transferred into easily cultivated or surrogate microorganisms, and the resulting mixing and matching of genes encoding biosynthetic machinery is now offering the opportunity to discover new or modified molecules or drugs. This could be of great significance in antibiotic discovery.

While most high-value biotechnological compounds such as antibiotics and therapeutic proteins are produced in monoculture under strict conditions of asepsis, there are now new avenues of research exploring product formation from mixed-culture systems. Such systems may well produce different patterns of metabolites or, indeed, novel metabolites as a result of interactions which can occur between competing microorganisms. Because of the complexity of these mixed organism processes, they have all but been ignored by the scientific community. Monoculture under aseptic conditions is totally

unnatural and rarely, if ever, occurs in nature. The norm is for microorganisms to exist together in the environment and to compete and respond to substrate availability and prevailing environmental conditions.

4.4 Scale-up

Most biotechnological processes will have been identified at laboratory scale and ultimate commercial success will be dependent on the ability to scale-up the process first from laboratory to pilot plant level and then to full commercial scale. The achievement of successful process scale-up must fit within a range of physical and economic restraints. The identification of some of the controlling parameters can usually be made with laboratory-scale bioreactors (5–10 litres) and then moved to pilot-scale level. A pilot plant is, in reality, a large-scale laboratory which has been designed to give flexibility for equipment accommodation and adaptability for process operation. Pilot-plant bioreactors range from 100 liters to 10 000 litres total volume, and the larger pilot bioreactors can, on occasion, be used as production units. Full-scale industrial bioreactors can range between 20 000 and 400 000 litres in volume. The management of scale-up requires high capital investment in mixing and aeration, in monitoring and control devices, and in stringent maintenance of sterility.

4.5 Media design for fermentation processes

Water is at the centre of all biotechnological processes and in most cases will be the dominant component of the media in which microorganisms will grow. After liquid fermentation processes have achieved optimum production, the removal of water is a major factor in the cost of bioproduct recovery and downstream processing. The quality of water is highly relevant as it affects microbial growth and the production of specific bioproducts. In the past, traditional brewing centres were established in localities where natural sources provided water of high quality without having to resort to extensive pretreatment.

In media production there is usually quality control of the raw materials. It is increasingly being realised that, in respect of volume, water is one of the most important raw materials in many biotechnological processes and that its supply and use must be carefully monitored and controlled.

The basic nutritional requirements of microorganisms are an energy or carbon source, an available nitrogen source, inorganic elements and, for some

Table 4.8. Sources of carbohydrate and nitrogen for industrial media

Sources of carbohydrate	Sources of nitrogen (% nitrogen by weight)
Glucose	Barley (1.5–2.0)
Pure glucose monohydrate, hydrolysed starch	Beet molasses (1.5–2.0)
Lactose	Corn-steep liquor (4.5)
Pure lactose, whey powder	
Starch	Groundnut meal (8.0)
Barley, groundnut meal, oat flour, rye flour, soy bean meal	Oat flour (1.5–2.0)
	Pharmamedia (8.0)
Sucrose	Rye flour (1.5–2.0)
Beet molasses, cane molasses, crude brown sugar, pure white sugar	Soyabean meal (8.0)
	Whey powder (4.5)

cell types, specific growth factors. In most biotechnological processes carbon and nitrogen sources are more often derived from relatively complex mixtures of cheap natural products or by-products (Table 4.8).

Availability and type of nutrient can exert strong physiological control over fermentation reactors and product formation. Raw material input to a fermentation will be largely dependent on the cost of the material at a particular time since commodity market prices do alter with seasonal and other variables.

Sterilisation practices for biotechnological media must achieve maximum kill of contaminating microorganisms with minimum temperature damage to medium components. Mostly, batch-wise sterilisation in the bioreactor is still the most widely used method, although continuous methods are gaining increased acceptability.

Media preparation may seem to be a relatively uninteresting part of the overall bioprocess but it is in fact the cornerstone of the whole operation. Poor media design will lead to low efficiency of growth and concomitant poor product formation.

4.6 Solid-substrate fermentation

There are many biotechnological processes that involve the growth of microorganisms on solid substrates in the absence or near absence of free water (Table 4.9). The most regularly used solid substrates are cereal grains, legume seeds, wheat bran, lignocellulose materials such as straws, sawdust or wood

Table 4.9. Some examples of solid-substrate fermentations

Example	Substrate	Microorganism(s) involved
Mushroom production (European and Oriental)	Straw, manure	*Agaricus bisporus* *Lentinus edodes* *Volvariella volvacea*
Sauerkraut	Cabbage	Lactic acid bacteria
Soy sauce	Soya beans and wheat	*Aspergillus oryzae*
Tempeh	Soya beans	*Rhizopus oligosporus*
Ontjom	Peanut press cake	*Neurospora sitophila*
Cheeses	Milk curd	*Penicillium roquefortii*
Leaching of metals	Low-grade ores	*Thiobacillus* sp.
Organic acids	Cane sugar, molasses	*Aspergillus niger*
Enzymes	Wheat bran, etc.	*Aspergillus niger*
Composting	Mixed organic material	Fungi, bacteria, actinomycetes
Sewage treatment	Components of sewage	Bacteria, fungi and protozoa

shavings, and a wide range of plant and animal materials. Most of these compounds are invariably polymeric molecules – insoluble or sparingly soluble in water – but are mostly cheap and easily obtainable and represent a concentrated source of nutrients for microbial growth.

Many of these fermentations have great antiquity and, in many instances, there are records dating back hundreds of years. In the East, there is a wide array of food fermentations, including soy sauce and tempeh, as well as many large industrial enzyme processes. In the West, the fermentation processes have centred on the production of silage, mushroom cultivation, cheese and sauerkraut production, and the composting of plant and animal wastes. Solid-substrate fermentations using recyclable raw materials such as straw, wood and other waste materials could well be industries of the future, producing ethanol, methane and edible biomass.

The microbiological components of solid-substrate fermentations can occur as single pure cultures, mixed identifiable cultures or totally mixed indigenous microorganisms.

In many solid-substrate fermentations there is a need to pre-treat the substrate raw materials to enhance the availability of the bound nutrients and also to reduce the size of the components, e.g. pulverising straw and shredding vegetable materials in order to optimise the physical aspects of the process.

Table 4.10. Advantages and disadvantages of solid-substrate fermentations (compared with liquid fermentations)

Advantages	Disadvantages
Simple media with cheaper natural, rather than costly, fossil-derived components.	Processes limited mainly to moulds that tolerate low moisture levels.
Low moisture content of materials gives economy of bioreactor space, low liquid effluent treatment, less microbial contamination, often no need to sterilise, easier downstream processing.	Metabolic heat production in large-scale operation creates problems.
Aeration requirements can be met by simple gas diffusion or by aerating intermittently, rather than continuously.	Process monitoring, e.g. moisture levels, biomass, O_2 and CO_2 levels, is difficult to achieve accurately.
Yields of products can be high.	Bioreactor design not well developed.
Low energy expenditure compared with stirred tank bioreactors.	Product limitation. Slower growth rate of microorganisms.

However, cost aspects of pre-treatment must be balanced with eventual product value. Bioreactor designs for solid-substrate fermentations are inherently more simple than for liquid cultivations. They are classified into fermentations (a) without agitation, (b) with occasional agitation, and (c) with continuous agitation. The relative advantages and disadvantages of solid-substrate fermentations when compared with liquid fermentations are represented in Table 4.10.

4.7 Technology of mammalian and plant cell culture

The main impetus to achieve mass *in vitro* cultivation of mammalian cells dates from the early 1950s with the need to produce large quantities of polio vaccine. During the second half of the twentieth century there was a major drive to develop media and cultivation practices to produce viable and actively proliferating cell cultures from a wide range of different organisms – from mammals such as humans, rats, mice, hampsters, monkeys, cattle, sheep and horses, and, more recently, from fish and insects. Specific cell lines have been obtained from human organs such as the liver, kidney, lungs, lymph nodes,

lung, heart and ovaries, together with an extensive range of various cancer cell lines.

In their natural environment, mammalian cells will obtain the necessary nutrients for metabolism and growth by way of blood circulation. To mimic the complexity of the blood supply has been a continuing area of study and now many successful media formulations have been achieved which will vary in make-up depending on the cell type. Most media will normally contain a complex mixture of organic compounds, such as amino acids, vitamins, organic acids and others, together with buffering inorganic salts. Some media still contain blood serum (5–20%) for the supply of growth factors, trace elements, lipids and other unknown factors. However, the use of serum creates many problems, including variability of nutrient content between batches, irregularity of supply, and now more recently the concern that serum may be contaminated with virions or prion particles.

When mammalian cells are cultured, they grow as unicellular organisms, multiplying by division provided that suitable nutrient and correct environmental conditions are available. Such cells differ from microbial and plant cells in lacking a rigid outer cell wall, making them vulnerable to shear forces and to changes in osmolarity. Furthermore, they are extremely sensitive to impurities in water, to the cost and quality control of media, and the need to avoid contamination by more rapidly growing microorganisms.

Freshly isolated cultures from mammalian systems are known as '*primary cultures*' until subcultured. At this stage they are usually heterogeneous, but still closely representative of the parent cell types and in the expression of tissue-specific properties. After several subcultures onto fresh media, the cell line will either die out or 'transform' to become a *continuous* or *immortalised cell line*. Such cell lines show many alterations from the primary cultures, including changes in cytomorphology, increased growth rate, increase in chromosome variation and increase in tumorigenicity. *In vitro* transformation is primarily the acquisition of an infinite lifespan.

Mammalian cells can be grown either in an unattached suspension culture or attached to a solid surface. Cells such as HeLa cells (cells derived from a human malignancy) can grow in either state, lymphoblastoid cells can grow in suspension culture, while primary or normal diploid cells will only grow when they are attached to a solid surface. Most future commercial development with mammalian cells will be dominated by the cultivation of anchorage-dependent cell types.

Monolayer cultivation of animal cells is governed by the surface area available for attachment, and design considerations have been directed to methods of increasing surface area. Early designs relied mainly on roller tubes or bottles

to ensure the exchange of nutrients and gases. A recent sophisticated system supports the growth of cells in coils of gas-permeable Teflon tubing, each tube having a surface area of 10 000 cm^2; up to 20 such coils can be incorporated into an incubator chamber. A wide range of cells have been successfully cultured under these conditions.

Suspension cultures have been successfully developed to quite large bioreactor volumes, thus allowing all the engineering advantages of the stirred tank bioreactor, which have accrued from microbial studies, to be used to advantage. Such studies have only been on a batch-culture basis.

A combination of attachment culture and suspension culture by the use of microcarrier and porous microcarrier beads has been a major recent innovation in this area. In principle, the anchorage-dependent cells attach to special DEAE-Sephadex beads (having a surface area of 7 cm^2/mg), which are able to float in suspension. In this way the engineering advantages of the stirred tank bioreactor may be used with anchored cells. Many cell types have been grown in this manner, with successful production of viruses and human interferon. The undoubted success of the microcarrier beads may eventually lead to the demise of conventional monolayer systems. New bioreactor designs involving the microcarrier bead concept will surely create a wider commercial development of animal and human cell types.

While such cell lines have allowed extensive studies in mammalian cell biochemistry, the major practical applications have included: vaccine production (polio, mumps, rabies, etc.), toxicological and pharmaceutical research with the aim of reducing animal testing, the production of artificial organs and skin, and the extensive use of mammalian cell lines as producers of proteins for diagnostic (monoclonal antibodies) and for therapeutic applications (interferons, hormones, insulin, etc.). The introduction of foreign genes into mammalian cell lines is now relatively commonplace and will be relevant to improving cell lines in many ways, such as extending productivity, the ability to grow on serum-free media, and to increasing the range of productivity of human therapeutic molecules.

The use of plant cell culture techniques for the micropropagation of certain plants is discussed in Chapter 10. In such cases, plant cell cultures will progress through organogenesis, plantlet amplification and eventual establishment in soil. However, large-scale production of suspension cell cultures of many plant species has now been achieved and yields of products typical of the whole plant have been impressive, e.g. nicotine, alkaloids and ginseng. It is now envisaged that large-scale fermentation programmes may be able to produce commercially acceptable levels of certain high-value plant products, e.g. digitalis, jasmine, spearmint and codeine.

The fermentation methods used to cultivate plant cells in liquid-agitated culture have been largely derived from microbial techniques. Plant cell culture is much slower than with microorganisms, though most of the other characteristics of fermentation are quite similar. The volume of an average cultured plant cell can be up to 200 000 times that of a bacterial cell. Although some plant products are now appearing on the market, it is not expected to be commercially attractive for some time.

4.8 Downstream processing

Downstream processing refers to the isolation and purification of a biotechnologically formed product to a state suitable for the intended use. In most, but not all, biotechnology processes the desired product(s) will be in dilute aqueous solution and the ultimate level of downstream processing will mirror the type of product and required degree of purity. The range of products is considerable and varied in form and can include whole cells, amino acids, vitamins, organic acids, solvents, enzymes, vaccines, therapeutic proteins and monoclonal antibodies. Within these products there will be considerable variation in molecular size and chemical complexity, and a wide range of separation methods will be required for recovery and purification. While many of the products are relatively stable in structure, others can be highly labile and require careful application of the methodology.

The design and efficient operation of downstream processing operations are vital elements in getting the required products into commercial use and should reflect the need not to lose more of the desired product than is absolutely necessary. An example of the effort expended in downstream processing is provided by the plant Eli Lilly built to produce human insulin (Humulin). Over 90% of the 200 staff are involved in recovery processes. Thus, downstream processing of biotechnological processes represents a major part of the overall costs of most processes but is also the least-heralded aspect of biotechnology. Improvements in downstream processing will benefit the overall efficiency and costs of the processes.

Downstream processing will primarily be concerned with initial separation of the bioreactor broth into a liquid phase and a solids phase and subsequent concentration and purification of the product. Downstream processing is a multistage operation (Table 4.11). Methods in use or proposed range from conventional to the arcane, including distillation, centrifuging, filtration, ultrafiltration, solvent extraction, adsorption, selective membrane technology, reverse osmosis, molecular sieves, electrophoresis and affinity

Table 4.11. Downstream processing operations

Operation	Method
Separation	Filtration Centrifugation Flotation Disruption
Concentration	Solubilisation Extraction Thermal processing Membrane filtration Precipitation
Purification	Crystallisation Chromatography
Modification	
Drying	

chromatography. It is in this area that several potential industrial applications of modern biotechnology have come to grief either because the extraction has defeated the ingenuity of the designers or, more probably, because the extraction process has required so much energy input as to render it uneconomic.

Final products of the downstream purification stages should have some degree of stability for commercial distribution. Stability is best achieved for most products by using some form of drying. In practice, this is achieved by spray drying, fluidised-bed drying or by freeze drying. The method of choice is product- and cost-dependent. Products sold in the dry form include organic acids, amino acids, antibiotics, polysaccharides, enzymes, SCP and many others. Many products cannot be supplied easily in a dried form and must be sold in liquid preparations. Care must be taken to avoid microbial contamination and deterioration and, when the product is proteinaceous, to avoid denaturation.

The role of downstream processing will continue to be one of the most challenging and demanding parts of many biotechnological processes. Purity and stability are the hallmarks of most high-value biotechnological products.

It can be said that biotechnological processes will, in most part, need to be contained within a defined area or bioreactor and, to a large extent, the ultimate success of most of the processes will depend on the correct choice and operation

of these systems. For most high-value products, cultivation of the producer organism will normally be by monoculture, requiring complete asepsis to maximise product formation. On the industrial side, the scale of operation will, for economic reasons, mainly be very large, and in almost all cases the final success will require the closest cooperation between the bioscientist, the chemist and the process or biochemical engineer – in this way demonstrating the truly interdisciplinary nature of biotechnological processes.

4.9 Postscript

It is now recognised that the production of microorganisms and their products for a multitude of purposes is now a worldwide activity. The know-how technology, equipment and materials are now routinely used for entirely legitimate, peaceful and creative purposes. Regrettably, they can also be used for the production of biological weapons. In biological warfare, specific microorganisms or derived toxins which can cause disease in humans, animals or plants or which harm the environment can be used to achieve military and/or political objectives. Furthermore, unlike nuclear and chemical weapons, biological weapons are relatively easy and cheap to produce and manufacture and can also be carried out on a small scale. Such sinister use of microbial biotechnology must be totally outlawed by world governments.