CHAPTER 1

INTRODUCTION TO BIOTECHNOLOGY

LEARNING OBJECTIVES

After studying the chapter the students familiarize themselves with the following concepts:

- *Introducing the subject Biotechnology and significance in various Fields in Pharmaceutical Industry.*
- *Basic Concepts on Biosensors Principles*
- *Protein Engineering*
- *Genetic Engineering*

Biotechnology is a multidisciplinary subject that combines biological sciences with engineering technologies to change live organisms and biological systems in order to create products that advance healthcare, medicine, agriculture, food, medicines, and environmental control. R&D in Biological Sciences and Industrial Processes are the two primary categories of biotechnology. The biological sciences section is concerned with research and development in areas such as microbiology, cell biology, genetics, and molecular biology, among others, in order to better understand disease occurrence and treatment, agricultural development, food production, environmental protection, and so on. In biological sciences, the majority of R&D is done in the laboratory. The industrial processes section is concerned with the large-scale manufacture of medications, vaccines, biofuels, and pharmaceuticals employing biochemical processes and techniques.

1.1 History of Biotechnology

The origins of biotechnology can be traced back to zymotechnology, which began with a focus on brewing procedures for beer. However, by the end of World War I, zymotechnology had expanded to address bigger industrial concerns, and the potential of industrial fermentation had given rise to biotechnology. Microbial fermentations are the oldest biotechnological processes, as evidenced by a Babylonian tablet dating from around 6000 B.C. that was discovered in 1881 and described the creation of beer.

Leavened bread was first made with the help of yeast around 4000 B.C. In the third millennium B.C., the Sumerians were able to brew up to

twenty different types of beer. The first vinegar production company was developed in France near Orleans in the 14th century.

With his newly developed microscope, Antony Van Leeuwenhoek examined yeast cells for the first time in 1680. Louis Pasteur first mentioned lactic acid fermentation by microbes in 1857.

By the end of the nineteenth century, a great number of enterprises and groups of scientists had been active in the field of biotechnology, and Germany and France had created large-scale sewage purification systems using bacteria.

Delbruck, Heyduck, and Hennerberg found the large-scale application of yeast in the food business between 1914 and 1916. Bacteria were used to produce acetone, butanol, and glycerine within the same time period.

Alexander Fleming developed penicillin in 1920, and large-scale production of the antibiotic began in 1944.

A timeline of modern biotechnology's development

500 B.C.: In China, the first antibiotic, mouldy soybean curds, is put to use to treat boils.

A.D.100: The first insecticide is produced in China from powdered chrysanthemums.

1761: English surgeon Edward Jenner pioneer's vaccination, inoculating a child with a viral smallpox vaccine.

1870: Breeders crossbreed cotton, developing hundreds of varieties with superior qualities.

1870: The first experimental corn hybrid is produced in a laboratory.

1911: American pathologist Peyton Rous discovers the first cancer-causing virus.

1928: Scottish scientist Alexander Fleming discovers penicillin.

1933: Hybrid corn is commercialized.

1942: Penicillin is mass-produced in microbes for the first time.

1950s: The first synthetic antibiotic is created.

1951: Artificial insemination of livestock is accomplished using frozen semen.

1958: DNA is made in a test tube for the first time.

1978: Recombinant human insulin is produced for the first time.

1979: Human growth hormone is synthesized for the first time.

1980: Smallpox is globally eradicated following 20-year mass vaccination effort.

1980: The U.S. Supreme Court approves the principle of patenting organisms, which allows the Exxon oil company to patent an oil-eating microorganism.

1981: Scientists at Ohio University produce the first transgenic animals by transferring genes from other animals into mice.

1982: The first recombinant DNA vaccine for livestock is developed.

1982: The first biotech drug, human insulin produced in genetically modified bacteria, is approved by FDA. Genentech and Eli Lilly developed the product.

1985: Genetic markers are found for kidney disease and cystic fibrosis.

1986: The first recombinant vaccine for humans, a vaccine for hepatitis B, is approved.

1986: Interferon becomes the first anticancer drug produced through biotech.

1988: The first pest-resistant corn, Bt corn, is produced.

1990: The first successful gene therapy is performed on a 4-year-old girl suffering from an immune disorder.

1992: FDA approves bovine somatotropin (BST) for increased milk production in dairy cows.

1993: FDA approves Betaseron®, the first of several biotech products that have had a major impact on multiple sclerosis treatment.

1994: The first breast cancer gene is discovered.

1994: The Americas are certified polio-free by the International Commission for the Certification of Polio Eradication.

1995: Gene therapy, immune-system modulation and recombinantly produced antibodies enter the clinic in the war against cancer.

1996: A gene associated with Parkinson's disease is discovered.

1996: The first genetically engineered crop is commercialized.

1997: A sheep named Dolly in Scotland becomes the first animal cloned from an adult cell.

1998: FDA approves Herceptin®, a pharmacogenomic breast cancer drug for patients whose cancer overexpresses the HER2 receptor.

1999: A diagnostic test allows quick identification of Bovine Spongicorm Encephalopathy (BSE, also known as "mad cow" disease) and Creutzfeldt - Jakob disease (CJD)

2000: Kenya field-tests its first biotech crop, virus-resistant sweet potato.

2001: FDA approves Gleevec® (imatinib), a gene-targeted drug for patients with chronic myeloid leukemia. Gleevec is the first gene-targeted drug to receive FDA approval.

2002: EPA approves the first transgenic rootworm-resistant corn.

2002: The banteng, an endangered species, is cloned for the first time.

2003: China grants the world's first regulatory approval of a gene therapy product, Gendicine (Shenzhen SiBiono GenTech), which delivers the p53 gene as a therapy for squamous cell head and neck cancer.

2003: The Human Genome Project completes sequencing of the human genome.

2004: UN Food and Agriculture Organization endorse biotech crops, stating biotechnology is a complementary tool to traditional farming methods that can help poor farmers and consumers in developing nations.

2004: FDA approves the first antiangiogenic drug for cancer, Avastin®.

2005: The Energy Policy Act is passed and signed into law, authorizing numerous incentives for bioethanol development.

2006: FDA approves the recombinant vaccine Gardasil®, the first vaccine developed against human papillomavirus (HPV), an infection implicated in cervical and throat cancers, and the first preventative cancer vaccine.

2006: USDA grants Dow Agro Sciences the first regulatory approval for a plant-made vaccine.

2007: FDA approves the H5N1 vaccine, the first vaccine approved for avian flu.

2009: Global biotech crop acreage reaches 330 million acres.

2009: FDA approves the first genetically engineered animal for production of a recombinant form of human antithrombin.

1.2 Scope of Biotechnology

Hereditary engineering has raised hopes for therapeutic proteins, medicines, and biological creatures such as seeds, insecticides, altered yeasts, and genetically modified human cells to treat genetic illnesses. With the introduction of gene therapy, stem cell research, cloning, and genetically modified food, the science of genetic engineering remains a hot topic of debate in today's culture.

Traditional biotechnology approaches such as plant and animal breeding, food production, fermentation products and processes, and pharmaceutical and fertiliser manufacture should all be seen as part of modern biotechnology.

1.2.1 The Key Components of Modern Biotechnology are as follows

(i) **Genomics:** The study of all genes in a species at a molecular level.

(ii) **Bioinformatics:** The application of information technology to evaluate and manage enormous data sets resulting from gene sequencing or related procedures, involving the assembling of data from genomic analysis into accessible formats.

(iii) **Transformation:** Incorporation of one or more genes providing potentially valuable features into plants, animals, fish, or tree species.

(iv) Organisms with enhanced genetics

(v) Organisms that have been genetically changed (GMO).

(vi) Living organisms that have been changed (LMO).

(vii) **Molecular breeding:** Using marker-assisted selection (MAS) to identify and evaluate useful features in breeding programmes;

(viii) **Diagnostics:** Using molecular characterization to enable more accurate and faster pathogen identification; and

(ix) **Vaccine technology:** the use of modern immunology to the development of recombinant deoxyribonucleic acid (rDNA) vaccines for improved disease management in cattle and fish.

Biotechnology encompasses a spectrum of technologies, ranging from old biotechnology's long-established and commonly utilised procedures to unique and rapidly evolving modern biotechnology techniques (Fig.1.1).

During the 1970s, scientists developed novel technologies for exact recombination of parts of deoxyribonucleic acid (DNA), the biological material that determines hereditary features in all living cells, and for transferring portions of DNA from one organism to another. rDNA technology, often known as genetic engineering, is a set of enabling techniques.

Gradient of Biotechnologies

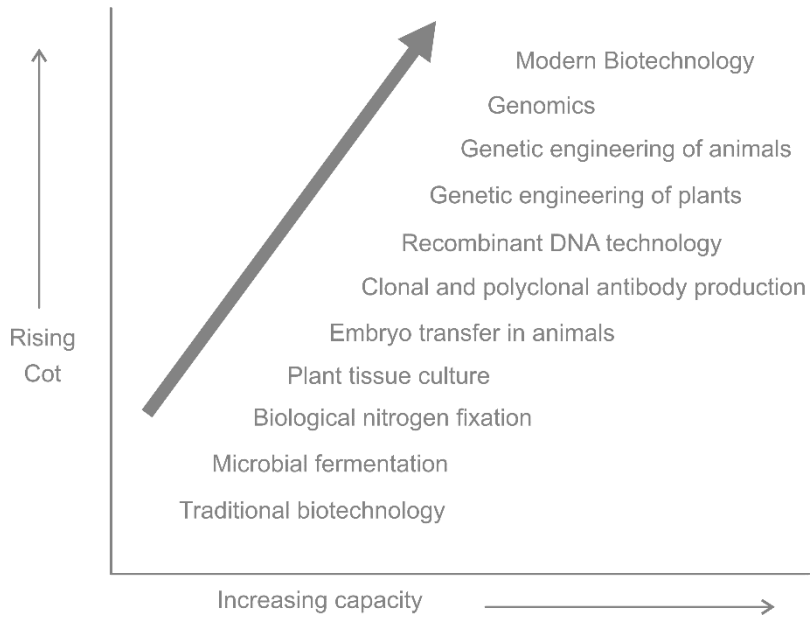


Fig. 1.1 Gradient of Biotechnologies

New techniques in rDNA technology, monoclonal and polyclonal antibodies, and new cell and tissue culture technologies are all used in modern biotechnology.

1.3 Applications of Biotechnology

(i) Therapeutics
(ii) Diagnostics
(iii) Genetically modified crops for agriculture
(iv) Processed food
(v) Waste disposal are examples of biotechnology applications.
(vi) Providing the best catalyst in the form of an improved organism, usually in the form of a microbe or pure enzyme
(vii) Downstream processing technologies to purify the protein/organic molecule by engineering ideal conditions for a catalyst to work.

Agriculture and Biotechnology

There are three possibilities for increasing food production:

(i) Agriculture based on agrochemicals.
(ii) Genetically altered crop-based agriculture and
(iii) Organic agriculture

Green biotechnology is the use of biotech methods in agriculture and food production. The Green Revolution was successful in raising crop yields, owing to the use of improved crop types and agrochemicals (fertilizers and pesticides).

The following are some of the benefits of using genetically engineered plants

(i) Crops have been genetically modified to be more resistant to abiotic conditions such as cold, heat, drought, salinity, and so on.
(ii) It has reduced crop reliance on chemical pesticides by making them pest-resistant.
(iii) Post-harvest losses are significantly minimized.
(iv) The early exhaustion of soil fertility is averted as plant efficiency in mineral utilization increases.

(v) The nutritional value of food generated from GM (Genetically Modified) crops has increased.

(vi) Genetic engineering has been utilised to develop custom-made plants for businesses such as starch, fuel, pharmaceuticals, and others.

Plants that are resistant to pests are grown.

(a) Bt Cotton is a type of cotton that has been genetically modified.

Bacillus thuringiensis, a soil bacterium, produces Cry proteins, which are harmful to insect larvae such as Tobacco budworm, armyworm, beetles, and mosquitoes. Because the alkaline pH of the gut solubilizes the crystals, the Cry proteins exist as inactive protoxins that are transformed into active toxin when swallowed by the insect. The active toxin attaches to the surface of midgut epithelial cells and causes holes to form. This induces swelling and cell lysis, resulting in the insect's death (Larva). The bacterium's genes (cry genes) producing this protein have been extracted and integrated into a variety of crop plants, including cotton, tomato, corn, rice, soybean, and others. Cry I Ac and cry II Ab control cotton bollworms, cry I Ab controls corn borer, cry III Ab controls Colorado potato beetle, and cry III Bb controls corn rootworm, respectively.

(b) Nematode Resistance: The nematode Meloidogyne incognita infects tobacco plants, reducing production. Using Agrobacterium as a vector, the parasite's particular genes (in the form of c DNA) are transferred into the plant. The genes are inserted in such a way that they produce both sense/coding RNA and antisense RNA (complimentary to sense/coding RNA). Because these two RNAs are complementary, they form a double-stranded RNA (ds RNA), which neutralizes the nematode's particular RNA through RNA interference. As a result, the parasite is unable to develop in the transgenic host, leaving the transgenic plant pest-free.

Biotechnology's application in medicine

The rDNA technology has been used to create more effective and safe therapeutic medications. The recombinant medicines did not cause an undesirable immune response, as is frequent with comparable compounds obtained from nonhuman sources.

(1) Insulin that has been genetically modified (Humulin)

Chain A and chain B are two short polypeptide chains connected by disulphide bridges in human insulin. Insulin is produced as a prohormone that must be converted into a mature and functioning hormone. Another polypeptide in the prohormone termed C-peptide is eliminated during maturation. In 1983, the American corporation Eli Lilly generated two DNA sequences coding for human insulin chains A and B and inserted them into plasmids of E. coli to create insulin. Disulphide bridges were used to connect the two chains that were formed.

(2) Gene therapy

Genes are injected into an individual's cells and tissues in this procedure to repair specific genetic disorders. It entails inserting a normal gene into a human or embryo to replace the gene's faulty mutant allele. Viruses that attack the host and infect it with their genetic material are all vectors. In 1990, a four-year-old girl with adenosine deaminase (ADA) deficiency received the first clinical gene therapy. In some children, bone marrow transplantation can cure ADA deficiency, however it is not totally curative. Lymphocytes were produced in a cultural and functional ADA for gene therapy. The lymphocytes are subsequently inoculated with cDNA. These lymphocytes are subsequently infused into the patient's body; the patient will need these genetically altered cells on a regular basis. It would be a permanent treatment if a functioning gene was delivered into the bone marrow cells at an early embryonic stage.

(3) Molecular diagnostics

For early diagnosis of illnesses, recombinant DNA molecules and procedures such as PCR (Polymerase Chain Reaction) are used. When the cloned gene is expressed to make recombinant proteins, it aids in the development of sensitive diagnostic tools like ELISA. The cloned genes can also be employed as 'probes' to identify complementary DNA strands. A probe is a single-stranded segment of DNA that has been labelled with a radioactive molecule and is used to hybridize with its complementary DNA to find it. After that, autoradiography is used to detect radioactivity. A approach like this can be used to detect the presence of a normal or mutant gene. PCR is used to detect HIV as well as gene mutations.

Production of Transgenic Animals

(i) Transgenic animals are those whose DNA has been modified to allow them to possess and express a foreign gene. The following are examples of how transgenic animals are used:

(ii) Transgenic animals can be produced specifically to allow researchers to investigate how genes are regulated and how they affect the body's regular functioning and development, for example. Information about the biological role of insulin-like growth factor is gathered.

(iii) The transgenic animals are engineered to act as models for human diseases in order to improve our understanding of how genes contribute to disease development.

(iv) Transgenic animals that generate useful biological products can be developed by inserting a fragment of DNA from an organism (s) that codes for that product, such as alpha-1 antitrypsin, a human protein that is used to treat emphysema. Rosie, the first transgenic cow, produced milk high in human protein (2.4g/ltr) as well as human alpha-lactalbumin, a more nutritionally balanced product for human new-borns.

(v) Transgenic mice are being generated for the purpose of assessing vaccination safety. (For example, polio vaccination).

(vi) To assess the toxicity of pharmaceuticals, transgenic animals with increased sensitivity to harmful chemicals are being produced.

Ethical Issues

When creatures are genetically modified, their consequences can be unpredictable /undesirable when they are introduced into the ecosystem. Patent issues have arisen as a result of the modification and usage of such organisms for public service. As a result, the Indian government has established an institution that is tasked with determining the legality of genetic alteration and the safety of integrating genetically modified organisms into public services. The Genetic Engineering Approval Committee is one such body (GEAC).

Biopiracy

The developed/industrialized countries are wealthy financially, but they lack biodiversity and traditional knowledge, whereas emerging and underdeveloped countries have a wealth of bioresearch and traditional knowledge. Some industrialized countries use other countries' bio

resources and traditional knowledge without their permission or pay (Biopiracy). Basmati rice, which is grown in India, is known for its particular flavour and aroma. However, an American firm obtained intellectual rights to Basmati through the US copyright and trademark office, and this corporation developed a new variety of Basmati by crossing an Indian variety with semi-dwarf kinds. Some countries are currently enacting legislation to prevent such illicit use of their bioresources and traditional knowledge.

1.4 Introduction to Enzyme Biotechnology

Enzymes are biological catalysts that speed up reactions but are not consumed in the process; they can be employed over and over again as long as they are active. The term "enzyme" was coined in 1878 by German scientist Wilhelm Kuhne to describe yeast's ability to make alcohol from carbohydrates, and it is derived from the Greek words en (meaning "inside") and zume (meaning "yeast"). Many advancements were achieved in the extraction, characterisation, and commercial exploitation of enzymes in the late nineteenth and early twentieth centuries, but it wasn't until the 1920s that enzymes were crystallised, indicating that catalytic activity is related with protein molecules. For the following 60 years or so, all enzymes were thought to be proteins, but in the 1980s, it was discovered that some ribonucleic acid (RNA) molecules can also catalyse reactions. These RNAs, known as ribozymes, play a crucial function in gene expression. Biochemists developed the technology to create antibodies with catalytic characteristics throughout the same decade. These so-called 'abzymes' hold a lot of promise as new industrial catalysts and medicines. The immense catalytic activity of enzymes is likely best described by the constant kcat, often known as the turnover rate, turnover frequency, or turnover number. This constant denotes the number of substrate molecules that a single enzyme molecule may convert to product in a given amount of time (usually per minute or per second). A single molecule of carbonic anhydrase, for example, may catalyse the conversion of almost half a million molecules of its substrates, carbon dioxide (CO2) and water (H2O), into the product, bicarbonate (HCO3), in less than a second—an incredible feat. Enzymes are extraordinarily potent catalysts, but they also have exceptional selectivity, catalysing the conversion of only one type (or a small number of comparable types) of substrate molecule into product molecule. Group specificity is demonstrated by some enzymes. Alkaline phosphates, for

example, can remove a phosphate group from a variety of substrates (an enzyme that is typically met in first-year laboratory sessions on enzyme kinetics).

Other enzymes have substantially higher absolute specificity, which is a term used to characterise how particular they are. Glucose oxidase, for example, is almost completely selective for its substrate, -D-glucose, and has little activity with other monosaccharides. Many analytical assays and devices (biosensors) that assess a specific substrate (e.g. glucose) in a complicated mixture place a premium on specificity (e.g. a blood or urine sample).

1.4.1 Names and Classifications of Enzymes

Although individual proteolytic enzymes have the suffix -in, enzymes commonly have common names (sometimes referred to as 'trivial names') that allude to the reaction that they catalyse, with the suffix -ase (e.g. oxidase, dehydrogenase, carboxylase) (e.g. trypsin, chymotrypsin, papain). Frequently, the enzyme's common name also denotes the substrate on which it works (e.g. glucose oxidase, alcohol dehydrogenase, pyruvate decarboxylase). However, certain common names (for example, invertase, diastase, and catalase) reveal little about the substrate, product, or reaction.

The International Union of Biochemistry established the Enzyme Commission to address the growing complexity and inconsistencies in enzyme name. The first Enzyme Commission Report was released in 1961, and it outlined a method for identifying enzymes. The sixth edition, issued in 1992, had information on almost 3200 distinct enzymes, with annual supplements bringing the total to over 5000.

All enzymes are given a four-part Enzyme Commission (EC) number in this system. The enzyme lactate dehydrogenase, for example, has the EC number 1.1.1.27 and is more properly known as l–lactate: NAD+ oxidoreductase.

1.4.2 Immobilization of Enzymes

Enzymes are combined in a solution with substrates in most procedures, and they can't be economically recovered after the reaction, so they're usually thrown away. As a result, there is an incentive to use enzymes

that are immobilized or insolubilized in order to keep them in a biochemical reactor for further catalysis. Enzyme immobilization is used to accomplish this.

"Enzymes physically contained or localized in a definite defined region of space with retention of their catalytic activity, and which can be used repeatedly and continuously," according to the definition of immobilized enzymes. Immobilization techniques form the foundation for a variety of biotechnology products, including diagnostics, bio-affinity chromatography, and biosensors, in addition to its use in industrial processes.

Only single immobilized enzymes were utilized at first, but from the 1970s, more complicated systems with two-enzyme reactions, cofactor regeneration, and living cells were produced. Interactions ranging from reversible physical adsorption and ionic connections to stable covalent bonds can be used to attach enzymes to the support. Although the most effective immobilization technique depends on the nature of the enzyme and the carrier, immobilization technology has become more of a question of rational design in recent years. Some features, such as catalytic activity and thermal stability, are altered as a result of enzyme immobilization. These effects have been proven and exploited in the past. For immobilizing enzymes, the concept of stabilization has been a major driving force. Furthermore, genuine molecular stabilization has been proven, such as proteins immobilized through multipoint covalent binding.

1.4.3 Enzyme Immobilization Salient Features

- The enzyme phase is known as the carrier phase, and it is a water insoluble but hydrophilic porous polymeric matrix, such as agarose or cellulose.
- Fine particulate, membranous, or microcapsule enzyme phases are all possibilities.
- Cross-linking allows the enzyme to bind to another enzyme.
- Using immobilization techniques, a unique module is created that allows fluid to readily pass through, changing the substrate into product while also allowing the catalyst to be easily removed from the product as it exits the reactor.

- At certain pH, ionic strength, or solvent conditions, the support or carrier used in the immobilization approach is not stable. As a result, it's possible that the enzyme component will be broken or dissolved after the reaction, releasing the enzyme component.
- Multiple or repetitive use of a single batch of enzymes Immobilized enzymes are usually more stable Ability to stop the reaction quickly by removing the enzyme from the reaction solution Product is not contaminated with the enzyme Easy separation of the enzyme from the product Allows development of a multienzyme reaction system Reduces effluent disposal problems.

Immobilization of enzymes has the following disadvantages

- It adds to the cost, and it invariably affects the stability and activity of enzymes.
- When one of the substrates is proven to be insoluble, the approach may not be beneficial.
- Certain immobilization procedures have major issues with substrate diffusion, which makes it difficult to get to the enzyme.

1.4.4 Immobilization methods for Enzymes

1. **Physical method:** Physical forces such as Vander Waals forces, hydrophobic interactions, and hydrogen bonding are used to attach enzymes to various matrices. Controlling physicochemical parameters allows the process to be reversed in nature. It consists of the methods listed below.
   - **(i) Entrapment:** Physical entrapment of enzymes inside a polymer or gel matrix can immobilize them. The matrix holes are large enough to hold the enzyme while allowing the substrate and product molecules to flow through. The enzyme (or cell) is not subjected to severe binding pressures or structural distortions in this approach, known as lattice entrapment.

Changes in pH or temperature, as well as the addition of solvents, may cause some deactivation throughout the immobilization process. Polyacrylamide gel, collagen, gelatin, starch, cellulose, silicone, and rubber are some of the matrices used to entrap enzymes. This approach has multiple advantages, including simplicity, no change in intrinsic enzyme characteristics, no chemical modification, low enzyme demand,

and a variety of matrices. The following are some of the method's drawbacks: enzyme leakage, the ability to use only modest substrate/product sizes, the need for a delicate balance between the matrix's mechanical qualities and their effect on enzyme activity, and the presence of a diffusional constraint.

Enzymes can be trapped in a variety of ways

1. Inclusion of enzymes in gels

This is an enzyme entrapment within the gels.

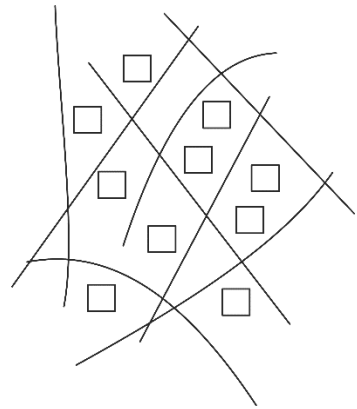


Fig. 1.2 Inclusion of enzyme in gel

2. Enzyme inclusion in fibres

The enzymes are trapped in a fibre format of the matrix

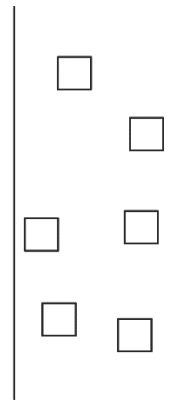


Fig. 1.3 Inclusion of enzyme in fibres

3. Enzyme inclusion in microcapsules

The enzymes are encapsulated within a microcapsule matrix in this situation. The matrix's hydrophobic and hydrophilic forms polymerize to form a microcapsule with enzyme molecules within. Enzyme entrapment is hampered by the leakage of enzymes from the matrix. For the immobilization of entire cells, most workers choose to employ the entrapment technique. In the industrial manufacture of amino acids (L-isoleucine, L-aspartic acid), L-malic acid, and hydroquinone, entrapped cells are used.

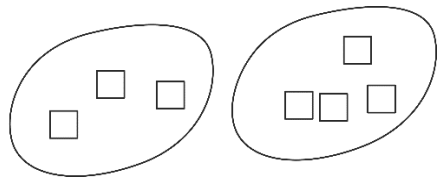


Fig. 1.4 Inclusion in microcapsules

(ii) Adsorption: Non-covalent linkages such as ionic or hydrophobic contacts, hydrogen bonding, and van der Waals forces attach the enzyme to the support material without any pre-activation of the support. Ceramic, alumina, activated carbon, kaolinite, bentonite, porous glass, chitosan, dextran, gelatin, cellulose, and starch are some of the organic and inorganic matrices that have been employed. pH, temperature, solvent type, ionic strength, enzyme concentration, and adsorbent concentration are all variables that must be optimized in the immobilization technique. The enzyme is applied directly to the surface (active adsorbent) without any non-adsorbed enzyme being removed during the washing process. The approach is straightforward and gentle, with a wide range of carriers available for simultaneous purification and enzyme immobilization (e.g., Asparginase on CM-cellulose) with no conformational changes. However, because a lot of parameters play a role in enzyme desorption in response to minor changes in its microenvironment, it necessitates extensive tuning. (e.g., pH, temperature, solvent, ionic strength and high substrate concentrations).

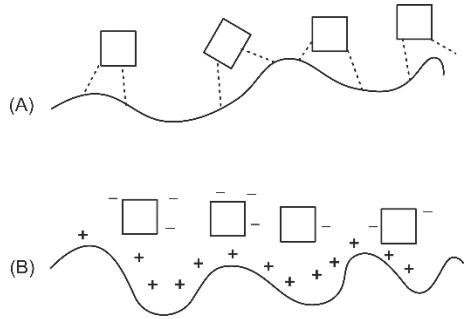


Fig. 1.5 Inclusion of Enzyme through Adsorption

(A) Immobilized enzymes by vanderwaal forces

(B) Immobilized enzymes by hydrogen bonds

(iii) Microencapsulation: Enzymes are immobilized by encapsulating them in spherical semi-permeable polymer membranes with regulated porosity (1–100m). Depending on the contents, semi-permeable membranes can be either permanent or non-permanent. Non-permanent membranes are formed of liquid surfactant, while permanent membranes are made of cellulose nitrate and polystyrene. Encapsulation of colours, medicines, and other substances is also done with these membranes. Because enzymes immobilized by encapsulation have such huge surface areas, they have a higher catalytic efficiency. Microencapsulation can be done in three different methods.

1. The construction of unique membrane reactors.
2. Emulsion formation is the second step.
3. Emulsion stabilization to create microcapsules

Recently, microencapsulation has been utilized to immobilize enzymes and mammalian cells. Microencapsulation, for example, can immobilize pancreatic cells growing in vitro. This method has also been used to successfully immobilize hybridoma cells.

2. Using Chemicals

This involves the irreversible attachment of enzymes to various matrices via covalent or ionic bonds.

(i) Covalent attachment: The enzyme is attached to the matrix via covalent bonds (diazotation, amino bond, Schiff's base formation, amidation reactions, thiol-disulfide, peptide bond, and alkylation

reactions). Enzyme molecules are connected to the matrix's reactive groups (e.g., hydroxyl, amide, amino, carboxyl groups) either directly or through a spacer arm, which is artificially bonded to the matrix using various chemical reactions (e.g., diazotization, etc. imine bond formation, Schiff base). Natural (e.g., glass, Sephadex, Agarose, Sepharose) or synthetic (e.g., glass, Sephadex, Agarose, Sepharose) matrices are often utilized (e.g., acrylamide, methacrylic acid, and styrene). The cost, availability, binding capacity, hydrophilicity, structural rigidity, and durability of a matrix are all factors to consider when choosing one for a certain application. Non-essential amino acids (other than active site groups) are used in this method of immobilization, resulting in minimal conformational alterations. It contributes to immobilized enzymes' increased tolerance to harsh physical and chemical environments (e.g., temperature, denaturants, organic solvents). Due to harsh immobilization circumstances and concurrence of comparable amino-groups at the active site being involved during enzyme contact with the matrix, this kind of immobilization puts more strain on the enzyme and can cause significant changes in conformational and catalytic properties.

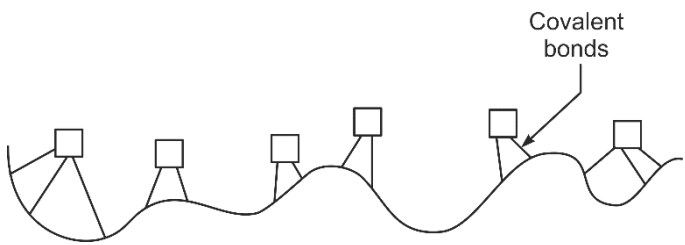


Fig. 1.6 Inclusion of Enzyme through Covalent Bonding

(ii) Cross-Linking: Using bi- or multi-functional reagents, a number of covalent links are formed between the enzyme and the matrix (e.g., glutardialdehyde, glutaraldehyde, glyoxal, diisocyanates, hexamethylene diisocyanate, toluene diisocyanate). Under mild conditions, lysine amino groups, cysteine sulfhydryl groups, phenolic OH groups of tyrosine, or imidazole groups of histidine are commonly employed for enzyme binding. The simplicity of this procedure is its greatest advantage. However, because to the non-regulation of the reaction, a significant amount of enzyme is lost. Furthermore, diffusion limits the effectiveness of this method of enzyme immobilization.

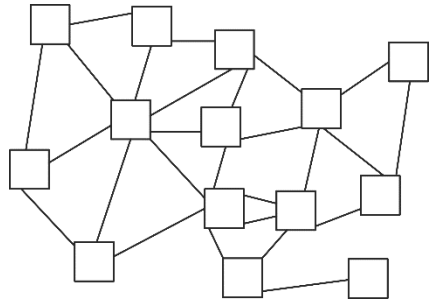


Fig. 1.7 Inclusion of Enzyme through Cross Linking

(iii) Ionic binding: Ionic interactions between enzyme molecules and a charged matrix are the basis for this method. The bigger the amount of enzyme attached to the matrix, the higher the surface charge density on the matrix. Enzyme molecules are sometimes physically adsorbed to the matrix in addition to ionic interactions. The method of enzyme immobilization is similar to that of physical adsorption, as explained before. The pH of the solution, the concentration of the enzyme, and the temperature all affect enzyme binding via ionic interactions during immobilization. Polysaccharide derivatives (e.g., diethylaminoethylcellulose, dextran, carboxymethylcellulose, chitosan), synthetic polymers (e.g., polyethylene vinyl alcohol), and inorganic materials are all often used matrices (e.g., Amberlite, alumina, silicates, bentonite, sepiolite, silica gel). Because of this approach of immobilization, only minor modifications in enzyme structure occur.

However, because there is a higher risk of enzyme detachment from the matrix under suboptimal conditions, extra attention is paid to maintaining proper ionic strength and pH of the fluid in which the immobilized enzyme conducts catalysis.

(iv) Affinity ligands: Specific ligands are employed to attach the enzyme to the matrix, such as the his-tag on the enzyme to a metal-containing matrix, the lectin-containing domain to carbohydrate moieties on the matrix, or sometimes substrate-mimicking chemical compounds. In some situations, ligands are naturally present on the enzyme; in others, they are intentionally attached by fusing a nucleotide sequence corresponding to the tag with the DNA encoding a polypeptide of the given enzyme. Due to the non-involvement of active site residues and higher immobilization efficiency due to the presence of high densities of

ligands on the matrix, this method of immobilization results in minimal changes in enzyme configuration, with high stability and catalytic efficiency of the immobilized enzyme. This approach can be used to immobilize a variety of proteins, including antibodies, cytokines, streptavidin, and others. Enzymes that have been immobilized via this approach have been used in biotechnology, diagnostics, and medicine. This approach has also been utilized in animal cell culture to connect several types of mammalian cells to diverse matrices containing a range of peptides, growth factors, and cytokines to a specific binding domain located on the cell, as well as to activate them.

1.4.5 Matrices Employed in the Immobilization of Enzymes

Enzymes are either adhered to the surface of matrices or entrapped inside them by physical or chemical means during enzyme immobilization.

The following are the matrices that were utilized to immobilize enzymes:

Surface-Bound Enzymes

The chemical, biological, mechanical, and kinetic properties of immobilized enzymes are all governed by the physical and chemical properties of the matrices employed for enzyme immobilization. Biopolymer, synthetic organic polymer, hydrogels, smart polymer, or inorganic solid can all be used as the matrix.

- Biopolymers are polysaccharides that are insoluble in water (e.g., cellulose, starch, agarose, chitosan, and proteins such as gelatin and albumin).
- **Synthetic organic polymers**

  Eupergit-C (acrylic resins), Sepa beads FP-EP, Amberlite XAD-7 (porous acrylic resins), and other synthetic organic polymers are utilised for enzyme immobilisation. Eupergit-C has a 170 mm surface diameter and a 25 nm pore diameter. N,N′-methylene-bi-(methacrylamide), methacrylamide, allyl glycidyl ether, and glycidyl methacrylate are used to make it. It's hydrophilic and chemically and mechanically stable across a pH range of 0–14. It possesses a high density of oxirane moieties on its surface, which allows it to bind several enzymes via covalent connections at neutral or alkaline pH. As a result, it provides long-term operating stability throughout a broad pH range (1–12).

- Alumina, silica, zeolites, and mesoporous silicas are examples of inorganic solids (MCM-41, and SBA-15). They are known to be the most cost-effective matrix for immobilising a variety of industrial and non-industrial enzymes.
- **Smart Polymer**

  The thermostable biocompatible polymer [poly-N-isopropylacrylamide (polyNIPAM)] is the best studied example of smart polymer. PolyNIPAM has the unusual feature of existing in two states: solution when the temperature is below 32 °C and polymer when the temperature is above 32 °C.

1.4.6 Immobilized Enzymes and Cells Have a Wide Range of Applications

(i) Application in Biomedicine: In medicine, immobilised enzymes are used to diagnose and cure illnesses. The inborn metabolic insufficiency can be remedied by substituting waste metabolites with encapsulated enzymes (i.e., enzymes encapsulated by erythrocytes). The RBC works as a carrier for exogenous enzyme medicines, and the enzymes are biocompatible in nature, thus there is no immune response. Enzyme encapsulation through electroporation is the simplest method of immobilisation in the biomedical area, and it is a reversible procedure with the ability to renew the enzyme. When enzymes are coupled with biomaterials, biological and functional systems are created. Biomaterials are utilised to mend defects in tissue engineering applications. In biomedicine, the advantage of enzyme immobilisation is that free enzymes are devoured by cells and are not active for long periods of time, thus the immobilised enzymes remain stable, stimulating growth and repairing defects. The distribution of enzymes to oncogenic regions in cancer therapy has been improved with innovative approaches. Nanoparticles and nanospheres are frequently utilised as enzyme carriers for therapeutic drug delivery.

(ii) Application in the food industry: Purified enzymes are employed in the food business, but the enzymes denature throughout the purifying process. As a result of the immobilisation approach, the enzymes are stable. Syrups are made with the enzymes that have

been immobilised. Immobilized beta-galactosidase is used in the manufacturing of baker's yeast to hydrolyze lactose in whey.

(iii) Production of Biodiesel: Biodiesel is made up of monoalkyl esters of long-chain fatty acids. Biodiesel is made by esterifying alcohol (methanol, ethanol) with triglycerides (vegetable oil, animal fat) in the presence of a catalyst. High energy consumption, glycerol recovery, and side reactions that may pollute the environment are among disadvantages of catalyst manufacture. As a result, the biological synthesis of liquid fuel with lipases is currently receiving a lot of attention and is rapidly improving. Lipase catalyses the reaction with less energy and at more benign conditions. However, lipase manufacturing is expensive, resulting in lipase immobilisation, which allows for recurrent use and stability. Because methanol inactivates lipase in the biological generation of biodiesel, the immobilisation approach is advantageous for biodiesel production.

(iv) Wastewater Treatment: As the consumption of fresh water and water bodies rises, they are more mixed with polluted industrial waste water, necessitating waste water treatment. Textile, paper, and leather industries are all sources of dye effluents, and the effluents are high in dye colourants. These effluents are hazardous to the environment and are carcinogenic even at low concentrations. Enzymes are being utilised to breakdown colouring materials. Preoxidases, laccase, and azo reductases are enzymes used in wastewater treatment. Extreme temperatures, low or high pH, and high ionic strength can cause these enzymes to lose their function; to alleviate this problem, immobilised enzymes are utilised.

(v) Textile Industry: Microbially generated enzymes are of tremendous interest in the textile industry. Cellulase, amylase, liccase, pectinase, cutinase, and other enzymes are employed in a variety of textile applications, including scouring, biopolishing, desizing, denim finishing, and wool treatment. Cellulase is one of these enzymes that has been widely employed from the beginning to the present. Instead of employing harsh chemicals that pollute the environment and harm materials, the textile industry has moved to enzyme processes. The processing of fabrics with enzymes necessitates high temperatures and elevated pH, which free enzymes are unable to handle. As a result, enzyme

immobilisation for this technique can tolerate high temperatures and keep its activity for more than 5-6 cycles.

(vi) Detergent Industry: Enzymes are also used in the detergent industry to remove stains. Protease is an enzyme used in the detergent business to remove stains such as blood, egg, grass, and human sweat. Amylase is a starch-based enzyme that can be used to remove stains from potatoes, gravies, and chocolate. Lipase is an enzyme that is used to remove oil and fat stains as well as stains in cuffs and collars. For cotton-based materials, cellulase is used to increase softening, colour brightening, and soil stain removal. In today's detergent industry, biotech cleaning ingredients are frequently employed. Biobased detergents have superior cleaning properties as compared to synthetic detergents. When opposed to synthetic detergents, enzyme-based detergents can be used in lower quantities, have greater biodegradability, do not harm the environment, and operate well at low temperatures; these are the advantages of enzymes in the detergent sector.

1.4.7 Manufacture of Commercial Products

A selected list of important immobilized enzymes and their industrial applications is given in the following Table. 1.1

Table 1.1 List of Immobilized Enzymes

Immobilized enzyme	*Application(s)*
Aminoacylase	Production of L-amino acids from D, L-acyl amino acids
Glucose isomerase	Production of high fructose syrup from glucose (or starch)
Amylase	Production of glucose from starch
Invertase	Splitting of sucrose to glucose and fructose
β-Galactosidase	Splitting of lactose to glucose and galactose
Penicillin acylase	Commercial production of semi-synthetic penicillins
Aspartase	Production of aspartic acid from fumaric acid
Fumarase	Synthesis of malic acid from fumaric acid
Histidine ammonia lyase	Production of urocanic acid from histidine
Ribonuclease	Synthesis of nucleotides from RNA
Nitrilase	Production of acrylamide from acrylonitrile

Here are some specifics on the production of L-amino acids and high fructose syrup.

L-Amino Acid Production

L-amino acids (rather than D-amino acids) are essential for usage in food and feed supplements, as well as medical applications. The chemical processes used to make them produce a racemic combination of D- and L-amino acids. D, L-acyl amino acids can be formed by acylation. Aminoacylase, an immobilised enzyme (often found on DEAE sephadex), may hydrolyze D, L-acyl amino acids to generate L-amino acids.

$$\text{D, L-Acyl amino acids} \xrightarrow{\text{Aminoacylase}} \text{L-Amino acids} + \text{D, L-Acyl amino acids}$$

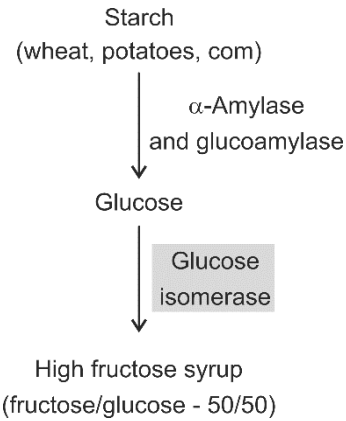
The unhydrolyzed D-acyl amino acids can be isolated from the free L-amino acids. The latter can be degraded into D, L-acyl amino acids and recycled using an enzyme reactor with immobilised Aminoacylase. This method produces massive amounts of L-methionine, L-phenylalanine, L-tryptophan, and L-valine all over the world.

High Fructose Syrup Production

Fructose is the sweetest of the monosaccharides, with sweetening power twice that of sucrose. Glucose is around 75% sweeter than sucrose. As a result, glucose (the most prevalent monosaccharide) cannot be used as a sweetener in place of sucrose. As a result, there is a high need for fructose, a sweet sugar that has the same calorific value as glucose or sucrose.

The quantities of glucose and fructose in high fructose syrup (HFS) are nearly equal. From a nutritional standpoint, HFS is essentially identical to sugar. In the manufacture of soft beverages and processed foods and baking, HFS is a good substitute for sugar.

Using an immobilised enzyme called glucose isomerase, high fructose syrup can be made from glucose. Hydrolysis is used to create glucose from starch-containing raw materials (wheat, potato, corn). Following that, glucose isomerase isomerizes glucose to fructose. HFS, which contains around 50% fructose, is the end product. (Note: Some publications refer to HFS as high fructose corn syrup, abbreviated as HFCS.)



Production of high fructose syrup from starch (glucose isomerase is the immobilized enzyme)

Glucose isomerise The intracellular enzyme glucose isomerase is generated by a variety of bacteria. The ideal sources include Arthrobacter, Bacillus, and Streptomyces species. Because glucose isomerase is an intracellular enzyme, isolating it without losing its biological function necessitates the use of specialised and expensive procedures. Whole or partially fragmented cells are frequently immobilised and employed.

1.4.8 Analytical Applications of Immobilized Enzymes and Cell

In Biochemical Analysis

Immobilized enzymes (or cells) can be utilised in biochemical analysis to build precise and specialised analytical techniques for the quantification of a variety of biochemical substances.

Table 1.2 Immobilized enzymes used in analytical biochemistry

Immobilized enzyme	Substance assayed
Glucose oxidase	Glucose
Urease	Urea
Cholesterol oxidase	Cholesterol

Table 1.2 *contd*…

Immobilized enzyme	Substance assayed
Lactate dehydrogenase	Lactate
Alcohol oxidase	Alcohol
Hexokinase	ATP
Galactose oxidase	Galactose
Penicillinase	Penicillin
Ascorbic acid oxidase	Ascorbic acid
L-Amino acid oxidase	L-Amino acids
Cephalosporinase	Cephalosporin
Monoamine oxidase	Monoamine

Examples of immobilized enzymes used in analytical biochemistry

The action of the immobilised enzyme on the substrate is crucial to the analytical assay principle. The assay can be performed with a drop in substrate concentration, a rise in product level, or a change in cofactor concentration. There are two types of detection systems that are routinely used.

Thermistors are heat measuring devices that can record the heat created in a reaction catalysed by an enzyme. Potential changes in the reaction system are measured using electrode devices. An enzyme thermistor and an enzyme electrode, as well as a specialised urease electrode, are shown in the diagram below (Fig. 1.1).

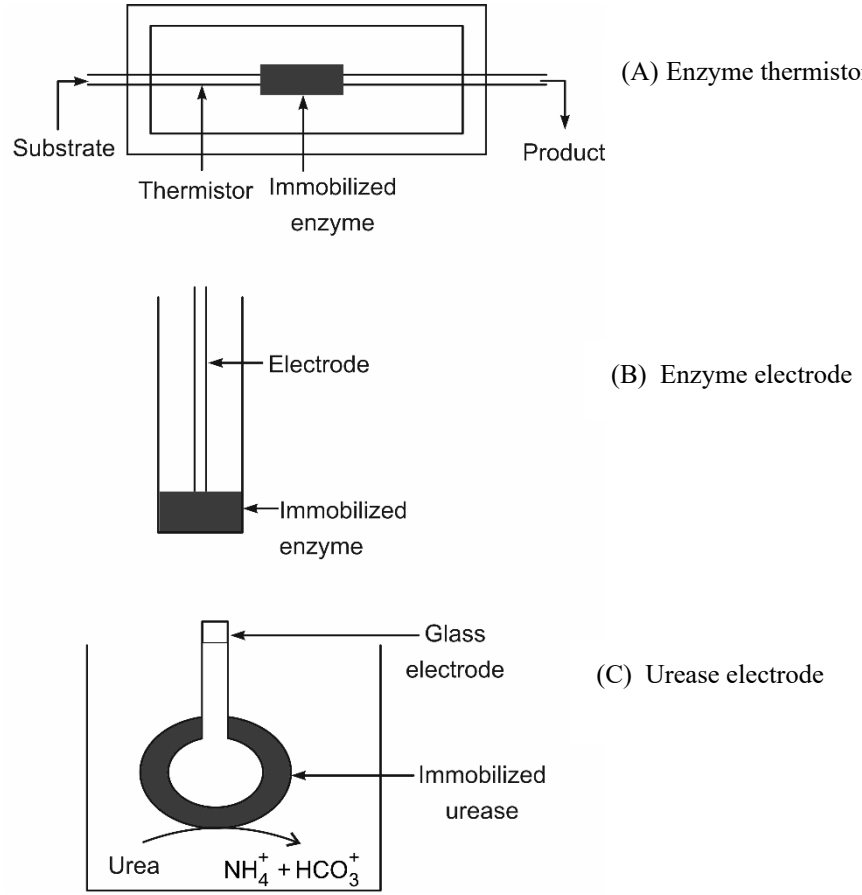


Fig. 1.8 Immobilized enzymes or cells in analytical biochemistry

In Affinity Chromatography and Purification

Immobilized enzymes can be employed in affinity chromatography and purification. It is feasible to purify a variety of chemicals using affinity, such as antigens, antibodies, and cofactors.

1.5 Biosensors

A biosensor is an analytical device which converts a biological response into an electrical signal (Fig. 1.**9**). The term 'biosensor' is often used to cover sensor devices used in order to determine the concentration of substances and other parameters of biological interest even where they do not utilize a biological system directly.

Biosensors represent a rapidly expanding field, at the present time, with an estimated 60% annual growth rate; the major impetus coming from the health-care industry (e.g. 6% of the western world are diabetic and would benefit from the availability of a rapid, accurate and simple biosensor for glucose) but with some pressure from other areas, such as food quality appraisal and environmental monitoring. Research and development in this field is wide and multidisciplinary, spanning biochemistry, bioreactor science, physical chemistry, electrochemistry, electronics and software engineering. Most of this current approach concerns potentiometric and amperometric biosensors and colorimetric paper enzyme strips. A successful biosensor must possess at least some of the following beneficial features:

1. The biocatalyst must be highly specific for the purpose of the analyses, be stable under normal storage conditions and, show good stability over a large number of assays (i.e. much greater than 100).
2. The reaction should be as independent of such physical parameters as stirring, pH and temperature as is manageable.
3. The response should be accurate, precise, reproducible and linear over the useful analytical range, without dilution or concentration. It should also be free from electrical noise.
4. If the biosensor is to be used for invasive monitoring in clinical situations, the probe must be tiny and biocompatible, having no toxic or antigenic effects. If it is to be used in fermenters it should be sterilisable. This is preferably performed by autoclaving but no biosensor enzymes can presently withstand such drastic wet-heat treatment. In either case, the biosensor should not be prone to fouling or proteolysis.
5. The complete biosensor should be cheap, small, portable and capable of being used by semi-skilled operators.
6. There should be a market for the biosensor

The biological response of the biosensor is determined by the biocatalytic membrane which accomplishes the conversion of reactant to product. Immobilised enzymes possess a number of advantageous features which makes them particularly applicable for use in such systems. They may be re-used, which ensures that the same catalytic activity is present for a series of analyses. This is an important factor in securing reproducible results and avoids the pitfalls associated with the replicate pipetting of free enzyme proportionality constant, k_L, in equation. Even if total dependence on the external diffusional rate is not achieved (or achievable), any increase in the dependence of the reaction rate on external or internal diffusion will cause a reduction in the dependence on the pH, ionic strength, temperature and inhibitor concentrations.

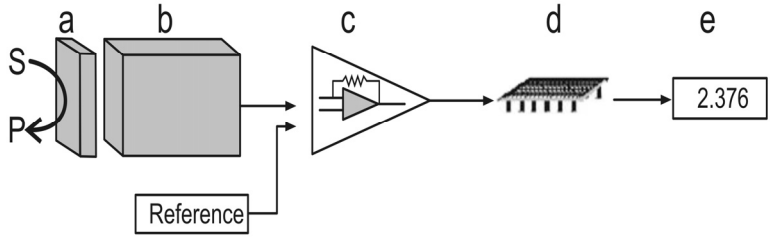


Fig. 1.9 Schematic diagram showing the main components of a biosensor. The biocatalyst (a) converts the substrate to product. This reaction is determined by the transducer (b) which converts it to an electrical signal. The output from the transducer is amplified (c) processed (d) and displayed (e).

The key part of a biosensor is the transducer (shown as the 'black box' in Fig. 1.9) which makes use of a physical change accompanying the reaction. This may be

1. The heat output (or absorbed) by the reaction (calorimetric biosensors),
2. Changes in the distribution of charges causing an electrical potential to be produced (potentiometric biosensors),
3. Movement of electrons produced in a redox reaction (amperometric biosensors),
4. Light output during the reaction or a light absorbance difference between the reactants and products (optical biosensors), or

5. Effects due to the mass of the reactants or products (piezo-electric biosensors).

There are three so-called 'generations' of biosensors;

First generation biosensors where the normal product of the reaction diffuses to the transducer and causes the electrical response, second generation biosensors which involve specific 'mediators' between the reaction and the transducer in order to generate improved response, and third generation biosensors where the reaction itself causes the response and no product or mediator diffusion is directly involved.

The electrical signal from the transducer is often low and superimposed upon a relatively high and noisy (i.e. containing a high frequency signal component of an apparently random nature, due to electrical interference or generated within the electronic components of the transducer) baseline.

The signal processing normally involves subtracting a 'reference' baseline signal, derived from a similar transducer without any biocatalytic membrane, from the sample signal, amplifying the resultant signal difference and electronically filtering (smoothing) out the unwanted signal noise. The relatively slow nature of the biosensor response considerably eases the problem of electrical noise filtration. The analogue signal produced at this stage may be output directly but is usually converted to a digital signal and passed to a microprocessor stage where the data is processed, converted to concentration units and output to a display device or data store.

1.5.1 Calorimetric Biosensors

Many enzyme catalyzed reactions are exothermic, generating heat (Table 1.3) which may be used as a basis for measuring the rate of reaction and, hence, the analyte concentration. This represents the most generally applicable type of biosensor. The temperature changes are usually determined by means of thermistors at the entrance and exit of small packed bed columns containing immobilized enzymes within a constant temperature environment (Fig. 1.10. Under such closely controlled conditions, up to 80% of the heat generated in the reaction may be registered as a temperature change in the sample stream. This may be simply calculated from the enthalpy change and the amount reacted. If a 1 mM reactant is completely converted to product in a

reaction generating 100 kJ mole^{-1} then each ml of solution generates 0.1 J of heat. At 80% efficiency, this will cause a change in temperature of the solution amounting to approximately 0.02 C. This is about the temperature change commonly encountered and necessitates a temperature resolution of 0.0001 C for the biosensor to be generally useful.

Table 1.3 Heat Output (Molar enthalpies) of Enzyme Catalyzed Reactions

Reactant	Enzyme	Heat output -DH (kJ mole^{-1})
Cholesterol	Cholesterol oxidase	53
Esters	Chymotrypsin	4 - 16
Glucose	Glucose oxidase	80
Hydrogen peroxide	Catalase	100
Penicillin G	Penicillinase	67
Peptides	Trypsin	10 - 30
Starch	Amylase	8
Sucrose	Invertase	20
Urea	Urease	61
Uric acid	Uricase	49

1.5.2 Potentiometric Biosensors

Potentiometric biosensors make use of ion-selective electrodes in order to transduce the biological reaction into an electrical signal. In the simplest terms this consists of an immobilised enzyme membrane surrounding the probe from a pH-meter (Fig. 1.11), where the catalysed reaction generates or absorbs hydrogen ions (Table 1.4). The reaction occurring next to the thin sensing glass membrane causes a change in pH which may be read directly from the pH-meter's display. Typical of the use of such electrodes is that the electrical potential is determined at very high impedance allowing effectively zero current flow and causing no interference with the reaction.

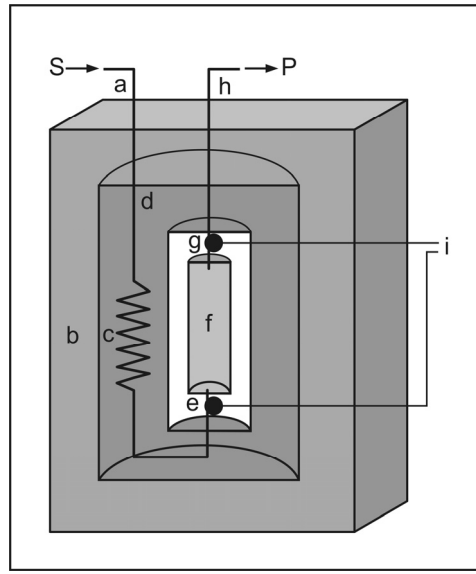


Fig. 1.10 Schematic diagram of a calorimetric biosensor. The sample stream (a) passes through the outer insulated box (b) to the heat exchanger (c) within an aluminium block (d). From there, it flows past the reference thermistor (e) and into the packed bed bioreactor (f) 1ml volume), containing the biocatalyst, where the reaction occurs. The change in temperature is determined by the thermistor (g) and the solution passed to waste (h). External electronics (l) determines the difference in the resistance, and hence temperature, between the thermistors.

There are three types of ion-selective electrodes which are of use in biosensors:

Glass electrodes for cations (e.g. normal pH electrodes) in which the sensing element is a very thin hydrated glass membrane which generates a transverse electrical potential due to the concentration-dependent competition between the cations for

1. Specific binding sites. The selectivity of this membrane is determined by the composition of the glass. The sensitivity to H^+ is greater than that achievable for NH_4^+,
2. Glass pH electrodes coated with a gas-permeable membrane selective for CO_2, NH_3 or H_2S. The diffusion of the gas through this membrane causes a change in pH of a sensing solution between the membrane and the electrode which is then determined.

3. Solid-state electrodes where the glass membrane is replaced by a thin membrane of a specific ion conductor made from a mixture of silver sulphide and a silver halide. The iodide electrode is useful for the determination of I^- in the peroxidase reaction (Table 1.4) and also responds to cyanide ions.

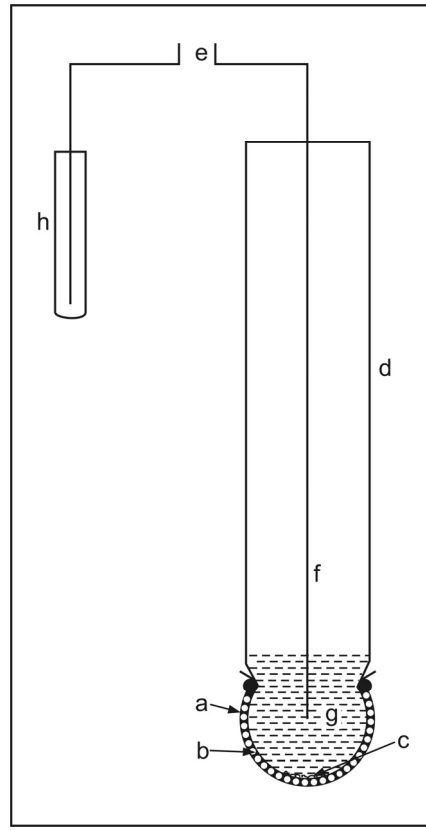


Fig. 1.11 A simple potentiometric biosensor. A semi-permeable membrane (a) surrounds the biocatalyst (b) entrapped next to the active glass membrane (c) of a pH probe (d). The electrical potential (e) is generated between the internal Ag/AgCl electrode (f) bathed in dilute HCl (g) and an external reference electrode (h)

Table 1.4 Reactions involving the Release or Absorption of Ions that may be utilized by Potentiometric Biosensors.

(a) H^+ cation,

glucose oxidase H_2O
D-glucose + O_2 → D-glucono-1,5-lactone + H_2O_2 → D-gluconate + H^+

penicillinase
penicillin → penicilloic acid + H^+

urease (pH 6.0)[a]
$H_2NCONH_2 + H_2O + 2H^+ \rightarrow 2NH_4^+ + CO_2$

urease (pH 9.5)[b]
$H_2NCONH_2 + 2H_2O \rightarrow 2NH_3 + HCO_3^- + H^+$

lipase
neutral lipids + H_2O → glycerol + fatty acids + H^+

(b) NH_4^+ cation,

L-amino acid oxidase
L-amino acid + O_2 + H_2O → keto acid + NH_4^+ + H_2O_2

asparaginase `
L-asparagine + H_2O → L-aspartate + NH_4^+

urease (pH 7.5)
$H_2NCONH_2 + 2H_2O + H^+ \rightarrow 2NH_4^+ + HCO_3^-$

(c) I^- anion,

peroxidase
$H_2O_2 + 2H^+ + 2I^- \rightarrow I_2 + 2H_2O$

(d) CN^-anion,

b-glucosidase
amygdalin + $2H_2O$ → 2glucose + benzaldehyde + H^+ + CN^-

[a] Can also be used in NH_4^+ and CO_2 (gas) potentiometric biosensors.

[b] Can also be used in an NH_3 (gas) potentiometric biosensor.es80ll66bp

A recent development from ion-selective electrodes is the production of ion-selective field effect transistors (ISFETs) and their biosensor use as enzyme-linked field effect transistors (**ENFETs**, **Fig. 1.14**). Enzyme membranes are coated on the ion-selective gates of these electronic

devices, the biosensor responding to the electrical potential change via the current output. Thus, these are potentiometric devices although they directly produce changes in the electric current. The main advantage of such devices is their extremely small size (<< 0.1 mm^2) which allows cheap mass-produced fabrication using integrated circuit technology. As an example, a urea-sensitive FET (ENFET containing bound urease with a reference electrode containing bound glycine) has been shown to show only a 15% variation in response to urea (0.05 - 10.0 mg ml^{-1}) during its active lifetime of a month. Several analytes may be determined by miniaturised biosensors containing arrays of ISFETs and ENFETs. The sensitivity of FETs, however, may be affected by the composition, ionic strength and concentrations of the solutions analyzed.

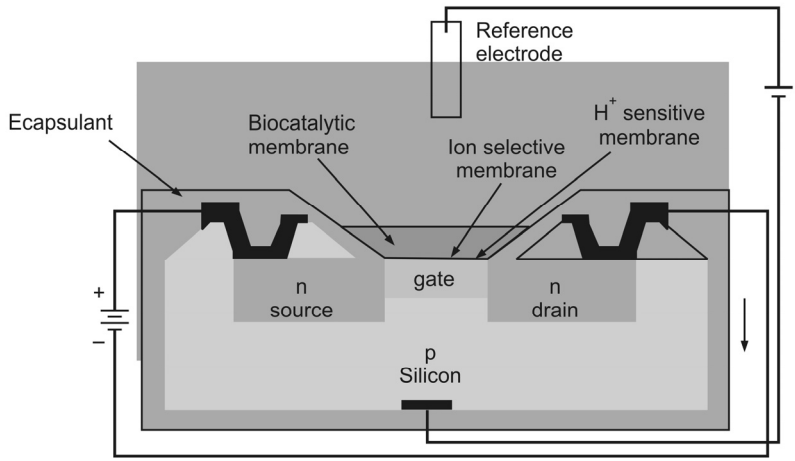


Fig. 1.12 Schematic diagram of the section across the width of an ENFET

The actual dimensions of the active area are about 500 mm long by 50 mm wide by 300 mm thick. The main body of the biosensor is a p-type silicon chip with two n-type silicon areas; the negative source and the positive drain. The chip is insulated by a thin layer (0.1 mm thick) of silica (SiO_2) which forms the gate of the FET. Above this gate is an equally thin layer of H^+-sensitive material (e.g. tantalum oxide), a protective ion selective membrane, the biocatalyst and the analyte solution, which is separated from sensitive parts of the FET by an inert encapsulating polyimide photopolymer. When a potential is applied between the electrodes, a current flows through the FET dependent upon the positive potential detected at the ion-selective gate and its consequent

attraction of electrons into the depletion layer. This current (I) is compared with that from a similar, but non-catalytic ISFET immersed in the same solution. (Note that the electric current is, by convention, in the opposite direction to the flow of electrons).

1.5.3 Amperometric Biosensors

Amperometric biosensors function by the production of a current when a potential is applied between two electrodes. They generally have response times, dynamic ranges and sensitivities similar to the potentiometric biosensors. The simplest amperometric biosensors in common usage involve the Clark oxygen electrode (Fig. 1.13). This consists of a platinum cathode at which oxygen is reduced and a silver/silver chloride reference electrode. When a potential of -0.6 V, relative to the Ag/AgCl electrode is applied to the platinum cathode, a current proportional to the oxygen concentration is produced. Normally both electrodes are bathed in a solution of saturated potassium chloride and separated from the bulk solution by an oxygen-permeable plastic membrane (e.g. Teflon, polytetrafluoroethylene).

The following reactions occur:

$$\text{Ag anode } 4Ag^0 + 4Cl^- \rightarrow 4AgCl + 4e^-$$

$$\text{Pt cathode } O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$

The efficient reduction of oxygen at the surface of the cathode causes the oxygen concentration there to be effectively zero. The rate of this electrochemical reduction therefore depends on the rate of diffusion of the oxygen from the bulk solution, which is dependent on the concentration gradient and hence the bulk oxygen concentration. It is clear that a small, but significant, proportion of the oxygen present in the bulk is consumed by this process; the oxygen electrode measuring the rate of a process which is far from equilibrium, whereas ion-selective electrodes are used close to equilibrium conditions. This causes the oxygen electrode to be much more sensitive to changes in the temperature than potentiometric sensors. A typical application for this simple type of biosensor is the determination of glucose concentrations by the use of an immobilized glucose oxidase membrane.

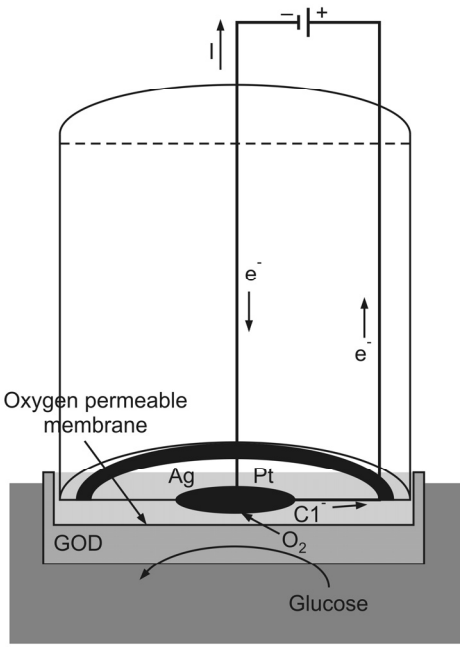


Fig. 1.13 Schematic diagram of a simple amperometric biosensor

A potential is applied between the central platinum cathode and the annular silver anode. This generates a current (I) which is carried between the electrodes by means of a saturated solution of KCl. This electrode compartment is separated from the biocatalyst (here shown glucose oxidase, GOD) by a thin plastic membrane, permeable only to oxygen. The analyte solution is separated from the biocatalyst by another membrane, permeable to the substrate(s) and product(s). This biosensor is normally about 1 cm in diameter but has been scaled down to 0.25 mm diameter using a Pt wire cathode within a silver plated steel needle anode and utilizing dip-coated membranes.

An alternative method for determining the rate of this reaction is to measure the production of hydrogen peroxide directly by applying a potential of +0.68 V to the platinum electrode, relative to the Ag/AgCl electrode, and causing the reactions:

$$\text{Pt anode } H_2O_2 \rightarrow O_2 + 2H^+ + 2e^-$$

$$\text{Ag cathode } 2AgCl + 2e^- \rightarrow 2Ag^0 + 2Cl$$

1.5.4 Optical Biosensors

There are two main areas of development in optical biosensors.

These involve determining changes in light absorption between the reactants and products of a reaction, or measuring the light output by a luminescent process. The former usually involve the widely established, if rather low technology, use of colorimetric test strips. These are disposable single-use cellulose pads impregnated with enzyme and reagents. The most common use of this technology is for whole-blood monitoring in diabetes control. In this case, the strips include glucose oxidase, horseradish peroxidase (EC 1.11.1.7) and a chromogen (e.g. *o*-toluidine or 3,3',5,5'-tetramethylbenzidine). The hydrogen peroxide, produced by the aerobic oxidation of glucose), oxidising the weakly colored chromogen to a highly coloured dye.

1.5.4.1 Peroxidase

$$\text{Chromogen(2H)} + H_2O_2 \rightarrow \text{dye} + 2H_2O$$

The evaluation of the dyed strips is best achieved by the use of portable reflectance meters, although direct visual comparison with a colored chart is often used. A wide variety of test strips involving other enzymes are commercially available at the present time. A most promising biosensor involving luminescence uses firefly luciferase (*Photinus*-luciferin 4-monooxygenase (ATP-hydrolysing), EC 1.13.12.7) to detect the presence of bacteria in food or clinical samples. Bacteria are specifically lysed and the ATP released (roughly proportional to the number of bacteria present) reacted with D-luciferin and oxygen in a reaction which produces yellow light in high quantum yield.

1.5.4.2 Luciferase

$$\text{ATP} + \text{D-luciferin} + O_2 \rightarrow \text{oxyluciferin} + \text{AMP} + \text{pyrophosphate} + CO_2 + \text{light (562 nm)}$$

The light produced may be detected photometrically by use of high-voltage, and expensive, photomultiplier tubes or low-voltage cheap photodiode systems. The sensitivity of the photomultiplier-containing systems is, at present, somewhat greater ($< 10^4$ cells ml^{-1}, $< 10^{-12}$ M ATP) than the simpler photon detectors which use photodiodes. Firefly luciferase is a very expensive enzyme, only obtainable from the tails of

wild fireflies. Use of immobilized luciferase greatly reduces the cost of these analyses.

1.5.5 Piezo-Electric Biosensors

Piezo-electric crystals (e.g. quartz) vibrate under the influence of an electric field. The frequency of this oscillation (f) depends on their thickness and cut, each crystal having a characteristic resonant frequency. This resonant frequency changes as molecules adsorb or desorb from the surface of the crystal, obeying the relationships

$$\Delta f = \frac{k f^2 \Delta m}{A}$$

Where Δ**f** is the change in resonant frequency (Hz), Δ**m** is the change in mass of adsorbed material (g), **K** is a constant for the particular crystal dependent on such factors as its density and cut, and **A** is the adsorbing surface area (cm^2). f = frequency of piezoelectric quartz crystal in MHz, for any piezo-electric crystal, the change in frequency is proportional to the mass of absorbed material, up to about a 2% change. This frequency change is easily detected by relatively unsophisticated electronic circuits. A simple use of such a transducer is a formaldehyde biosensor, utilizing a formaldehyde dehydrogenase coating immobilized to a quartz crystal and sensitive to gaseous formaldehyde.

The major drawbacks: These devices are the interference from atmospheric humidity and the difficulty in using them for the determination of material in solution.

Advantages: They are, however, inexpensive, small and robust, and capable of giving a rapid response.

1.5.6 Immunosensors

Biosensors may be used in conjunction with enzyme-linked immunosorbent assays (**ELISA**). The principles behind the ELISA technique are shown in Fig. 1.14. ELISA is used to detect and amplify an antigen-antibody reaction; the amount of enzyme-linked antigen bound to the immobilized antibody being determined by the relative concentration of the free and conjugated antigen and quantified by the rate of enzymic reaction. Enzymes with high turnover numbers are used in order to achieve rapid response. The sensitivity of such assays may be

further enhanced by utilizing enzyme-catalyzed reactions which give intrinsically greater response; for instance, those giving rise to highly colored, fluorescent or bioluminescent products. Assay kits using this technique are now available for a vast range of analyses.

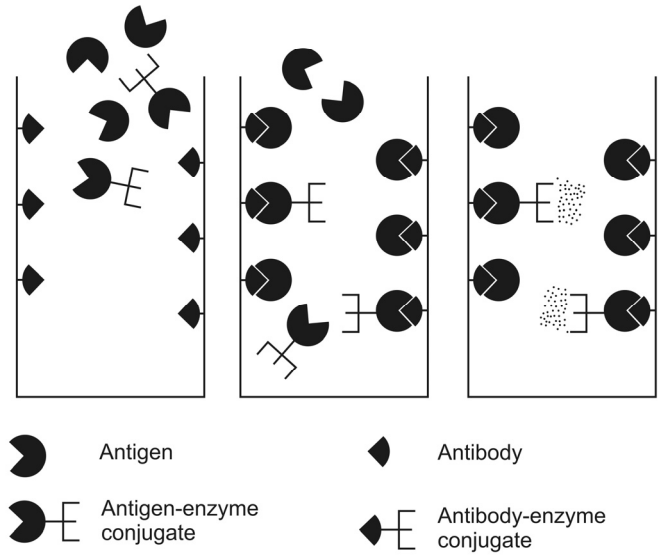


Fig. 1.14 Principles of a direct competitive ELISA. (i) Antibody, specific for the antigen of interest is immobilized on the surface of a tube. A mixture of a known amount of antigen-enzyme conjugate plus unknown concentration of sample antigen is placed in the tube and allowed to equilibrate. (ii) After a suitable period the antigen and antigen-enzyme conjugate will be distributed between the bound and free states dependent upon their relative concentrations. (iii) Unbound material is washed off and discarded. The amount of antigen-enzyme conjugate that is bound may be determined by the rate of the subsequent enzymic reaction.

Recently ELISA techniques have been combined with biosensors, to form **immunosensors**, in order to increase their range, speed and sensitivity. A simple immunosensor configuration is shown in Fig. 1.15, where the biosensor merely replaces the traditional colorimetric detection system. However more advanced immunosensors are being developed (Fig. 1.15 (b)) which rely on the direct detection of antigen bound to the antibody-coated surface of the biosensor. Piezoelectric and FET-based biosensors are particularly suited to such applications.

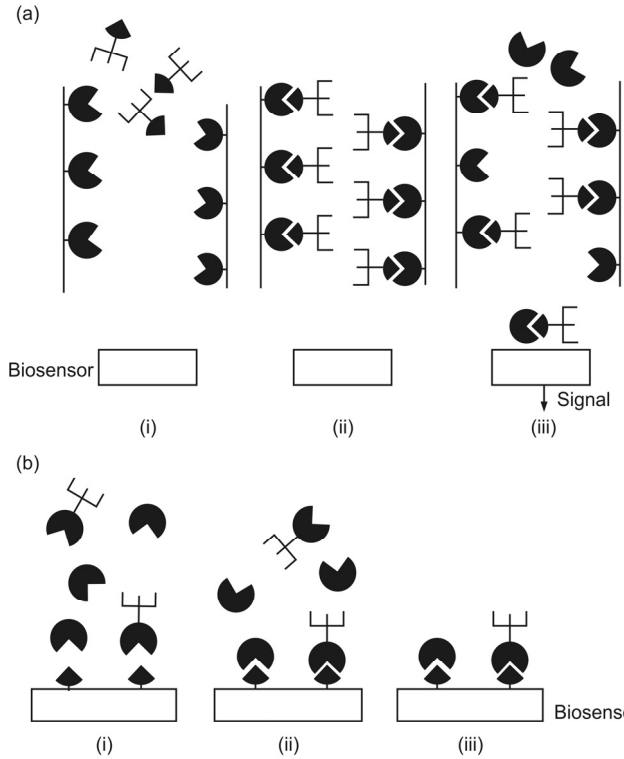


Fig. 1.15 Principles of immunosensors. (a)(i) A tube is coated with (immobilized) antigen. An excess of specific antibody-enzyme conjugate is placed in the tube and allowed to bind. (a)(ii) After a suitable period any unbound material is washed off. (a)(iii) The analyte antigen solution is passed into the tube, binding and releasing some of the antibody-enzyme conjugate dependent upon the antigen's concentration. The amount of antibody-enzyme conjugate released is determined by the response from the biosensor. (b)(i) A transducer is coated with (immobilized) antibody, specific for the antigen of interest. The transducer is immersed in a solution containing a mixture of a known amount of antigen-enzyme conjugate plus unknown concentration of sample antigen. (b)(ii) After a suitable period the antigen and antigen-enzyme conjugate will be distributed between the bound and free states dependent upon their relative concentrations. (b)(iii) Unbound material is washed off and discarded. The amount of antigen-enzyme conjugate bound is determined directly from the transduced signal.

1.5.7 Microbial Biosensors

- A biosensor is a device that detects, transmits and records information regarding a physiological or biochemical change.
- Technically, it is a probe that integrates a biological component with an electronic transducer thereby converting a biochemical signal into a quantifiable electrical response.
- Biosensors make use of a variety of transducers such as electrochemical, optical, acoustic and electronic.
- The function of a biosensor depends on the biochemical specificity of the biologically active material.
- The choice of the biological material will depend on a number of factors viz the specificity.
- storage, operational and environmental stability.
- Selection also depends on the analyte to be detected such as chemical compounds antigens, microbes, hormones, nucleic acids or any subjective parameters like smell and taste.
- Enzymes, antibodies, DNA, receptors, organelles and micro-organisms as well as animal and plant cells or tissues have been used as biological sensing elements.
- Some of the major attributes of a good biosensing system are its specificity, sensitivity, reliability, portability, (in most cases) ability to function even in optically opaque solutions, real-time analysis and simplicity of operation.

1.5.8 Use of Microbial Cells as Biosensing Elements

- Advantages of microbes as biological sensing materials in the fabrication of biosensors
- Present ubiquitously
- Able to metabolise a wide range of chemical compounds
- Great capacity to adapt to adverse conditions
- Develop the ability to degrade new molecules with time
- One of the ways to obviate this problem is to use permeabilised cells.
- Permeablisation can be achieved via Physical (freezing and thawing),

- Chemical (organic solvents/detergents) and
- Enzymatic (lysozyme, papain) approaches
- The most common technique uses organic solvents such as toluene, chloroform, ethanol and butanol or detergents like

N-cetyl-N, N, N-trim ethyl ammonium bromide (CTAB), Na-deoxycholate and digitonin (Patil and D'Souza, 1997).

- Such chemical treatment creates minute pores by removing some of the lipids from the cell membranes, thereby allowing for the free diffusion of small molecular weight substrates/products across the cell membrane while retaining most of the macromolecular compounds like the enzymes inside the cell.
- The Permeablisation process, however, renders the cell non-viable but can serve as an economical source of intracellular enzymes.
- In the case of periplasmic enzymes such as invertase and catalase in yeast (D'Souza and Nadkarni, 1980; Svitel et al., 1998) and uniease and phosphatases in bacteria (Kamath and D'Souza, 1992; Macaskie et al.,1992) whole cells can be used without Permeablisation
- One of the recent advances is to engineer the cell to transport the intra cellular enzyme and anchor it into the peri-plasmic space.
- Such an approach has been applied to obtain recombinant *Escherichia coli* cells with surface expressed oragno phosphorous hydrolase (OPH), an enzyme useful in the fabrication of biosensors for the detection of organophosphate compounds (Mulchandani et al., 1998a,b).
- These cells could degrade the organophosphates more efficiently (Mulchandani et. al., 1998a,b) without the diffusional limitations otherwise observed in engineered cells expressing OPH intra-cellular (Rainina et al., 1996).
- The above approach is an important development in the field of microbial-biosensors as it provides a cell system with no membrane transport problems and at the same time will not affect the cellular structure and activity.
- This is in contrast to chemically permeabilised cells which result in loss of cell viability
- These types of genetic approaches may have major significance in the future, especially for sensors like BOD wherein polymers such as

protein, starch, lipid etc., have to be broken down to monomers before they can be metabolised.

- Another limitation in using whole cells is the low specificity as compared to biosensors containing pure enzymes.
- This is mainly due to the unwanted side reactions catalyzed by other enzymes in a cell.
- Several approaches are being investigated to minimize such non-specific reactions.
- Permeabilisation of the cell empties it of most of the small molecular weight cofactors etc., thus minimizing the unwanted side reactions (D'Souza, 1989a).
- Thus a whole cell of yeast containing intracellular –galactosidase converts lactose to ethanol and CO_2 whereas the same cell on permeabilisation converts lactose only to glucose and galactose due to the loss of cofactors from the cell (Rao et al., 1988; Joshi et al.,1989).
- Side reactions, which can occur due to the presence of other enzymes in a cell, can also be minimised by inactivating such enzymes either by physical (heat) or chemical means when non-viable cells are used (Godboleet al., 1983; Di Paolantonio and Rechnitz,1983; D'Souza, 1989a; Riedel, 1998).
- Another approach that is of significance in viable cell-based biosensors is the blockage of unwanted metabolic pathways or transport systems.
- Thus, for the determination of glutamic acid in the presence of glucose by Bacillus subtilis, the glucose up take carrier system of the cell was blocked using a thiol inhibitor like chloromercuri benzoate and also the glycolysis was reversibly inhibited by NaF (Riedel and Scheller, 1987). .
- Microbial biosensors based on light emission from luminescent bacteria are being applied as a sensitive, rapid and non-invasive assay in several biological systems (Burlage and Kuo, 1994; Matrubutham and Sayler, 1998).
- Bioluminescent bacteria are found in nature, their habitat ranging from marine (Vibrio fischeri) to terrestrial (Photorhabdus luminescens) environments.

- Bioluminescent whole cell biosensors have also been developed using genetically engineered micro-organisms (GEM) for the monitoring of organic, pesticide and heavy metal contamination.
- The micro-organisms used in these biosensors are typically produced with a constructed plasmid in which genes that code for luciferase are placed under the control of a promoter that recognizes the analyte of interest.
- When such microbes metabolise the organic pollutants, the genetic control mechanism also turns on the synthesis of luciferase, which produces light that can be detected by luminometers.
- One approach to environmental monitoring is to detect changes in gene expression patterns induced by adverse conditions.
- Bacterial strains that increase light production in the presence of specific chemicals have been constructed using bioluminescence genes (lux) as reporters of transcriptional responses.
- A typical example is the *Pseudomonas fluorescens* HK44, a lux - based bioluminescent bio-reporter that is capable of emitting light upon exposure to naphthalene, salicylate and other substitute danalogues.

1.5.9 Immobilization of Bio Materials

- The basic requirement of a biosensor is that the biological material should bring the physico-chemical changes in close proximity of a transducer.
- Immobilisation not only helps in forming the required close proximity between the biomaterial and the transducer, but also helps in stabilising it for reuse.
- The biological material has been immobilised directly on the transducer or in most cases, in membranes, which can subsequently be mounted on the transducer.
- Biomaterials can be immobilised either through adsorption, entrapment, covalent binding, cross-linking or a combination of all these techniques (D'Souza, 1989a, 1999; Bickerstaff, 1997).
- *e.g.*, Covalent binding, commonly used technique for the immobilisation of enzymes and antibodies.

1.5.10 Microbial Biosensors for Environmental Applications

Table 1.5 Microbial Biosensors for Environmental Applications

Analysis	Microorganism	Transducer/ immobilization	Detection limit	Reference
BOD	*Trichosperum cutaneum*	Miniature oxygen electrode (UV cross-linking resin (ENT – 3400)	0.2 – 18 mg/*l*	Yang et.al (1996)
BOD	*T. cuteneum*	Miniature oxygen electrode array (photo cross-linkable resin)	<32 mg/*l*	Yang et.al (1997)
BOD	*T.cutaneum*	Oxygen electrode (entrapment)	10-70 mg/*l*	Marty et. al (1997)
BOD	*P.putidex*	Oxygen electrode (adsorption on porous nitro cellulose membrane)	> 0.5 mg/*l*	Chee et. al (1999)
BOD	Activated sludge (mixed microbial consortium)	Oxygen electrode/flow injection system (entrapped in dialysis membrane)	> 3.5 mg/*l*	Lin et. al (2000)
BOD	Salt tolerant mycelia yeast A. adeinvarans 1.53	Oxygen electrode (PVA)	2.61-524 mg/*l*	Tag et. al (2000)
Bioavailable organic carbon in oxic sediments	Yeast cells	Oxygen electrode (PVA)	Microscale	Neudoerfer and Meyer (1997)

Table 1.5 *contd…*

Analysis	Microorganism	Transducer/ immobilization	Detection limit	Reference
Aniomic surfactants (linear alky benzene sulfonates (LAS)	LAS degrading bacteria isolated from activated sludge	Oxygen electrode, (reactor type sensor, ca-alginate)	< 6 mg/*l*	Nomura et. al (1994)
Acrylamide acrylic acid	*Broxibacterium*.sp	Oxygen electrode (free celis)	0.01-0.075 and 0.01-0.1 g/*l*	Ignatov et. al. (1997)
Phenotic compounds	*Ps. parido*	Oxygen electrode (reactor with cells adsorbed on PEI glass)	100 uM	Nandakumar and Mattiason (1999 a)
Nitrite	*Nurobacter vulgaris* DSM 10236 *S.cerevisine*	Oxygen electrode (adsorption on Whatman paper)	> 10 µm	Reshetilov et. al (2000)
Cyanide	*S.cerevisiae*	Oxygen electrode (PVA)	0.15-15 nM	Tkebukaro et. al (1996)
Chlorophenols	*Rhodococcus sp., Trichosporon beigelli Ps. Putida*	Oxygen electrode (PVA)	0.004-0.04 and 0.002-0.04 mM	Riedel et. al (1993, 1995)
3-Chloro-benzoate	*Ps. putida*	Oxygen electrode (PVA)	40-200 µN	Riedel et. al (1991)
Chlorinated and brominated hydrocarbons (I-chlorobutane and ethylenebromide)	*Rhodococcus sp*. DSM 6344	Ion selective electrodes (alginate)	0.22 and 0.04 mg/*l*	Peter et. al (1996)
Polycyclic aromatic hydrocarbons (Naphthalene)	*Sphingomonus yanolkuvate* B1 or *Ps. Fluorescens* WW 4	Oxygen electrode	0.01-3.0 mg/*l*	Keenig et al. (1996, 1997a)

Table 1.5 *contd…*

Analysis	Microorganism	Transducer/ immobilization	Detection limit	Reference
Organophosphate nerve agents (paraxon, methyl parathion, diazinon)	GEM[b] *E.coli* (organophosphorous hydrolase)	Potentiometric (adsorption on electrode surface)	0.055-1.8, 0.06-0.91 and 0.46-8.5 µM	Mulchandani et. al. (1998a)
Organophosphate nerve agents (paraxon, parathion, coumaphos)	GEM[b] *E.coli* (organophosphorous hydrolase)	Fiber-optic (agarose)	0.0-0.6, 0.0-0.03 and 0.0-0.075 µM	Mulchandani et.al. (1998b)
Pollutants such as diuron and mercuric chloride	*Synechacaccus sp.* PCC 7942	Photoelectrochemical (photo cross linkable PVA bearing styrylpyridium group)	0.2 and 0.06 µM	Rouillon et al (1999)
Herbicides (diuron and atrazine)	Chloroplast/thylakoid membranes	Pt-electrode in microelectrochemical cell (photo cross linkable PVA bearing styrylpyridium group	2×10^{-5} and 2×10^{-4} µM	Rouition et al (1995)
Mono and polyphenols (atrazine)	Potato (S. tuberosum) slices (polyphenol oxidase inhibition)	Oxygen electrode (tissue slice sandwitched between membranes)	20-130 µM	Mazzel et. al (1993)

Table 1.6 Applications of Bioluminescence-based Biosensors

Application	Microorganism	Reference
Monitoring toxicity of compounds to eukaryoles	*S. cerevislae* was genetically modified to express firefly luciferase	Hollis et. al. (2000)
On-line monitoring of microbial growth	*E.coli* engineered for constitutive bioluminescence	Marincs (2000)
Toxicity of Zn, Cu and Cd, alone or in combination	*E.coli* HB101 and Ps. Fluorescens 10586 genetically modified with luxCDABE	Presion et. al (2000)
Polycyclic aromatic hydrocarbons	Ps. Fluorescens HK44 genetically modified with luxCDABE	Webb et.al. (1997), Sayler et al. (1999), Ripp et. al. (2000)
Exotoxicity assessment of organotins and their initial breakdown products (tributyltin, dibutyltin, triphenyltin and diphenyltin)	Microtox and hexCDABE modified *Ps. Fluorescens*	Bundy et. al. (1997)
Ethanol as a model toxicant	*E.coli TV* 1061, *harboring* the plasmid pGrpELux5	Gu et. al. (1996), Rupani et al. (1996)
Monitoring of biocides	Bioluminescent strain of E.coli produced by recombinant DNA technology.	Fabricant et. al. (1995)
Metals, solvents, crop protection chemicals etc	*E.coli* heat shock promoters, dnak and grpE were fused with lux genes of V,fischeri	Van Dyk et. al (1994)
Identifying constraints to bioremediation of BTEX contaminated sites	luxCDABE modified Ps. Fluorescens	Sousa et. al. (1998)
Assessment of the toxicity of metals in soils amended with sewage sludge	luxCDABE modified Ps. Fluorescens	McGrath et. al. (1999)

1.6 Protein Engineering

Protein Engineering is a term that refers to the study of proteins. The design of novel enzymes or proteins with new or desirable functionalities is known as protein engineering. It is based on the modification of amino acid sequences using recombinant DNA technology. Within the broader area of genetic engineering, protein engineering might be regarded a sub-discipline. Protein engineering is distinguished by the end product, which is a protein with a modified amino acid sequence rather than a new (or modified) live organism. Many of the concerns raised in the broader field of genetic engineering (e.g., the current debate over genetically modified species) do not apply to protein engineering because proteins do not reproduce. In this way, designed proteins are more akin to new chemical compounds derived from non-biological sources, which raise safety and toxicity issues but are biodegradable by their very nature.

1.6.1 The following are the Steps in Protein Engineering

(i) Identification: Protein engineering begins with the discovery of a protein with a specific function that can be altered to meet a specific purpose.

(ii) Protein Isolation and Characterization: Identified proteins are isolated and biochemically characterised. After that, the 3D structure and function of the protein, as well as the link between structure and function, are discovered.

(iii) Protein Modification: Based on the foregoing facts and established principles of protein conformation, protein modification may be suggested as a means of achieving desired results.

(iv) Change Incorporation: Changes are incorporated into the protein using side-directed mutagenesis, biochemical, or molecular methods. A new protein's activity must be evaluated. Protein design begins with an understanding of protein structural fundamentals.

A sequence of amino acids is created using these principles with the goal of causing the protein to take up a specific 3D structure and perform the required function. DNA is produced according to the amino acid of choice. Then, using recombinant DNA technology, it is cloned into an expression vector system. Tests and the next stage of the design process will be performed on the expressed protein.

1.6.2 Methods of Protein Engineering

There are two main approaches for protein engineering, rational design and directed evolution (irrational design).

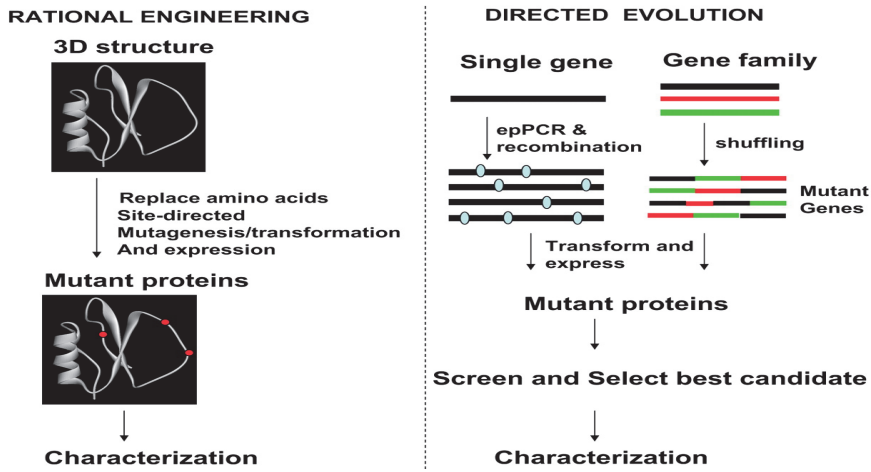


Fig. 1.16 Schematic Diagram of Protein Engineering

In the case of rational design, the protein's structure and function are taken into account, and a reasonable gene mutation is designed. This is usually accomplished by making rationally designed alterations to the gene of the protein cloned in a heterologous translation expression vector. Site directed or site-specific mutagenesis of protein genes alters the synthesis of protein molecules. However, in other circumstances, protein structure is unavailable, necessitating the use of a directed evolution approach. Random changes (mutation) are made to the protein in this process, and a mutant version with desired features is chosen.

1. **Rational design:** The so-called "rational design" approach, which incorporates "site-directed mutagenesis" of proteins, is the most well-known method in protein engineering. Site-directed mutagenesis allows specific amino acids to be introduced into a target gene. The "overlap extension" method and the "whole plasmid single round PCR" method are two common methods for site-directed mutagenesis.

   (i) The "overlap extension" approach: This method employs two primer pairs, one of which carries the mutant codon with the mismatched sequence. These four primers are used in the first

polymerase chain reaction (PCR), which involves two PCRs and the production of two double-stranded DNA products. Two heteroduplexes are generated during denaturation and annealing, and each strand of the heteroduplex contains the desired mutagenesis codon. The non-mutated primer set is then used to amplify the mutagenic Protein Engineering DNA, and DNA polymerase is employed to fill in the overlapping 3' and 5' ends of each heteroduplex.

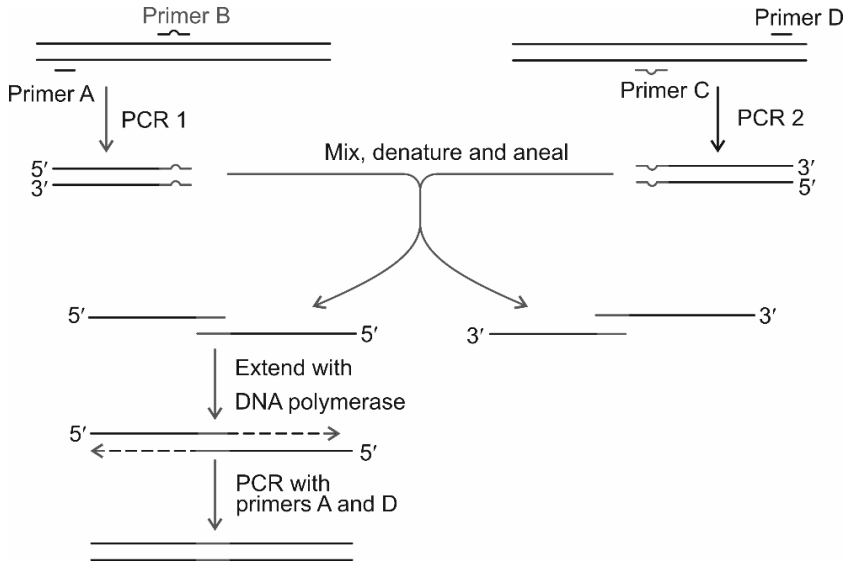


Fig. 1.17 Schematic Diagram of Rational design Protein Engineering

(ii) The complete plasmid single round PCR: Stratagene's commercial "Quick-change Site-Directed Mutagenesis Kit" is based on this technology. It requires two oligonucleotide primers that are complementary to the opposite strands of a double-stranded DNA plasmid template and contain the desired mutation(s). PCR is performed with DNA polymerase, and both strands of the template are reproduced without displacing the primers, yielding a modified plasmid with non-overlapping breaks. After that, DpnI methylase is employed to selectively digest the vector to generate a circular, nicked vector containing the mutant gene. The nick in the DNA is repaired when the nicked vector is transformed into competent cells, yielding a circular, altered plasmid.

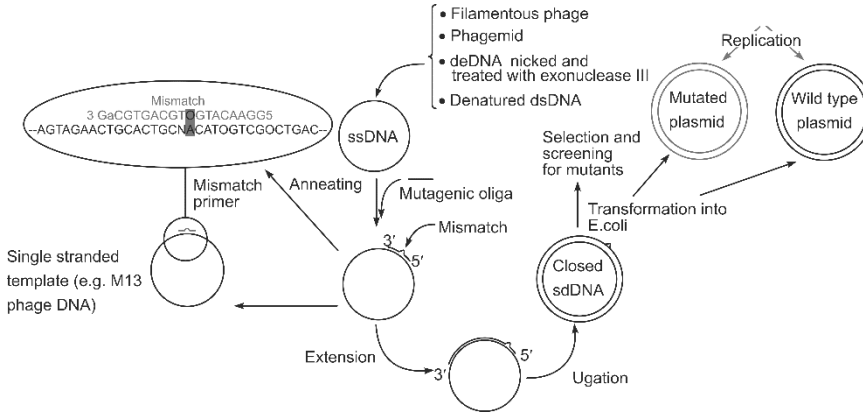


Fig. 1.18 Schematic Diagram plasmid single round PCR

When the structure and mechanism of the protein of interest are well-known, rational design is a viable option. However, in many cases of protein engineering, there is a scarcity of information about the structure and processes of the protein in question. As an alternative, the use of "evolutionary approaches" such as "random mutagenesis and selection" for the desired protein qualities was introduced. Random mutagenesis could be a useful tool, especially when there is little knowledge about the structure and mechanism of a protein. The sole stipulation is that a sufficient selection technique that favours the desired protein characteristics be available. "Saturation mutagenesis" is a simple and widespread approach for random mutagenesis.

Saturation mutagenesis

Saturation mutagenesis is when a single amino acid in a protein is replaced with each of the natural amino acids, resulting in all conceivable variants at that point. Another strategy that combines rational and random techniques to protein engineering is "localised or region-specific random mutagenesis." It entails replacing a few amino acid residues in a specific location at the same time to produce proteins with new specificities. As with site-directed mutagenesis, this approach makes use of overlap extension and whole-plasmid, single-round PCR mutagenesis. However, the codons for the selected amino acids are randomised, resulting in the employment of a variety of 64 distinct forward and reverse primers, depending on a statistical mixture of four bases and three nucleotides in a randomised codon.

Random Mutagenesis (PCR based) with degenerated primers (Saturation mutagenesis)

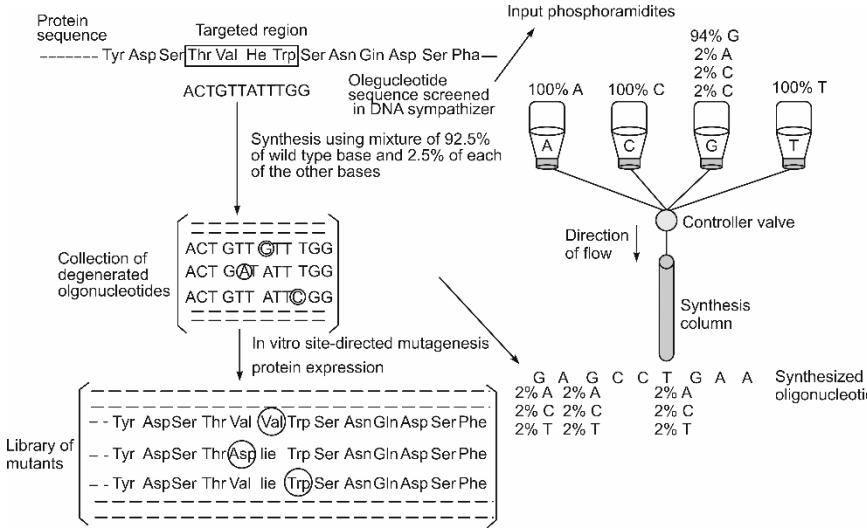


Fig. 1.19 Schematic Diagram of Saturation mutagenesis

2. **Irrational design (directed evolution):** "In vitro protein evolution systems" are based on the notion of gene hierarchy evolution. Modern genes are thought to have evolved from tiny genetic units through hierarchical and combinatorial processes. MolCraft, an in silico developed microgene, is an example.

DNA shuffling method

A set of genes with double-stranded DNA and similar sequences is collected from various organisms or created by error-prone PCR in the DNA shuffling method. These genes are digested with DNase I, which results in randomly cleaved tiny pieces that are purified and re-joined using an error-prone and thermostable DNA polymerase. The fragments are employed as PCR primers, aligning and cross-priming one another. As a result, a hybrid DNA with portions from many parent genes is created.

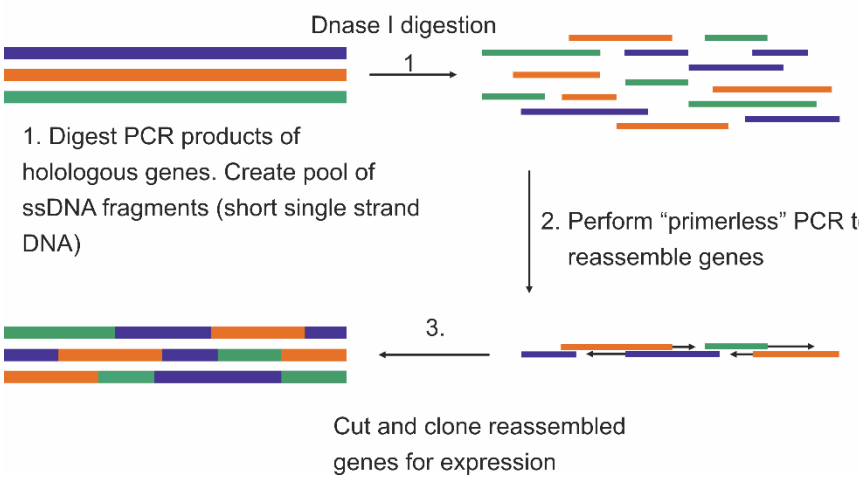


Fig. 1.20 DNA Shuffling

1.6.3 Protein Engineering's Applications

Protein engineering is being used to:

(a) Create superior enzymes with the ability to catalyse the production of high-value specific chemicals;

(b) Produce enzymes for large-scale use in the chemical industry; and

(c) Produce biological compounds that are superior to natural ones, such as synthetic peptides, storage proteins, and specific drugs.

Protein engineering technology has been used to change a long variety of proteins. A mutation of isoleucine to cystine in 'T4 Lysozyme' results in the creation of a disulphide bridge, resulting in thermal stability and a 200-fold increase in enzyme activity at 670°C. The stability of the human beta interferon enzyme was improved by removing one of the three cysteine residues. It's possible that the enzyme 'Trypsin' might be modified to have a different substrate selectivity. By substituting the active site methionine with alanine, the substrate selectivity of lactic protease (in E. coli) was drastically altered.

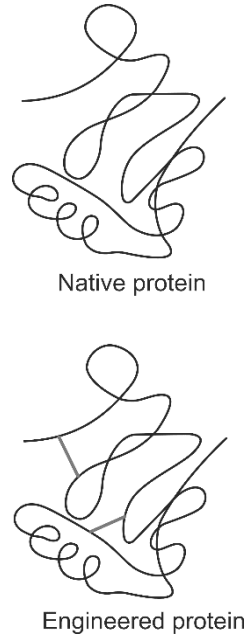


Fig. 1.21 Structure of Protein

- Immunotoxins, which are conjugates of cell-binding antibodies or antigens covalently attached to a plant or bacterial toxin, are another application of protein engineering. These immunotoxins are made through the fusion of genes with sequences coding for antibodies and hazardous peptides. When a patient is given immunotoxins, the antibody or antigen aids in the recognition of the cells that need to be killed, and the toxin component aids in the destruction of those cells. So immunotoxins are made up of two parts: a) a toxin polypeptide or a portion of it with toxin activity, called A chain, and b) a cell binding recognition polypeptide or antibody, called B chain, or a portion of it with binding site. For example, the plant toxin ricin was utilised as an immunotoxin in a study of its effect on mice tumour cells.
- Another emerging field in biotechnology is drug design. Drug design can be altered by inhibiting enzyme activity, depending on the mode of action used by the medications. Trimethoprim (TMP), for example, is a therapeutically relevant antibacterial medication that works by inhibiting the enzyme dihydrofolate reductase (dHFR) in bacteria, and is used to treat urinary tract infections. However, at high doses, it begins to damage human dHFR, making it toxic. TMP has been

synthesised, and it will have a rigid three-dimensional structure in connection with the bacterial enzyme dHFR, preventing it from attacking human dHFR.

- Renin is another example of an enzyme. Modelling of inhibitors of the enzyme ‘renin' is also underway. The enzyme catalyses the first step in a chain of events that results in high blood pressure. Nonpeptide inhibitors that imitate the intermediate products in the reaction of renin with its substrate and thereby halt renin's function are being developed. These inhibitors will aid in the treatment of high blood pressure.

1.6.4 Production of Enzyme

Microbial enzymes have been used for ages without being fully understood. Taka-diastase (a fungus amylase) was the first enzyme produced commercially in the United States in 1896. It was used to treat digestive problems as a medicinal agent.

Before tanning, softening the hides with the faeces of dogs and pigeons was a century-old technique in Europe. In 1905, a German scientist (Otto Rohm) demonstrated that extracts from animal organs (pig and cow pancreases) may be utilised as a source of enzymes (proteases) for leather softening.

In 1915, enzymes (mostly proteases) were first used for washing applications. However, due to allergic reactions to contaminants in enzymes, it was not maintained. Special procedures for the manufacturing and application of enzymes in washing powders are now available (without allergic reactions). Enzymes for commercial use can be made from a variety of biological sources. At the moment, microbial sources account for the vast bulk (80%).

The following are the many organisms and their proportionate contributions to the creation of commercial enzymes:

Fungi – 60%

Bacteria – 24%

Yeast – 4%

Streptomyces – 2%

Higher animals – 6%

Higher plants – 4%

Enzymes derived from microorganisms: Commercial enzymes are derived from microorganisms, which are the most important and convenient sources. They can be induced to produce large amounts of enzymes under the right conditions. Microorganisms can be cultured on low-cost media, and manufacturing can be completed quickly.

Furthermore, using genetic engineering techniques, it is simple to control microbes to boost the production of desired enzymes. Microbial enzymes are easier to recover, isolate, and purify than enzymes derived from animals or plants.

In reality, microorganisms have effectively manufactured the majority of industrial enzymes. This is accomplished using a variety of fungi, bacteria, and yeasts.

Table 1.7 List of Industrially Produced Enzymes

Enzyme	Source(s)	Application(s)
α-Amylase	Asperigilus cryzae Aspergillus niger Bacilus subilus Bacillus icenforms	Production of bear and alcohol Preparation of glucose syrups As a digestive aid Removal of starch sizes
Amyloglucosidase	Aspergilus niger Rhizopus niveus	Starch hydrolysis
Cellulase	Aspergillus niger Tricoderma koningi	Alcohol and glucose production
Glucoamylase	Aspergillus niger	Production of beer and alcohol Starch hydrolysis
Glucoamylase	Aspergillus niger Bacillus amyloliquefaciens	Production of beer and alcohol Starch hydrolysis
Glucose Isomerase	Arthrobacter sp Bacillus sp	Manufacture of high fructose syrups
Glucose oxidase	Aspergillus niger	Antioxidant in prepared foods
Invertase	Saccharomyces cerevisiae	Sucrose inversion Preparation of artificial honey confectionaries

Table 1.7 *contd…*

Enzyme	Source(s)	Application(s)
Keratinase	Streptomyces fradiae	Removal of hair from hides
Lactase	Kluyveromyus sp Saccaromyces fragilis	Lactose hydrolysis Removal of lactose from whey
Lactase	Kluyveromyus sp Saccharomyces fragilis	Lactose hydrolysis Removal of lactose from whey
Lipase	Candida lipolytica Asperigilus niger	Preparation of cheese Flavour production
Pectinase	Aspergilus sp Sclerotina libertine	Clarification of fruit juices and wines Alcohol production, coffee concentration
Penicillin acylase	Escherichia coli	Production of 6-aminopenicillanic acid
Penicillanase	Bacillus subtilis	Removal of penicillin
Protease, acid	Aspergillus niger	Digestive aid Substitute for calf rennet
Protease, neutral	Bacillus amyloliquefaciens	Fish and meat tenderizer
Protease, alkaline	Aspergilus oryzae Streptomyces griseus Bacillus sp	Meat tenderize Detergent additive Beer stabilizer
Pollulanase	Klebsiella aerogens	Hydrolysis of starch
Takadiastase	Aspergillus oryzae	Supplement to bread Digestive aid

Niger (a fungus) is unique among microorganisms in that it is capable of producing a vast variety of enzymes in high amounts. A. Niger is capable of producing well over 40 commercial enzymes. A-amylase, cellulase, protease, lipase, pectinase, phytase, catalase, and insulinase are examples of these enzymes.

General consideration in the Production of Enzymes

The salient features for production of enzymes are as follows:

1. Selection of organisms
2. Formulation of medium
3. Production process
4. Recovery and purification of enzymes.

An outline of the flow chart for enzyme production by microorganisms

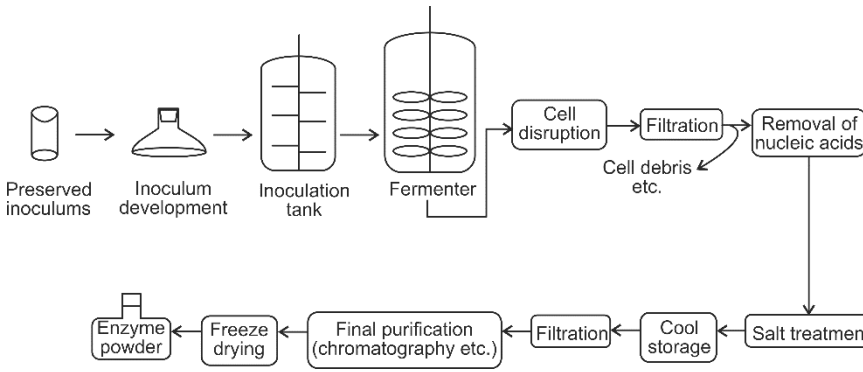


Fig. 1.22 Schematic outline of the flow chart for enzyme production by microorganisms

Selection of Organism

The most significant factors for choosing a microbe are that it should produce the largest amount of the required enzyme in the shortest amount of time while producing the least number of other metabolites. After the organism has been chosen, strain improvement can be done using appropriate methods to optimise enzyme production (mutagens, UV rays). Inoculum can be made in a liquid media from the organism chosen.

Formulation of medium

The culture medium used should contain all of the nutrients necessary to enable optimal microbe development, which will eventually result in large amounts of enzyme synthesis. The medium's elements should be easily accessible at a minimal cost and nutritionally sound. Starch hydrolysate, molasses, maize steep liquor, yeast extract, whey, and soy bean meal are some of the most often utilised substrates for the medium. There have also been some grains (wheat) and pulses (peanut) used. For optimal microbial growth and enzyme synthesis, the pH of the medium should be kept at 7.0.

Production process

Enzyme production in industry is generally done under submerged liquid conditions, with some solid-substrate fermentation thrown in for good measure. The yields are higher and the chances of infection are lower when using the submerged culture approach. As a result, this is the

favoured method. Solid substrate fermentation, on the other hand, has a long history and is still used to produce fungal enzymes such as amylases, cellulases, proteases, and pectinases.

Batch or continuous sterilisation techniques can be used to sanitise the medium. Inoculating the medium kicks off the fermentation process. The appropriate growth circumstances (pH, temperature, O2 supply, and nutrition input) are maintained. Antifoam substances can be used to reduce the production of froth.

Batch fermentation and, to a lesser extent, continuous fermentation are the most common methods for producing enzymes. Throughout the fermentation process, the bioreactor system must be kept sterile. Fermentation lasts anywhere from 2 to 7 days in most manufacturing processes. Several additional metabolites are created in addition to the intended enzyme(s). It is necessary to recover and purify the enzyme(s).

Enzyme recovery and purification

The desired enzyme generated may be expelled into the culture media (extracellular enzymes) or present within the cells (intracellular enzymes) (intracellular enzymes). The commercial enzyme can be crude or highly refined, depending on the use. It could also take the shape of a solid or a liquid. The downstream processing processes, such as recovery and purification, will be determined by the nature of the enzyme and the degree of purity desired.

Recovery of an extracellular enzyme present in the broth is generally easier than recovery of an intracellular enzyme. Special cell disruption techniques are required for the release of intracellular enzymes. Physical measures can be used to break down microbial cells (sonication, high pressure, glass beads). The enzyme lysozyme can lyse the cell walls of bacteria. The enzyme glucanase is employed in yeasts. Enzymatic approaches, on the other hand, are costly.

Once the cells are disturbed and intracellular enzymes are liberated, the recovery and purification stages will be the same for both intracellular and extracellular enzymes. The most crucial factor is to keep the amount of desired enzyme activity as low as possible.

Removal of cell debris

Cell debris removal can be accomplished using filtration or centrifugation.

Removal of nucleic acids

Nucleic acids must be removed because they obstruct the recovery and purification of enzymes. Poly-cations such as polyamines, streptomycin, and polyethyleneimine can be used to precipitate and remove them.

Enzyme precipitation

Precipitation of enzymes: Salts (ammonium sulphate) and organic solvents can be used to precipitate enzymes (isopropanol, ethanol, and acetone). Precipitation is useful because the enzyme can be dissolved in a small amount of water to concentrate it.

Liquid-liquid partition

Using polyethylene glycol or polyamines, liquid-liquid extraction can be used to increase the concentration of desired enzymes.

Separation by chromatography

Separation and purification of enzymes can be accomplished using a variety of chromatographic techniques. Ion exchange, size exclusion, affinity, hydrophobic interaction, and dye ligand chromatography are some of these techniques. Ion-exchange chromatography is the most widely used method for enzyme purification among these.

Drying and Packing

Drying produces a concentrated version of the enzyme. Film evaporators or freeze dryers can help with this (lyophilizers). The dried enzyme is ready to be packaged and sold. Stability can be achieved for some enzymes by storing them in ammonium sulphate suspensions.

All enzymes used in foods or medicinal treatments must be of high purity and meet regulatory criteria. These enzymes must be completely devoid of poisonous chemicals, dangerous bacteria, and allergic reactions.

General Considerations on Microbial Enzyme Production Regulation

By optimising the fermentation conditions, the maximum production of microbial enzymes can be attained (nutrients, pH, O2, temperature etc.). This necessitates a thorough grasp of the genetic regulation of enzyme synthesis. Some of the general aspects of microbial enzyme control are briefly explained.

Induction

Several enzymes are inducible, meaning they can only be generated in the presence of inducers. The inducer could be a substrate, a product, or an intermediary (sucrose, starch, galactosides) (fatty acid, phenyl acetate, xylobiose).

A list of inducible enzymes and their corresponding inducers.

Table 1.8 list of inducible enzymes

Enzyme	Inducer
Invertase	Sucrose
Amylase	Starch
Lipase	Fatty acids
β-Galactosidase	Galactosides
Penicillin G amidase	Phenylacetate
Xylanase	Xylobiose

Inducible Enzymes

Inducible enzymes are expensive and difficult to handle (sterilisation, adding at a specified time). In recent years, researchers have attempted to create microorganism mutants that are not dependent on inducers.

Feedback repression

The final product (typically a tiny molecule) regulates the enzyme synthesis in a major way. This happens when a huge quantity of the end product accumulates. The manufacture of feedback-regulated enzymes on a large scale is problematic. To circumvent this challenge, mutants lacking feedback repression have been produced.

Nutrient repression

The native metabolism of microorganisms is designed in such a way that no superfluous enzymes are produced. In other words, microbes do not produce enzymes that they do not require because it is a waste of time. Nutrient repression is used to stop the development of undesirable enzymes. In the growing media, the nutrients could be carbon, nitrogen, phosphate, or sulphate sources. Nutrient suppression must be overcome in order to produce enzymes on a big scale.

Repression of glucose is a classic example of nutritional (or, more accurately, catabolite) repression. That is, the enzymes required for the metabolism of the other chemicals are not produced in the presence of glucose. Glucose repression can be circumvented by providing carbohydrate to the fermentation medium at a pace that keeps the glucose concentration near zero at all times. Attempts have been made in recent years to select mutants that are resistant to glucose-induced catabolite suppression. Other carbon sources, such as pyruvate, lactate, citrate, and succinate, operate as catabolite repressors for some microbes.

In microorganisms, nitrogen source suppression is also found. Ammonium ions or amino acids could be to blame. As a nitrogen source, ammonium salts are most typically utilised. Ammonium salt repression can be circumvented by creating mutants resistant to this nitrogen source.

Microbial Enzyme Production by Genetic Engineering

Enzymes are the functional products of genes. As a result, enzymes are theoretically ideal candidates for genetic engineering-assisted manufacturing. Advances in recombinant DNA technology have undoubtedly aided in expanding microbial production of commercial enzymes over the last 15 years. The desired enzyme genes can now be transferred from one organism to another. The required gene can be cloned and introduced into a suitable production host once an enzyme with potential industrial use has been found.

Cloning strategies

Cloning procedures entail creating a cDNA library for the mRNA and designing oligonucleotide probes for the targeted enzyme. The specific cDNA clones can be identified by hybridization with oligonucleotide probes.

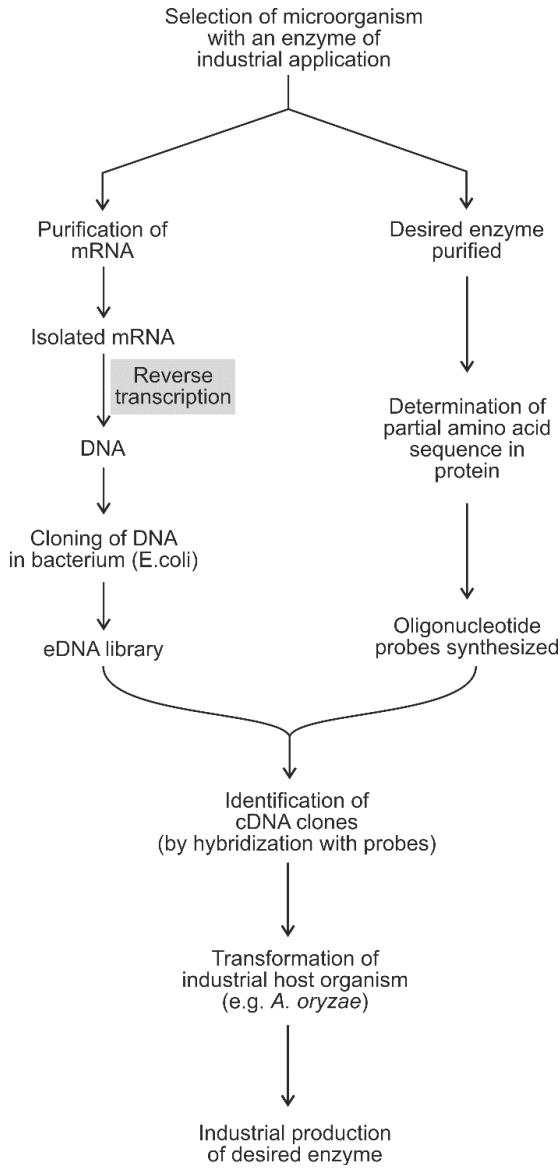


Fig. 1.23 Schematic diagram of Cloning strategies

Cloning Strategy for Industrial Production

The next stage is to turn an industrially relevant host organism (such as Aspergillus oryzae) into the enzyme of choice. It is possible to produce

high-quality industrial enzymes using this method. The following are some of the enzymes that have been created through cloning techniques:

Lipolase, an enzyme found in the fungus Humicola lanuginose, is particularly good at removing fat stains from fabrics. However, due to a relatively low degree of synthesis, industrial manufacturing of lipolase by this organism is not conceivable. The lipolase gene was extracted, cloned, and introduced into the Aspergillus oryzae fungus.

As a result, large-scale production of this enzyme has been realised. Lipolase is extremely stable and resistant to proteases, which are typically found in detergents. All of these characteristics make lipolase an excellent candidate for fabric washing.

Rennet (chymosin) is a widely utilised enzyme in the production of cheese. The stomachs of new born calves are the most common source. As a result, there is a scarcity in its supply. The gene for chymosin synthesis has been cloned, allowing for large-scale manufacture.

Protein engineering for industrial enzyme modification

Protein engineering and site-directed mutagenesis have now made it possible to change the structure of a protein/enzyme. Increased enzyme stability and catalytic performance, resistance to oxidation, modified substrate preference, and increased tolerance to alkali and organic solvents are all goals of the alterations to the enzymes.

Select amino acids at specified places (in an enzyme) can be modified using site-directed mutagenesis to make an enzyme with desired features. Protein engineering has been used to structurally change phospholipase A2 such that it can withstand high acid concentrations. As a food emulsifier, the modified enzyme performs better. Genetic engineering has had a huge impact on the cost-effective industrial manufacture of enzymes with desired characteristics.

1.7 Genetic Engineering Principles

Genetic engineering entails the direct and pre-determined alteration of genetic material to achieve a certain goal. Recombinant DNA technology or gene cloning are other terms for the same thing.

Basic Procedure

Gene cloning is the process of inserting a specific piece of 'desired DNA' into a host cell in such a way that the inserted DNA is duplicated and passed down to daughter cells during cell division.

The following are the most important factors in gene cloning

- Isolation of the gene to be cloned.
- Insertion of the gene into another piece of DNA called vector which will allow it to be taken by bacteria and replicated within them as the cells grow and divide.
- Transfer of the recombinant vector into bacterial cells, either by transformation or by infection using viruses.
- Selection of those cells which contain the desired recombinant vectors.
- Growth of the bacteria, that can be continued indefinitely, to give as much cloned DNA as needed.
- Expression of the gene to obtain the desired product.

Isolation of DNA Fragments

There are four techniques for isolating desired DNA fragments.

• **Restriction endonuclease digestion**

This method cleaves the desired area of DNA using restriction enzymes. They are a class of enzymes that detect nucleotide sequences in DNA, usually 4 or 6 base pairs long, and cut both strands of DNA within the recognition site. They are unique to each location.

These enzymes can do two sorts of cuts:

Blunt ends - If it cleaves both DNA strands at exactly opposite locations on both strands, it results in blunt end fragments that are difficult to ligate or attach to the vector in the next step.

Cohesive ends - In some situations, the two DNA strands are not severed at the same time, but rather staggered, resulting in cohesive ends (sticky ends). Sticky ends are ideal for cloning because the staggered ends make it easier to attach another bit of DNA.

- **Mechanical shearing**

Sonication (using sound waves to shear the DNA) or pushing the DNA molecule with a syringe are two methods for mechanical shearing.

- **Duplex cDNA synthesis**

Synthesis of a complementary DNA (cDNA) strand to the intended DNA: It is sometimes possible to synthesise a complementary DNA (cDNA) strand to the desired DNA.

There are two ways to do it:

Classical method: In this method, oligonucleotide dT primers, klenow fragment of T4 DNA polymerase and S1nuclease is used to synthesize cDNA.

New method: In this method, terminal transferase and dCTP primer is used. After removing any contaminating mRNA by sucrose gradient, oligo dGTP primer is added to synthesize the second DNA strand.

Direct chemical synthesis

The desired DNA fragment can be synthesized if the sequence of the desired DNA is known.

Vector Installation of the Desired Gene

After obtaining the necessary DNA fragment, it must be transmitted to the host cell. Small plasmids, phage, or (animal virus DNA molecules) are utilised as cloning vehicles to transport a DNA fragment into a live cell. Vehicles for cloning are also known as vectors. They must possess the following characteristics:

- Replication origin to allow for independent replication.
- The presence of restriction enzyme recognition sites for insertion of the DNA fragment.
- After transfer, the bacteria must be able to multiply in the host cell.
- The presence of many selection/screening markers.

Different approaches can be used to insert or ligate the desired gene into the vector

- **Homopolymer tailing:** Homopolymer tailing entails adding the identical bases to the terminal end of the polymer, for example, 8 mol of poly G tail is added using terminal transferase. As a result, the complementary strand produces a poly C tail. As a result, the entering DNA does not need to be chopped.
- **Linker molecule:** A brief sequence containing a site for a specific restriction enzyme is inserted and ligated to the DNA by the enzyme DNA ligase in the absence of restriction enzyme sites. Linkers and adaptors are utilised to create non-complementary single strands. Adaptors aim to complement each other.
- **Blunt end ligation:** When the DNA and the vector have blunt ends, a high concentration of both the plasmid and insert DNA is required,

and DNA ligase is utilised to ligate them. Low concentrations of self-ligation have been discovered.

- **Ligation of cohesive terminals:** is is more effective and occurs naturally.

The Host Cell: An Introduction

After the vector and desired DNA molecule have been ligated, the vector must be transferred to a host cell, where it will replicate and produce copies of the desired gene, as well as its products. The following approaches can be used to accomplish this transfer.

Transfection with recombinant phage DNA: If the vector is a phage, it can infect the host cell, allowing the gene to be transferred.

Recombinant plasmid transformation: If the vector is a plasmid, it can be transferred to the host by recombination.

Screening or Selection

After being delivered to the host via the vector, the recombinant DNA is integrated into the host cell DNA and begins reproducing alongside the host or independently with the phage within the host. In both circumstances, the host cells become factories for replicating and expressing the target gene. Following that, the host cells are screened to see if the target gene has been successfully integrated and replicated, as well as the expression of its products. The following strategies are used to do this:

Genetic Method

This entails the expression of specific characteristics. These qualities are usually encoded via the vector or, if a direct selection mechanism is available, by the desired cloned sequence. Antibiotics are one of the easiest strategies for selecting for the presence of vector molecules. For example, the Ampr and Tcr genes in pBR322 provide resistance to Ampicillin and Tetracycline, respectively.

Nucleic Acid Hybridization Screening

This is a very powerful method of screening clone banks and one of the most important approaches in gene manipulation. It employs a nucleic acid probe that detects the presence of a certain gene sequence. The power of nucleic acid hybridization comes from the fact that complementary sequences will attach to each other with extreme fidelity.

cDNA, genomic DNA, and oligonucleotides are the three basic types of probes employed.

Immunological Screening

An immunological approach is used to identify the protein product of a cloned gene. A particular antibody is utilised instead of a nucleic acid probe. The method of detection can be radioactive or non-radioactive.

Analysis of Cloned Genes: This method entails determining the protein product using two methods based on in vitro mRNA translation. Hybrid release translation (HRT) and hybrid arrest translation (HAT) are the terms for these techniques (HART). The preferred method is HRT.

Techniques for Blotting

The samples are first processed through a gel electrophoresis, after which the separated fragments are transferred to a nitrocellulose or nylon membrane using a blotting procedure. The capillary technique is used in the original procedure. A radioactive probe can then be hybridised with the filter. The filter is rinsed after hybridization, then exposed to X-ray film and an autoradiogram is created, which offers information on the clone's structure.

Southern Blotting: It's used to test DNA samples. E.D Southern was the first to develop it, hence the name. An agarose gel is utilised in this experiment.

Northern Blotting: Here RNA samples are been made to run. Here also agarose gel is used.

Western Blotting: It used to find the proteins. Here SDS PAGE method is followed. Membrane is then probed with an antibody to detect the protein.

Questions

1. Define the term "biotechnology." Explain branches, applications in detail.
2. Create an essay on enzyme biotechnology.
3. Explain the concept of enzyme immobilization and how it can be used.
4. Describe how biosensors works and its applications.
5. Have a conversation about protein engineering.
6. Describe genetic engineering and its applications in detail