1.1.1. Introduction

Biotechnology is a branch of biology involving the use of living organisms and bioprocesses in engineering, technology, medicine, and other fields using bioproducts. The term biotechnology indicates the use of living organisms or their products for modifying the human health and environment.

The field of biotechnology has been further expanded by a host of new molecular inventions, which permit making unique changes to the living systems. Transgenic plants and animals are widely used in agricultural field; in humans; devastating diseases are being treated by gene therapy; and in the environment, biotechnology is used in water and land management and also in the management of pollution resulting from over industrialisation. Therefore, biotechnology is a collection of biological, chemical, and engineering disciplines with various uses in agricultural, medical, industrial and environmental situations.

1.1.2. Historical Background

The earlier formative works which made significant contributions in the growth of biotechnology are enlisted in table 1.1:

Table 1.1: History of biotechnology

Year	History of Biotechnology
1665	Robert Hooke — observed tiny microscopic s _ tructures under the first microscope and coined the word cell.
1830	Robert Brown (a Scottish physician) identified an opaque area in plant cells and called it nucleus.
1857	Biologists observed small rod-like bodies in dividing cells, and called them chromosomes (coloured bodies) as they were found to be absorbing coloured dyes.
1869	Johann Friedrich Miescher (a Swiss biologist) isolated nuclein, which at present is called DNA. Watson and Crick later studied the DNA structure for more than a decade.
1879	Albrecht Kossel (a German biochemist) discovered the nucleic acid composition, i.e., adenine, thymine, cytosine, guanine, and uracil.
1882	Edouard van Beneden (a Belgian biologist) found out that every species characteristically possesses a specific number of chromosomes.
1902	Walter S. Sutton — studied grasshopper sperm and reported that each chromosome paired with another physically similar pair and then got separated during meiosis. The individual members of each pair then divided among the cells.
1907	Tissue culture techniques were developed
1909	Wilhelm Ludvig Johannsen (a Danish botanist) coined the term genes.
1919	Thomas Hunt Morgan identified XY male and XX female chromosomes and suggested that some traits are sex -linked. He also demonstrated the mutation of genes.
1930	Electron microscope was invented with the help of which the researchers viewed viruses. This microscope had better contrast and staining agents, thus macromolecules like DNA were exposed.
1944	Oswald T. Avery along with his colleagues demonstrated that DNA is the carrier of genetic information.
1953	James Watson and Francis Crick proposed a double helix molecular structure of DNA based on Rosalind Franklin's model.
1961	Francois Jacob and Jacques Monod (French molecular biolo gists) suggested that differential gene expression causes differences in the structure and function of cells.
1964	Marshall Nirenberg cracked genetic code.
1970	First restriction enzyme (a specialised protein used to cut DNA strands at specific locations) was isolated.

1973	Herbert W. Boyer and Stanford's Stanley N. Cohen performed the first genetic engineering experiment by linking toad genes with E. coli bacteria.
1976	J. Michael Bishop and Harold E. Varmus discovered oncogenes (which lead to cancer). Yuet Wai Kan established molecular techniques to allow the first fetal test for identifying sickle cell anaemia.
1979	Genentech Inc. (the biotechnology company) in collaboration with UCSF (University of California, San Francisco) developed synthetic hum an growth hormone. Yuet Wai Kan and Judy C. Chang (of UCSF's) used gene mapping techniques to discover the single genetic mutation causing _—__B-thalassemia (the most common form of life-shortening blood disease).
1980	Virus-fighting interferon was cloned.
1981	Transgenic mice and transgenic fruit flies were produced, which became the model systems for studying mutations, gene expression, and human diseases. After 7 years, a cancer prone transgenic mouse became the first patented life form.
1987	William J. Rutter (a biochemist) and his Emeryville Chiron Corporation produced the first commercial genetically engineered vaccine against hepatitis B.
1990	Chymosin (an enzyme used in cheese -making) became the first product of genetic engineering to be introduced into the food supply.
1991	The gene implicated in the inherited form of breast cancer was discovered. This paved the way for the treatment of other similar forms of cancer.
1992	Techniques for testing embryos for inherited diseases such as cystic fibrosis were developed.
1996	The first trial of gene therapy for a neurological disorder was conducted in New Zealand. Dolly, the sheep was clone d from a cell from an adult sheep making it the first cloned organism.
1999	New Zealand researchers developed a new vaccine for bovine and human tuberculosis. Chinese scientists cloned a giant panda embryo.
2003	The human genome was sequenced.
2004	UN Food and Agriculture Organisation endorsed biotech crops, and stated biotechnologyas a complementary tool for traditional farming methods. FDA approved Avastin (the first anti-angiogenic drug for cancer).
2005	The Energy Policy Act was passed and signed into law, authorizing numerous incentives for bioethanol development.
2006	FDA approved Gardasil recombinant vaccine, which is the first vaccine developed ag ainst Human Papillomavirus (HPV) that causes an infection implicated in cervical and throat cancers, and the first preventative cancer vaccine.
2007	USDA granted Dow AgroSciences, the first regulatory approval for a plant-made vaccine.
2009	FDA approved the HSN1 vac_ cine (the first vaccine approved for avian flu). Global biotech crop acreage reached 330 million acres. FDA approved the first genetically engineered animal for production of a recombinant form of human anti-thrombin.

1.1.3. Applications of Biotechnology in Pharmaceutical Sciences

The pharmaceutical applications of biotechnology are:

1) Medicine: Modern biotechnology finds promising applications in medicine as in:

i. Drug Production: Most of the traditional pharmaceutical drugs use d for treating the symptoms of a disease are simpler molecules found through trials and errors. Small molecules are manufactured chemically, but the larger ones are created by human cells, bacterial cells, yeast cells, and animal or plant cells. Modern bio technology involves the use of genetically altered microorganisms (e.g., FE. coli or yeast) for producing

insulin or antibiotics via synthetic means. Modern biotechnology can also be used for producing plant-made pharmaceuticals. Biotechnology is also used in the development of molecular diagnostic devices used to define the target patient population for a given biopharmaceutical. For example, herceptin was the first drug to be used with a matching diagnostic test for treating breast cancer in women whose cancer cells expressed HER; protein.

ii. Pharmacogenomics: It is the study of how the genetic inheritance of and individual affects his/her body's response to drugs. The term pharmacogenomics was derived from the words pharmacology and genomics, thus it involves studying the relationship between pharmaceuticals and genetics. Pharmacogenomics aim _ s _ to design and produce drugs adapted to each individual's genetic makeup.

iii. Gene Therapy: It is used for the treatment of gene __ tic and acquired diseases like cancer and AIDS. Gene therapy utilises normal genes for supplementing or replacing the defective genes or for strengthening immunity. This therapy targets either the somatic cells (i.e., body) or thegametes (i.e., egg and sp erm). In somatic gene therapy, the recipient's genome is altered; however, this alteration is not passed on to the next generation. On the other hand, in germline gene therapy , the egg and sperm cells of the parents are altered to be passed on to their offspring.

iv. Genetic Testing: This involves direct examination of the DNA, and is used for:

a) Carrier screening, or identifying unaffected individuals who carry one copy of a gene for a disease that requires two copies for the disease to manifest,

b) Confirming the diagnosis of symptomatic individuals,

c) Determining sex,

d) Forensic/identity testing,

e) New-born screening,

f) Prenatal diagnostic screening,

g) Pre-symptomatic testing for determining the risk of developing adult-onset cancers, and

h) Pre-symptomatic testing for predicting adult-onset disorders.

2) Cloning: In this method, nucleus from one cell is removed and is transferred to an unfertilised egg cell whose nucleus has either been deactivated or removed. Cloning can be done in the following two ways:

i. Reproductive Cloning: In this method, the egg cell after a few divisions is transferred to a uterus for its development into a foetus that is genetically identical to the donor of the original nucleus.

ii. Therapeutic Cloning: In this method, the egg is placed in a petridish for its development into embryonic stem cells that are potential for treating several ailments.

3) Agriculture: Biotechnology in agricultural field is used for the following purposes:

i) Crop Yield: For in creasing the crop yield, one or two genes are transferred to a highly developed crop variety for imparting a new character. The current techniques of genetic engineering are best for the

effects controlled by a single gene. Some genetic characteristics related to yield (e.g., enhanced growth) can be controlled by various genes, each posing a nominal effect on the yield.

ii. Reduced Vulnerability of Crops to Environmental Stresses: Such crops which can be made resistant to biotic and abiotic stresses can be developed with the help of genes; for example, drought and salty soil are the two limiting factors in crop productivity.

iii. Increased Nutritional Qualities: The nutritional value of proteins contained in foods can be enhanced; for example, proteins in legumes and cereals can be transformed such that they also provide amino acids required in the balanced diet of humans.

iv. Reduced Dependence on Fertilisers: Modern biotechnology can also be used to reduce the dependence of farmers on agrochemicals; for example, Bacillus thuringiensis (Bt) is a soil bacterium that produces a protein having insecticidal properties. Conventionally, these bacteria were used to produce an insecticidal spray by a fermentation process. In this form, the Bt toxin occurs as an inactive protoxin, which becomes effective when digested by an insect. There are several Bt toxins and each has a specificity for some target insects.

v) Production of Novel Substances in Crop Plants: Biotechnology is also

applied for novel uses apart from food; for example , oilseed is

genetically modified to produce fatty acids for detergents, substitute

fuels, and petrochemicals. Potatoes, tomatoes, rice tobacco, lettuce,

safflowers, and other plants are genetically engineered to produce insulin

and certain vaccines.

4) Biological Engineering: It is a branch of engineering that involves biotechnologies and biological science. It includes different disciplines such as biochemical engineering, biomedical engineering, bio –process engineering, biosystem engineering, etc.

1.2. ENZYME BIOTECHNOLOGY:

1.2.1. Introduction

Enzymes are non -toxic and biodegradable biocatalysts synthesised by the living cells. They are complex protein molecules that bring about chemical reactions associated with life. Enzymes function, i.e., they bring about catalysis even when separated from the cells. They can also be produced by microorganisms to be used in industries. Enzyme technology involves production, isolation, purification and use of soluble or immobilised form of enzymes for benefiting humankind.

Recombinant DNA technology and protein engineering involved in the production of more efficient and useful enzymes are also a part of enzyme technology.

1.2.2. Enzyme Immobilization

Enzyme immobilis ation is a technique of restraining the enzymes in or on an inert support for their stability and functional reuse. The immobilisation matrix or the support allows exchange with the bulk phase in which the substrate, effect or inhibitor molecules are dispersed, but remains separated from it. With the use of enzyme immobilisation, enzymes are made more efficient and cost-effective to be used industrially.

Advantages

1) Enzymes are used repeatedly only if they can be completely recovered from the accomplished reactionm ixtures. Thus, immo bilisation allows the repeated usage of enzymes as such enzyme preparation s can be separated from the reaction system involved.

2) The final desired product should be from the enzyme. It goes a long way in affecting reduction and saving upon the cost of downstream processing of the end-product.

3) Non-aqueous systems (that utilise organic solvents) and the immobilized enzymes are compatible, and this is extremely desirable in some typical and specific cases.

4) Immobilised enzymes can be used in most continuous production systems, which is not possible with free enzymes.

5) Some immobilised enzymes exhibit thermostability of the highest order; for example, glucose isomerase (free enzyme) denatures at 45°C temperature in solution; however, on immobilisation it remains stable fora year even at 65°C temperature.

6) The ultimate recovery of immobilised enzyme reduces the high effluent disposable problems (acute in several fermentation industries).

7) Immobilised enzymes can be used at a much higher concentration range than the corresponding free enzyme.

Disadvantages

1) There is a possibility of the loss of biological activity of an enzyme during immobilisation or while in use.

2) It is an expensive technique as it requires sophisticated equipment.

3) Immobilization of enzymes affects the stability and/or activity. Such conditions can be avoided by strictly following the developed immobilization protocols.

4) If one of the substrates is insoluble, the immobilised enzymes are not considered fit for practical utilisation.

5) Some immobilisation protocols give rise to problems related to the diffusion of the resultant substrate to have an access to the corresponding enzyme.

1.2.3. Methods of Enzyme Immobilization

Enzymes can be immobilised by the following four different methods depending on the physical relationship of the catalyst being used with the carrier matrix:

1) Adsorption method,

2) Covalent bonding,

3) Entrapment, and

4) Encapsulation.

1.2.3.1. Adsorption Method

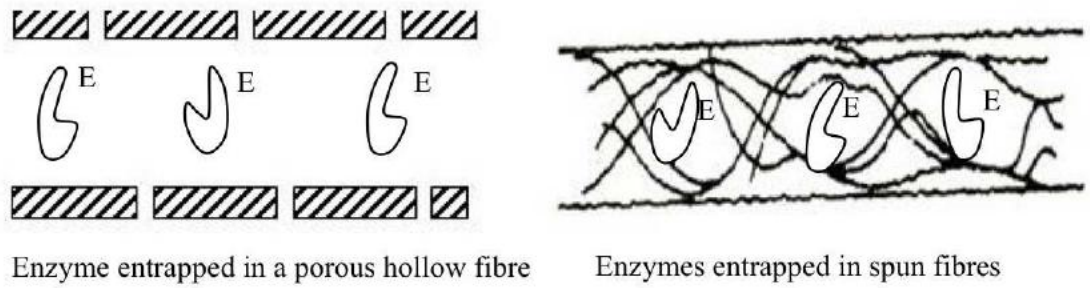
Adsorption of an enzyme is brought about by allowing the enzyme to come in contact with a polymer support. The enzyme molecules adhere to the surface of the carrier matrix with the help of hydrophobic bonds and __ by forming several salt-linkages per enzyme molecule. Various specific and non-specific forces, like hydrophobic bonds, electrostatic interactions, or affinity bondage to some specific ligands attached to the carrier matrix, involve in the adsorption method.

Examples of some enzymes that have been immobilized by adsorption on different carrier matrices are given below:

Enzymes	Carrier Matrices
α-Amylase	Calcium phosphate
Amyloglucosidase	Agarose gel; DEAE-Sephadex
Catalase	Activated charcoal
Glucose oxidase	Cellophane (followed cross -linking with glutaraldehyde, inorganic adsorbents)
Invertase	Activated charcoal; DEAE-Sephadex
Subtilisin	Cellulose

Methods for Immobilisation by Adsorption

The adsorptive immobilisation of enzymes (figure 1.1) can be carried out by the following ways:



Enzyme entrapped in a porous hollow fibre Enzymes entrapped in spun fibres

Figure 1.1: Representation of Immobilisation of Enzymes by Adsorption Methods

1) Static Pores: This is the most efficient technique, but is time consuming. In this process, the enzyme is immobilised on the carrier by bringing the enzyme containing solution in contact with the carrier without agitation or stirring.

2) Dynamic Pores: This technique is frequently used in the laboratories. In this process, the enzyme solution is admixed with the carrier with constant agitation or stirring with a mechanical shaker. The process is effective and results in uniform and high loading provided an adequate concentration of enzymes is used.

3) Reactor Loading: This technique is used for producing immobilized enzymes. In this process, the carrier is placed into the reactor and enzyme solution is transferred to the reactor loaded with carrier. Immobilization is accomplished via dynamic environment by either circulating the enzyme or by agitating the enzyme-carrier solution.

4) Electro-Deposition: In this process, the carrier is placed in proximal vicinity of one of the electrodes in an enzyme bath and then electric current is passed. As a result, the enzyme molecules migrate towards the carrier and deposit on its surface. The c arrier system used for immobilization by electro-deposition process should be stable in an electric field.

1.2.3.2. Covalent Bonding

In this method, the enzyme molecules adhere to the carrier matrix by forming covalent bonds (figure 1.2), which forms with the side chains of amino acids present in the enzyme; however, their actual strength of reactivity is entirely related to the status of 'charge' present in them as given below:

$$—S^- > —SH > —O^- > —NH_2 > —COO^- > —OH >> —NH_3^+$$

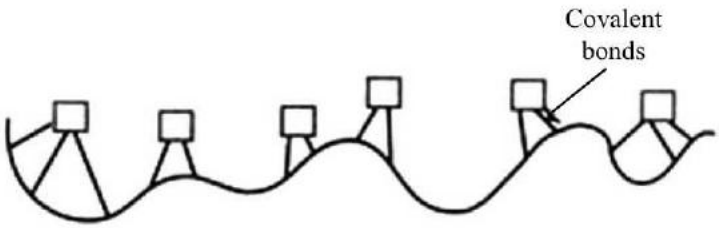


Figure 1.2: Representation of Immobilisation of Enzymes by Covalent Binding

The following common methods of covalent binding are in use:

1) Cyanogen Bromide (CNBr) Activation: In this method, the inert support materials (e.g., cellulose, sepharose, and sephadex) containing glycol groups are activated by CNBr, which then binds to the enzymes and immobilises

them (figure 1.3A).

2) Diazotation: In this method, the support materials (e.g., amino benzyl cellulose, amino derivatives of polystyrene, and aminosilanised porous glass) are diazotised with $NaNO_2$ and HCl, which then covalently bind to tyrosyl or histidyl groups of enzymes (figure 1.3B).

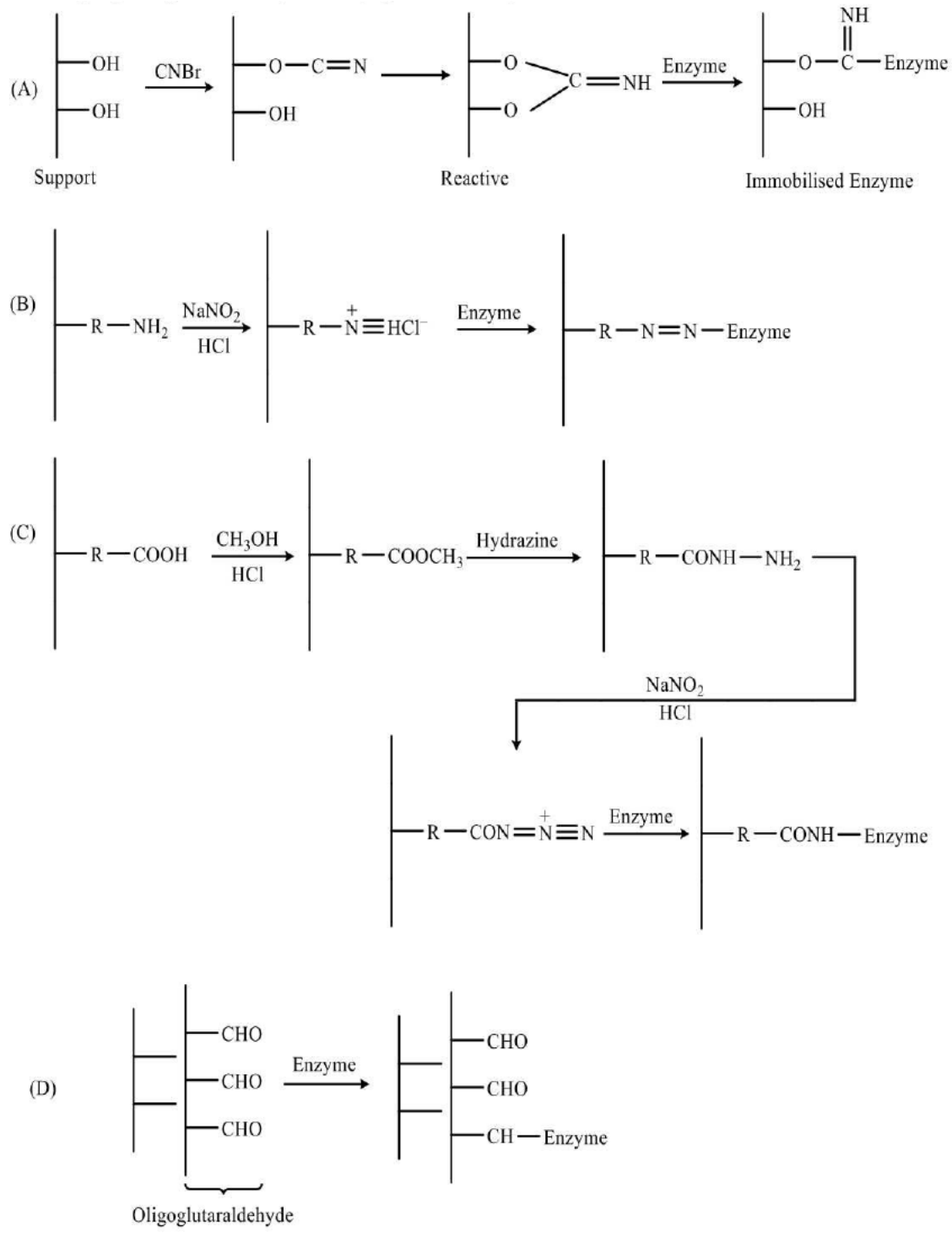


Figure 1.3: Immobilisation of Enzymes by Covalent Binding (A) Cyanogen Bromide Activation, (B) Diazotation, (C) Peptide Bond Formation, (D) Activation by Bifunctional Agent

Peptide Bond Formation: In this method, peptide bonds are formed between the amino (or carboxyl) groups of the support materials and the carboxyl (or amino) groups of the enzymes (figure 1.3C). The support material is previously treated chemically to form active functional groups.

4) Activation by Bi- or Poly-Functional Reagents: In this method, the reagent (e.g., glutaraldehyde) is used to form bonds between the amino groups of enzymes and amino groups of support materials (_ e.g., aminoethylcellulose,

albumin, and amino alkylated porous glass) (figure 1.3D).

Some enzymes immobilized by covalent bonding using typical carrier matrix and binding reaction are summarised in table 1.2:

Table 1.2: Enzymes Immobilised by Covalent Bonding

Enzymes	Carrier Matrix Used	Binding Reaction Involved
α-Amylase	DEAE-cellulose*	Direct coupling
Amyloglucosidase	—do—	Cyanuric chloride
Cellulase	Polyurethane	Isocyanate
Glucose isomerase	—do—	—do—
Glucose oxidase	Porous glass	Isothiocyanate
Pectinase	Polyurethane	Isocyanate
Pronase	CM-Sephadex	Carbodiimide activation

*Cellulose-2-(Diethylamino) ethyl ether.

1.2.3.3. Cross-Linking

In this method of enzyme immobilisation, a solid support is not required; instead cross-links are formed between the enzyme molecules. Poly -functional reagents (e.g., glutaraldehyde, diazobenzidine, hexamethylene diisocyanate, and toluene di-isothiocyanate) are used which react with the enzyme molecules and form bridges that act as a backbone to support the enzyme molecules (figure 1.4).

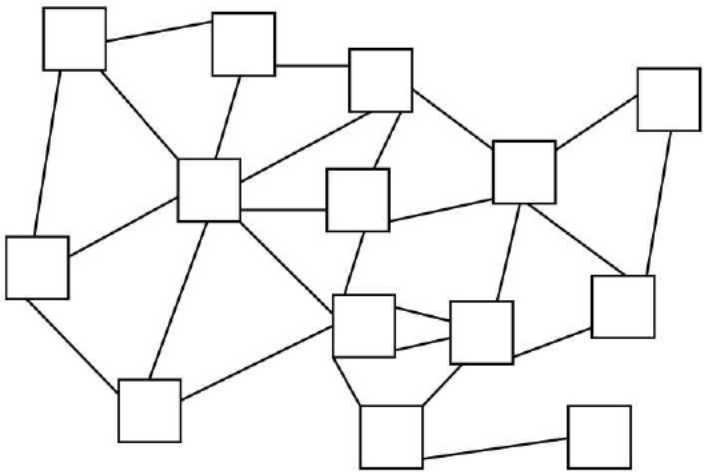


Figure 1.4: Representation of Immobilisation of Enzymes by Cross-Linking

Glutaraldehyde, the most commonly used cross -linking reagent , forms a Schiff's base by reacting with the lysyl residues of the enzymes. Irreversible cross-links are formed between the enzyme molecules and glutaraldehyde, and these cross-links can withstand extreme p_ H and temperature. Glutaraldehyde cross-linking has been used forimmobilis ing some industrial enzymes, e.g., glucose isomerase and penicillin amidase.

The cross-linking method is simple and cost -effective; but the enzyme may get denaturised by the poly-functional reagent.

1.2.3.4. Entrapment

Entrapment is a phenomenon in which the enzyme molecules are held or entrapped within appropriate fibres or gels. This entrapment, however, may or may not be the result of covalent bonding existing between the enzyme entities (molecules) and the carrier matrix. In this method of enzyme immobilization, the enzyme molecules are either held or entrapped within fibres or gels. The following methods are used for entrapment:

1) Three-Dimensional Gels: In this method, a large number of stable, three dimensional gels are formed under mild conditions so that the enzymes get entrapped without losing their activity. Hydrogels are used extensively as they provide maximum stabilization to the actives structure and get easily wetted allowing free access of the substrate (figure 1.5).

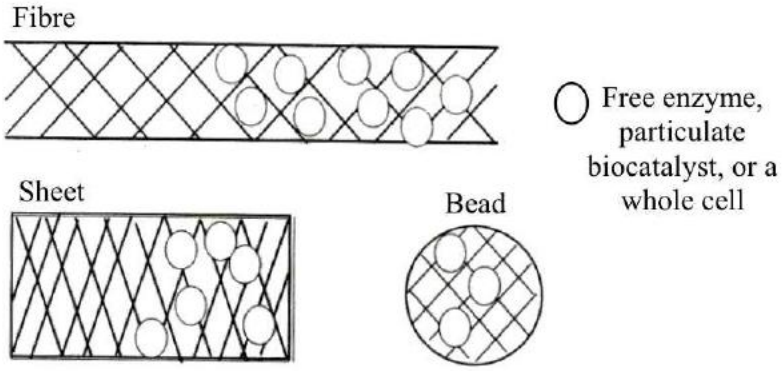


Figure 1.5: Entrapment of Enzymes in a Three-Dimensional Porous Matrix

2) In Situ Polymerization: In this method, pre -polymeric weak -ionic or adsorptive interactions occur with the enzymes. Therefore, the entrapped enzymes should be rapidly washed to remove the unreacted chemicals and other reagents. In the method of in situ polymerisation, the enzyme is suspended or dissolved in an aqueous buffer containing acrylamide and a bisacrylate as a cross -linker. The vinyl polymerisation of these two monomers within the solution is initiated by generating free radicals either by incorporating a redox couple or by energetic electromagnetic radiation (such as light or y-rays).

Cross-linked polymers of acrylamide are extensively used as a medium for chromatography and electrophoresis. The hydrophilic nature of gel, its high water regain capacity, high porosity, and low level of absorptivity for biochemicals, make it useful as a carrier for immobilis ing enzyme, and also in separation techniques. Other acrylates based on methacrylate monomers like Hydroxy Ethylmethacrylate (HEMA), bifunctional acrylates like PEG dimethacrylates can also be used as gel-forming polymeric matrices.

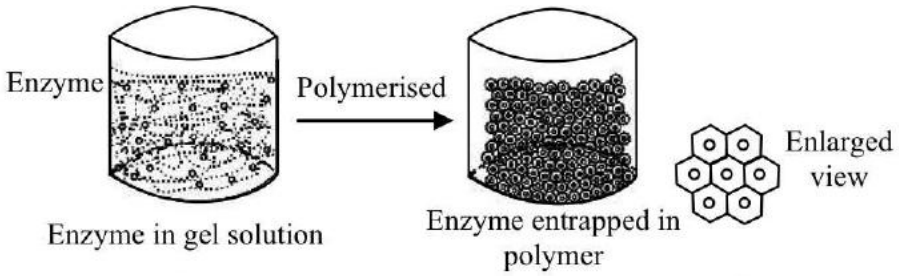


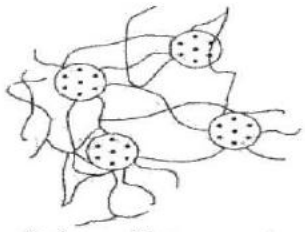
Figure 1.6: Entrapped Enzyme Inside a Polymerised Gel

Polycondensation: Polymerisation of bis-isocyanates with polyalcohols or polyamines to give urethanes and the cross -linking of inactive proteins with bifunctional glutaraldehyde are the common examples of polycondensation reactions. Streptomyces rimosus cells are immobilized in urethane pre -polymers and used for oxyt etracycline production. Bifunctional aldehyde like glutaraldehyde reacts with the amine groups of proteins to formacross -linked structure. Aqueous solution of gelatin containing urease can be entrapped by dipping dried film into glutaraldehyde to fix -up the matrix.

Immobilization of Enzymes in Gelatin: The protein nature of gelatin, its high hydrophilicity, and strong swelling power make it suitable for immobilising cells and enzymes. In this method, the cells are immobilised by suspending them in deionised water and dispersing in 10% w/v aqueous gelatin solution at35 -40°C temperature to give final cells to gelatin concentration ratio of 0.1%w/w. The so formed cell-gelatin suspension is mixed with hardening agents e.g., mixture of 20%w/v formaldehyde in 50 %v/v alcohol) and immediately poured into cylindrical moulds (0.3cm), which are then allowed to gel in deep freeze (at —25°C). After 4 hours, the moulds are kept at room temperature and cut into thin discs (0.2 -0.4cm), which can be stored for months under refrigerated conditions without the loss of enzyme activity (e.g., yeast cell invertase).

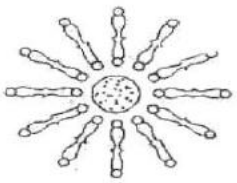
The enzyme can be immobilised using gelatin in the same manner as the cells, except that the gelatin and hardening agents are mixed first, and immediately added wit h defined volume of enzyme solution. This is advantageous as the exposure of enzyme to formaldehyde is reduced, and the active preparation can be obtained with no loss of enzyme activity.

For example, penicillin acylase is a fibre-entrapped enzyme that was immobilised by entrapment in the microcavities of the synthetic fibres.



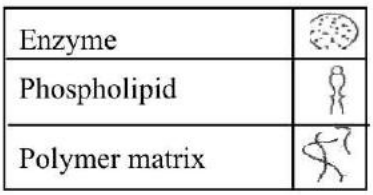
Polymer Entrapment

Enzyme Immobilisation via Polymer Entrapment



Liposome Entrapment

Enzyme Immobilisation via Liposome Entrapment



1.2.3.5. Encapsulation

Encapsulation (or microencapsulation or membrane confinement) is a versatile and effective method of enzyme in mobilisation by entrapping. In this method, the enzyme molecules are regularly taken up in an aqueous medium and strategically confined in a semi -permeable membrane, which allows an absolute free movement of the enzymes.

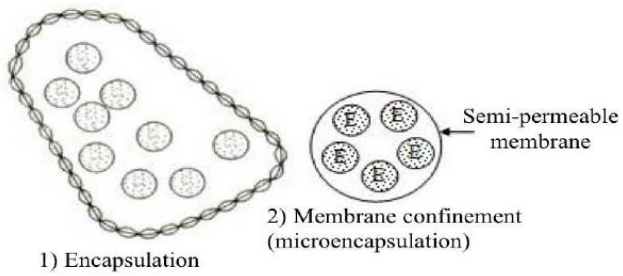


Figure 1.7: Enzyme Immobilisation by 1) Encapsulation and 2) Membrane Confinement (or Microencapsulation)

Encapsulation can be done by the following methods:

1) Membrane Reactors: Reverse osmosis and ultra -filtration can be operated on immobilized enzyme or cell reactors; however, the size of substrate molecules which must pass through the membrane is a limiting factor. The enzyme is retained behind the membrane, the substrate is introduced in the enclosed compartment, and the reaction products pass out as permeate or filtrate. It is certain that in membrane techniques the concentration of solutes results in flux of solvent and small solutes through the membrane and formation of larger solutes on the inner face as a polarization layer.

2) Microcapsules: In liquid membrane system (a biphasic system), an organic intermediate phase acts as a barrier to the diffusion of enzyme molecules, thereby keeping them immobilized in the inner phase. For substrate transportation, a specific carrier system can be used to facilitate an effective enzyme substrate interaction. Emulsions are stabilized permanently by hardening a polymer membrane at the interface, thus producing a microcapsule entrapping the enzyme droplet. This simplifies the transfer of these membranous capsules to a fresh, non-emulsified solution.

3) Dehydrated-Rehydrated Vesicles (DRVs): Gregoriadis et al suggested the encapsulated incorporatio n of proteins and enzymes in liposomal vesicles (dehydration-hydration vesicles). This method involves the formation of lipid vesicles in which hydration fluid contains protein or enzyme to be incorporated. The total content of the hydration stage is frozen and rehydrated using Plain Phosphate Saline (PBS) buffer.

The transformation stages involved in the form of rehydrated-dehydrated vesicles produce uni- or oligo-lamellar liposomes that can entrap up to 70% of therapeutic proteins and DNAse. This entrapment can be improved by increasing the concentration of sodium or potassium chloride; but accidental or intentional introduction of sugar or carbohydrates decreases the entrapment of macromolecular bioproteins. This happens due to cryoprotection caused by sugars; this process prevents the membrane rupture, thus, the vesicles undergo fusion during the dehydration phase. Fusion is essential for effective performance of this method during freeze drying.

Reverse Phase Evaporation Method (REVs) : Szoka and Papahadjopolous developed this method in which bioproteins (including enzymes) can be incorporated into liposomes . The method is based on the formation of w/o emulsion which was considered to be the reverse of o/w emulsion involving the evaporation of solvent from the emulsion. The water phase of the system contains the bioproteins to be incorporated.

On removal of solvent, droplets are formed which when subjected to sonication makes the emulsion dry to a semi - solid form in a rotary evaporation under reduced pressure. At this stage, the monolayers of phospholipids surrounding discrete water compartments are closely attached. In the second stage, vigorous mechanical shaking is applied and thus the water droplets collapse. These collapsed vesicles contribute their monolayers resulting into larger intact vesicles. The aqueous content of the droplets is the medium in which the newly formed liposomes are suspended.

Table: 1.3 Comparison of Characteristic Features of Immobilisation Techniques

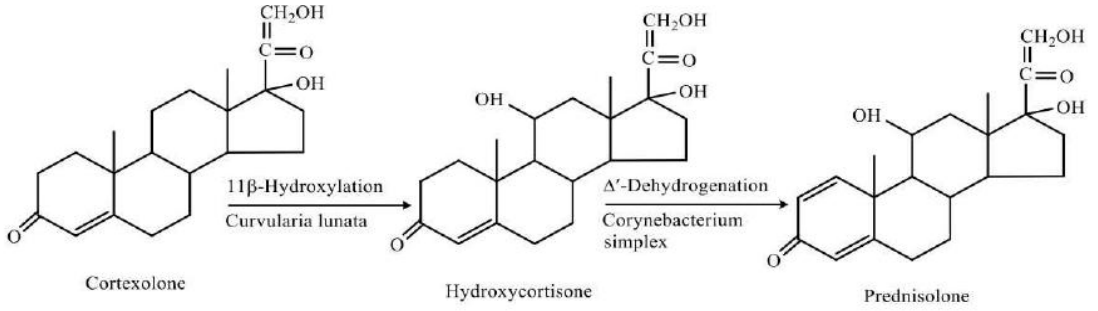
S. No.	Characteristic Features	Immobilisation Techniques			
		Adsorption	Covalent Bonding	Entrapment	Encapsulation
1.	Matrices	Clays, glasses, ion-exchange matrices	Acrylamide, cellulose, sepharse	Acrylamide, cellulose acetate etc.	Semipermeable membranes via., hollow fibres, liposomes etc.
2.	Preparation	Simple	Difficult	Difficult	Simple
3.	Immobilisation mechanism	Hydrophobic effects; salt links, etc.	Covalent bonds	Trapping in gel or fibre	Confinement in semipermeable membrane.
4.	Binding force	Variable	Strong	Weak	Strong
5.	Enzyme loading	High (Ca. 1g/g matrix)	Low (Ca. o.2g/g matrix)	—	—
6.	Enzyme leakage during usage	Yes	No	Yes	No
7.	Applicability	Wide	Selective	Wide	Very wide
8.	Problems encountered during operation	High	Low	High	High
9.	Matrix influences on enzyme	Yes	Yes	Yes	No
10.	Diffusional barriers to substrate and product molecules	Absent	Absent	Significantly large	Significantly large
11.	Protection from microbial attack	No	No	Yes	Yes
12.	Cost factor	Low	High	Moderate	High

1.2.4. Applications of Enzyme Immobilization in Pharmacy

Immobilised enzymes are used in pharmaceutical industries for manufacturing drugs. These enzymes can be used for:

1) Production of antibiotics: Immobilised enzymes are used for producing 6 -amino penicillanic acid, penicillins, cephalosporins, etc. on a commercial scale. This process is performed in a column, stirred tank, or a batch membrane reactor.

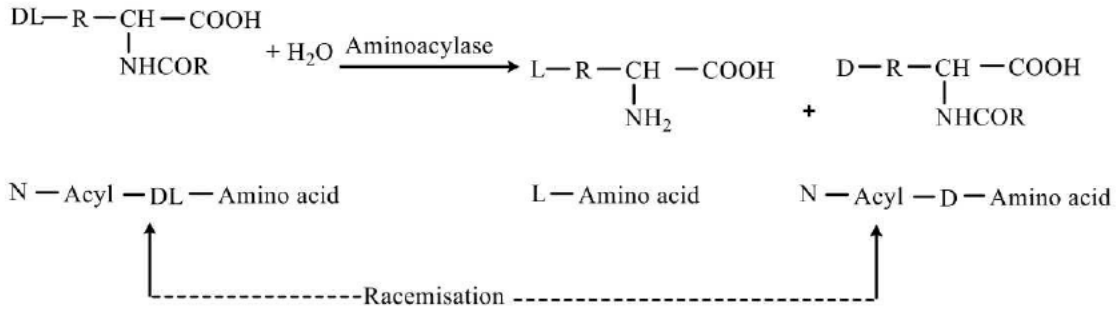
2) Production of steroids: Biocatalytic conversions are used in steroid transformations. Enzymes utilized for steroid transformations need cofactors. Whole cells of microorganisms with cofactors are immobilised to be used for large-scale steroid transformations. Hydrocortisone and prednisolone can be synthesized from cortexolone achieved by immobilizing the enzyme by entrapment with polyacrylamide. Whole cells of Corynebacterium simplex are used for A'-dehydration reaction; and for 11 {-hydroxylation reaction, mycelia of Curvularia lunata are used. Prednisolone can be produced from cortexolone by combining these immobilized systems.



3) Production of amino acids:

Pharmaceutical industries employ amino acids for various purposes. There are several existing large -scale processes employing immobilized enzymes. Production of amino acids by enzymatic resolution involving amino acid acylase and fructose syrups obtained from glucose isomerase is an example.

Optical Resolution of DL -Amino Acids: L-amino acid and unhydrolyzed acyl-D-amino acid is produced by the asymmetric hydrolysis of acyl-D L amino acid using aminoacylase. Both the products are separated through solubilisation. Unhydrolysed acyl -D-amino acid is further racemized to be reused in the resolution procedure.



4) Production of acids:

Immobilized enzymes yield better productivity of organic acids. There are many processes available for the production of acetic acid by Acetobacter aceti immobilised with porous ceramics, of citric acid by A. niger immobilized with calcium alginate, of lactic acid by L. casei immobilised with polyacrylamide, of L-malic acid by Bravibacterium flavum immobilized with carrageenan, of 12 - ketochenodeoxycholic acid by B. fuscum immobilised with carrageenan, of 2 - ketogluconic acid by Serratia marcescens immobilised with collagen, etc.

5) Production of other organic compounds:

Organic compounds that can be produced by immobilized enzymes are:

1) Coenzyme A: It can be produced continuously from pantothenic acid, L - cysteine, and ATP using Bravibacterium ammoniagenes cells immobilized on polyacrylamide gel.

2) Flavin Adenine Dinucleotide (FAD): It can be produced from flavin-mononucleotides and ATP using whole cells of Arthrobacter oxydans immobilized on a film of polyvinyl alcohol cross -linked with tetraethylsilicate. The F AD pyrophosphorylase activity is high in these cells.

3) Pyridoxol 5'-Phosphate: It can be produced from pyridoxine 5 '-phosphate using whole cells of Pseudomonas fluorescens 1mmobilised on a film of polyvinyl alcohol cross-linked with tetraethylsilicate. Pyridoxol 5'-phosphate has a high enzyme activity.

4) Adenine Arabinoside: The cells of Enterobacter aerogenes _ bear transglyosylation activity. These cells are immobilised with hydrophilic photocrosslinkable resin, and used to produce adenine arabinoside from uracil arabinoside and adenine in a proper water-organic solvent system.

5) Vitamin B-12: It can be produced using cells of immobilized Propionibacterium sp.

6) Dihydroxy Acetone: It is a pharmaceutical intermediate, and can be produced through glycerol oxidation with cells of Acetobacter xylinum immobilised on polyacrylamide gel.

7) Proinsulin: The plasmids of Bacillus subtilis cells having gene encoding for proinsulin can be immobilised on agarose beads. These immobilised cells can then be used for proinsulin produ ction in a small continuous stirred tank reactor.

8) Interleukin-2 and Monoclonal Antibodies: These can be produced using animal cells (lymphoblastoid MLA 144 cells and hybridoma cells) immobilised on agarose beads.

9) Prostaglandin: It can be produced from ar achidonic acids by using ram seminal microsomes immobilised on photocrosslinkable resin.

10) Ajmalicine Isomers: These can be produced from tryptamine using cells of the plant, Catharanthus roseus immobilised with calcium alginate.

11) Anthraquinones: These canbe produced by De novo synthesis from secologanin using cells of the plant, | Morinda citrifolia immobilized with calcium alginate.

6) Analytical applications:

Given below are the analytical applications of immobilised enzymes:

1) Enzyme Electrodes: These are probes which can generate an electrical potential when a reaction catalyzed by an immobilised enzyme (fixed on or around the probe) occurs. In automated analysis, immobilized enzymes are used for replacing the soluble enzyme in an existing automatic analyzer system.

2) Affinity Chromatography and Purification: Immobilised enzymes are also used in affinity chromatography. The species having a high affinity for the material to be removed from solution allows the purification or analysis of enzyme inhibitors, cofactors, antigens, antibodies, and other substances.

3) Biosensors: These are devices, probes, or electrodes having immobilized enzyme which on contact with a sample converts the presence of the desired analyte into physical, chemical or electrical signals which are measured. The sample concentration is measured in the form of electrical signals, or with a biological recognition system combined with an electrochemical transducer. Biosensors respond reversibly and specifically to the variation in the concentration of biochemical event of great practical utility.

BIOSENSORS

An analytical device used to change biological response into an electrical signal is called biosensor (figure 2.1). The term biosensor refers to sensor devices used for determining the concentration of su bstances and other biological parameters even where biological system is not directly involved.

Biosensors use a transducer to couple a biological sensing element with a detector. The first scientifically planned and successfully commercialized biosensors were the electrochemical sensors useful for multiple analytes.

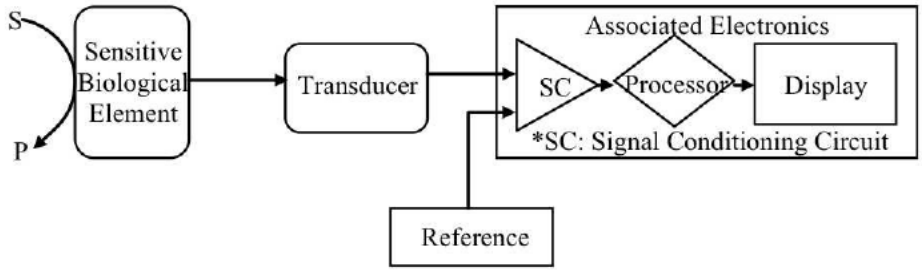


Figure 2.1: Schematic Diagram Showing Main Components of a Biosensor

The biosensor contains a biological sensing element (e.g., tissues, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids, natural products , etc.), a material obtained biologically(e.g., recombinant antibodies, engineered proteins, aptamers , etc.) or agents that mimic biological system (e.g., synthetic receptors, biomimetic catalysts, combinatorial ligands, imprinted polymers , etc.) either closely associated to or integrated in a

transducer.

2.1.2. Principle

The preferred biological material is generally a specific enzyme that is immobilized using conventional methods (e.g., physical or membrane entrapment, non- covalent or covalent binding) and brought in close contact with the transducer.

The analyte on binding to the biological material forms a bound analyte that produces a measurable electronic response. Sometimes due to the release of heat, gas (oxygen), electrons or hydrogen ions, the analyte converts into a product; and changes associated to this product is transformed by the transducer to electrical signals that are amplified and measured.

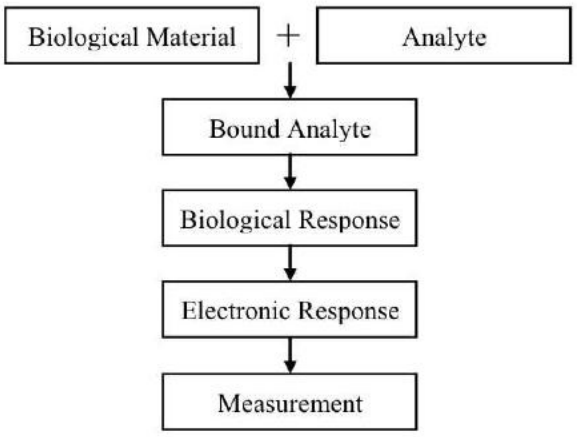


Figure 2.2: Measurement Flow for a Biosensor

2.1.3. Working

The electrical signal coming from the — transducer is low and superimposed by high and noisy baseline (could be due to high frequency signal component of random nature, or electrical interference generated in transducer electronic components). A baseline signal derived from a similar transducer without any biocatalyst membrane is called a reference baseline signal.

In signal processing, this reference baseline signal is subtracted from the sample signal; the signal difference obtained is amplified and the unwanted noise signals are electronically filtered (i.e. smoothened). The biosensor response is slow and eases the electrical noise filtration. The analogue signal produced directly is the output; however, it is converted to a digital signal, passed to a microprocessor for processing and manipulating the data to desired units , and then the output is displayed or stored.

2.1.4. Types

A biosensor is of the following different types based on the type of sensor devices and the biological materials:

1) Electrochemical Biosensor: It is a simple device used to measure electronic current, ionic or conductance changes carried by bio-electrodes.

2) Amperometric Biosensor: It determines the movement of electrons or electronic current due to a redox reaction catalysed by enzyme. Usually, a normal contact voltage moves along the electrodes to be analysed. In the enzyme-catalysed reaction, the substrate or product obtained can transfer the electrons with the surface of electrodes to be reduced; hence, an alternate current flow is measurable.

3) Blood Glucose Biosensor: It is employed extensively for diabetic patients. It contains a watch pen -shaped disposable electrode for single use. This electrode has glucose oxide and derivatives of a mediator (Ferrocene). The electrodes are converted using hydrophilic mesh.

4) Potentiometric Biosensor: It measures the changes in the concentration of ionic species withthe help of — ion-selective electrodes present in it. It generally employs pH electrodes, thus in the release of hydrogen ions a large amount of enzymatic reactions are involved.

5) Conductometric Biosensor: Many reactions occurring in the biological system bring about a change in the ionic species. This change is helpful in measuring the electronic conductivity. Urea biosensor which utilizes the immobilised areas is an example of conductometric biosensor.

6) Thermometric Biosensor: Several biological reactions involve production of heat and form the basis of thermometric — biosensors. The diagram representing a thermal biosensor consists of a heat insulated box fitted with a heat exchanger.

7) Optical Biosensor: It works on the principle of optical measurements, like fluorescence, absorbance , etc., and is utilis ed in fibre optics and optoelectronic transducers. Optical biosensor can even be safely used for non-electrical remote sensing of materials. Itis involved in enzymes and antibodies in the transducer elements. This biosensor generally does not require any reference sensor, and sampling sensor is used for generating comparative signals.

8) Fibre Optic Lactate Bio sensor: It measures the change in oxygen concentration at molecular level by identifying the effects of oxygen in fluorescent dye.

10) Optical Biosensor for Blood Glucose: In diabetic patients, the blood glucose level is important to be monitored. It is based on a simple technique in which paper strips saturated with reagents, like glucose oxide, Horseradish Peroxidase and a chromogen are used. The intensity of the dye colour is measured using a portable reflectance meter. The calorimetric test strips of cellulose layered with suitable enzymes and reagents are also widely used for testing blood and urine parameters.

10) Piezoelectric Biosensor: It is also called acoustic biosensor as its principle relies on sound vibrations. It contains piezoelectric crystals and the characteristic frequencies vibrate with the positively and negatively charged crystals. With the help of electronic devices, certain molecules on the crystal surface can be measured. The response frequencies can be changed by using these crystals with at tached inhibitors. For example , the b iosensor for cocaine (inthe gas phase) works by attaching the —- cocaine antibodies on crystal surface.

2.1.5. Applications in Pharmaceutical Industries

Biosensors aremade up ofa biological dement and a physiochemical detector used for detecting analytes. These devices have a wide range of applications in fields ranging from clinical to environmental to agricultural and to food industries. Given below are some of the fields in which biosensor technology is used

1. General healthcare monitoring,
2. Screening of diseases,
3. Clinical analysis and diagnosis of diseases,
4. Veterinary and agricultural applications,
5. Industrial processing and monitoring, and
6. Environmental pollution control.

Biosensors can be used for quantitative determination of numerous biologically important substances in body fluids , e.g., glucose, cholesterol, urea. Glucose biosensor is widely used for regular monitoring of blood glucose in diabetic patients. Also biosensors are used for blood gas monitoring for pH, pCO2 and pO2 during critical care and surgical monitoring of patients. Mutagenicity of a few chemicals can be determined by using biosensors. Presence of toxic compounds produced in the body can also be detected. Thus, biosensors possess many applications in biomedical sciences . Table 2.1 enlists some of the important applications:

Table 2.1: List of Biosensors with Their Applications

Transduction	Biosensor Type	Applications
Electrochemical	Acetylcholinesterase (AC hE) inhibition biosensors	Pesticidal study
	Hba1c biosensor	Determining glycated haemoglobin
	Piezoelectric biosensors	Detecting carbamate and organophosphate
	Uric acid biosensors	Diagnosis of various clinical abnormalities (**e.g.,** diagnosis of CVS diseases)
	Glucose oxidase electrode biosensors	Measuring of glucose in biological sample of diabetic patient
	Quartz-crystal biosensors	Detection of proteins at ultrahigh-sensitive level in liquids
Optical	Polyacrylamide-based hydrogel biosensors	Immobilisation of biomolecules
	Silicon biosensor	For biosensing, bioimaging, and in cancer therapy
	Microfabricated biosensor	In novel drug delivery (**e.g.,** in optical corrections)
	Fluorescence tagged or Genetically encoded biosensor	Investigation of vario us biological process and molecular systems in the cell
Electrochemical or optical	Nanomaterial biosensors	For diagnosis and in drug delivery

PROTEIN ENGINEERING

2.2.1. Introduction

The process of developing proteins having desired functions by manipulating their stability and specificity is termed protein engineering . This can be done by taking two main approaches, i.e., rational design and irrational design (or directed evolution). In the former case, knowledge of the protein structure and function is considered and a rational gene mutation is planned (figure 1).

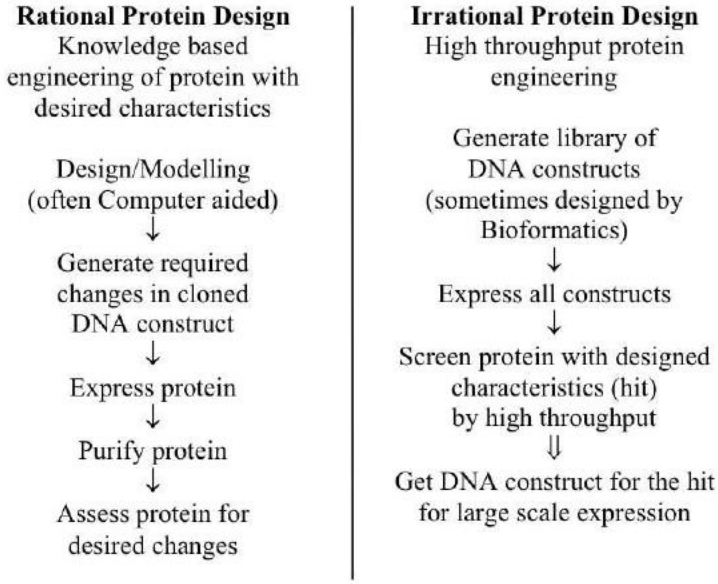


Figure 2.3: Two Different Approaches of Protein Engineering

This is done by making rationally designed changes in the gene of the protein cloned in expression vector of heterologous expression. The production of protein molecules is changed by site directed or site-specific mutagenesis of their genes. While there are cases in which protein structure is not available, and thus irrational design is required in which random changes (mutation) are made in the protein and a mutant with desired properties is selected.

2.2.2. Objectives

Enzymes, antibodies, hormones, receptor proteins, etc., are the protein classes chosen for engineering. Out of these, enzymes are the most widely used ones. Protein engineering mainly aims to get functionally more useful and efficient proteins and obtain economic gains from them.

Protein engineering has the objectives of:

1) Increasing the stability of enzymes,

2) Increasing the catalytic efficiency of enzymes,

3) Introducing new active sites and new catalytic activity in enzymes,

4) Altering pH dependence of enzymes,

5) Improving thermostability and modifying substrate specificity of enzymes,

6) Understanding structural and functional relationship for an enzyme for making predictions of actions,

7) Transferring the ability to make desirable enzyme in easy to cultivate and safer to use microbes,

8) Producing tailor -made enzymes in large quantities by increasing the expression of structural genes,

9) Producing hybrid enzymes,

10) Making isolation and purification of enzymes easy,

11) Making hormones resistant to attack by antibodies or stomach enzymes,

12) Getting more specific and potent biopharmaceuticals with altered pharmacological action, and

13) Getting humanized antibodies with less immunogenicity.

2.2.3. Increasing the Stability and Biological Activity of Proteins

The industrial applications or therapeutic uses of enzymes/proteins can be appropriately brought into use by increasing their half-lives or thermostability.

Proteins with enhanced stability can be obtained by the following methods:

1) Addition of disulfide bonds,

2) Changing asparagine to other amino acids,

3) Reducing the free sulfhydryl groups,

4) Single amino acid changes, and

5) Improving kinetic properties of enzymes.

2.2.3.1. Addition of Disulfide Bonds

Introduction of disulfide bonds significantly increases the thermostability of enzymes. The disulfide bonds added should not disturb the normal functioning of enzymes. The new protein obtained after the addition of disulfide bonds does not unfold at high temperatures and also does not denatures at non-physiological conditions (i.e., high pH and presence of organic solvents). These features of the new protein facilitate the use of certain enzymes for industrial applications.

T4 Lysozyme

This is an enzyme of bacteriophage T4. Disulfide bonds in T4 lysozyme were introduced by changing two, four or six amino acids (in close proximity) to cysteine residues to form one, two or three disulfide bonds, respectively. In the native T4 lysozyme, two cysteine residues (not held together by a disulfide bond) are present. By oligonucleotide - directed mutagenesis, cysteine residues created disulfide bonds between positions 3 and 97, 9 and 164, and 21 and 142 (numbered from N-terminal end) of the enzyme. On adding disulfide bonds, the folded structure and thermostability of the enzyme increases. Thus, it can be said that T4 lysozyme with three disulfide bonds has high stability and good biological activity.

Xylanase

This is an enzyme used for manufacturing paper from wood pulp. Xylanase should be catalytically active at high temperature. When added with disulfide bonds (one, two or three), it becomes thermostable and more functionally efficient.

2.2.3.2. Changing Asparagine to Other Amino Acids

The amino acids asparagine and glutamine undergo deamidation (i.e., release ammonia) to form aspartic acid and glutamic acid, respectively at high temperature. These alterations are associated with changes in the protein folding and loss of biological activity.

Triose Phosphate Isomerase

This is a dimeric enzyme with identical subunits, each having two thermosensitive asparagine residues which undergo deamidation. Oligo -nucleotide-directed mutagenesis was used for introducing threonine or isoleucine instead of asparagine to obtain a new thermostable enzyme. While on replacing the asparagine residues with aspartic acid, an enzyme unstable even at low temperature and having reduced activity is obtained.

2.2.3.3. Reducing the Free Sulfhydryl Groups

The presence of a large number of free sulfhydryl groups (contributed by cysteine residues) may lowe r the activity of proteins. In this case, the stability and activity of the protein or enzyme can be improved by reducing the number of sulfhydryl groups.

Human p-Interferon (IFN-B)

IFN-f is produced in E. coli by genetic engineering. Its antiviral activity was found to be only 10% of the original glycosylated form. It was found to exist as inactive dimers and oligomers. Cysteine residues were involved in intermolecular disulfide bonding, thus forming dimers and oligomers.

However, this is the case only in E. coli cells and not in human cells; and this problem was also overcome by replacing the cysteine residues with serine. The structures of these two amino acids (cysteine and serine) are also similar, with the only difference that the sulphur of cysteine has been replaced with oxygen in serine (and this consequently reduces free sulfhydryl groups). This process helps in the production of IFN-B with increased stability and good biological activity.

2.2.3.4. Single Amino Acid Changes

The stability and biological activity of recombinant proteins can be improved by a second generation variant. This is achieved by a single amino acid change.

Alpha-1-Antitrypsin

This amino acid binds to and inhibits the action of neutrophil elastase (an enzyme that damages the lung tissues , and causes emphysema, i.e., abnormal distension of lungs by air). In this process, a -antitrypsin is cleaved into serine and methionine (figure 2.4 a); and the free methionine is oxidised to methionine sulfoxide, thus making o,-antitrypsin a poor inhibitor of elastase. Methionine is replaced with valine (an oxidative -resistant variant of ayantitrypsin) (figure 2.4 b) and the new enzyme obtained is used for treating patients having genetic deficiency of a)-antitrypsin.

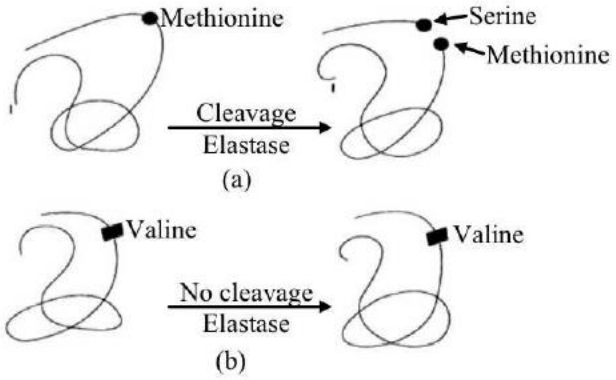


Figure 2.4: (a) Cleavage of α_1-Antitrypsin by Binding to Elastase; (b) No Cleavage Occurs when Methionine is Replaced by Valine

Insulin

In neutral solution, therapeutic insulin is present as zinc -containing hexamer. On introducing single amino acid substitutions, insulin exist s in monomeric state with good stability and biological activity.

Tissue Plasminogen Activator (tPA)

This is therapeutically use d for the lysis of blood clots causing myocardial infarction. The tPA has a shorter half -life of around 5 minutes, thus has to be administered recurrently. The half -life of tPA can be increased by replacing asparagine residue with glutamine as it is less glycosylated than asparagine, thus making a difference in the half-life of tPA.

2.2.3.5. Improving Kinetic Properties of Enzymes

The functional activities of enzymes can be improved by improving their kinetic properties (K ., specificity, etc.) through oligonucleoti de-directed mutagenesis. This is required for enzymes having industrial and therapeutic benefits.

Subtilisin

This is a serine protease enzyme secreted by gram _ -positive bacteria of Bacillus species. It is extensively used in industries as an enzyme detergent (i.e., cleaning agent in laundries). However, its large scale industrial use is restricted as it becomes inactive when the methionine lying close to the active site gets oxidised. This problem is overcome by replacing methionine with other amino acids. Subtilisin enzyme has also been used for genetic manipulations over the past 15 years. As a result of which about 50% of the native amino acids of this enzyme have been changed by in vitro mutagenesis, and most of the features of subtilisin (including its stability, substrate specificity, thermal and alkaline inactivation) has been altered.

Asparaginase

This enzyme is used for controlling leukaemia (i.e., uncontrolled growth of WBCs). On intravenous administration, asparaginase cleaves asparagine to aspartate (the reduced availability of asparagine restricts cell proliferation). Asparaginase obtained from different sources shows different effectiveness in controlling leukaemia due to their different kinetic properties. Asparaginase having alow Km (Michaelis constant) value has a high affinity for asparagines (hence more breakdown), thus should be selected to be used therapeutically for controlling leukaemia.

Tyrosyl t-RNA Synthetase

This enzyme is obtained from Bacillus stearothermophilus, and its Km value has been modified with regard to substrate binding. Tyrosyl t-RNA synthetase enzyme catalyses the following two reactions, to yield tyrosine t-RNA:

Tyrosine + ATP— Tyrosyladenylate + PP1

Tyrosyladenylate + t-RNA — Tyrosine t-RNA + AMP

Replacement of threonine with either alanine or proline has yielded variants of tyrosyl t-RNA synthetase. Alanine variant has a low Km value, thus possesses two fold binding affinity for ATP; while proline variant has a very low Km value, and thus ATP binds about 100-fold more tightly.

Restriction Endonucleases

This enzyme has been modified by oligonucleotide -directed mutagenesis. Most of the restriction endonucleases enzymes recognise the same DNA sequence for their action. There are only 200 different recognition sites, and thus an overlap in the recognition sites of several restriction enzymes has been observed. There are twotypes of restriction endonucleases, i.e., the frequent cutters which recognise a sequenceof 4 -6bp and rarecutters which recognise a sequence above 8 bp. The latter ones are more useful for producing large DNA fragments, and therefore protein engineering techniques have been utilized to modify the existing restriction endonucleases and produce rare cutters.

2.2.4. Applications

Protein engineering is used for cancer treatment studies. Pre –targeted radioimmunotherapy is a potential cancer treatment, as pre -targeting minimizes radiation toxicity by separating the rapidly cleared radionuclide and the long circulating antibody. Advances in protein engineering and recombinant DNA technology have increased the use of pre-targeted radioimmunotherapy. The use of novel antibodies as anti-cancer agents is also an important field, wherein the ability of antibodies to select the specific antigens with high affinity is employed; thus, protein engineering techniques are used for modifying the antibodies which can be targeted to cancer cells.

Protein engineering techniques are also used for producing therapeutic proteins. In 1996, recombinant protein production for therapeutic purposes was reviewed, and stated that protein engineering by mutation and deletion of fusion resulted in a second generation of therapeutic protein products with application - specific properties. Other studies on therapeutic protein production include single chain Fv designs for protein, cell and gene therapy; DNA shuffling and recursive genetic recombination studies to improve therapeutic proteins; development of secreted proteins such as insulin, interferon, erythropoietin as biotherapeutic agents; combinatorial protein biochemistry for therapeutics and proteomics; meganucleases and DNA double - strand break induced recombination for gene therapy; use of protein cationization techniques for future drug discovery and development; protein display scaffolds for protein engineering of new therapeutics; and polymer-based therapeutics for drug delivery and tissue regeneration.

Due to the advances in recombinant DNA technology, antibody engineering is possible. Combinational approaches (such as bacteriophage display libraries) have been introduced as an alternative to hybridoma technology for producing antibodies with desired antigen binding characteristi cs. Phage display has become a powerful technique in protein engineering, immunology, oncology, etc. Phage display of antibody fragments, mainly the production of artificial epitopes by phage antibodies is an important application. Antibody modelling studies to produce antibody - like molecules and increase their stability and specificity has also become common. Antibodies are also being

used as vectors for molecular imaging. Pharmacokinetic properties of antibodies have been improved by protein engineering and antibody variants of different size and antigen binding sites have been produced to be used as imaging probes specific to target tissues. Examples include antibody fragments conjugated to bioluminescence, fluorescence, quantum dots for optical imaging, and iron oxide nanoparticles for magnetic resonance imaging.

Use of Microbes in Industry

Enzymes can be produced commercially making use of various microbes, which are the most significant and convenient s ources. Rich quantities of enzymes can be produced under suitable growth conditions using the microorganisms. They can be produced using inexpensive media within a short time period. It is also easy to manipulate the microorganisms in genetic engineering techniques so that the production of desired enzymes can be increased. The processes of recovery, isolation, and purification are easy with microbial enzymes in comparison to that with enzymes obtained from animals and plants.

Most of the enzymes used indu _ strially are obtained from microbes. A large number of fungi, bacteria, and yeasts are used for this purpose. For example, Aspergillus niger (a fungus) is an organism used extensively for the production of bulk enzymes. There are around 40 commercial enzymes suitably produced by A. niger, like a-amylase, cellulase, protease, lipase, pectinase, phytase, catalase, and insulinase.

Production of Enzymes

The production and use of enzymes is a major part in biotechnology. Various fields like microbiology, chemistry, process engineering, and biochemistry have contributed extensively in the growth of enzyme technology. Microbial enzymes are in use since many centuries even if complete information on them was not known. In 1896, in US, taka-diastase (a fungal amylase) was produced; this was the first industrially produced enzyme, and was used as a pharmaceutical agent for the treatment of disorders related to digestion.

Table 1.4 enlists some of the enzymes obtained from microbial sources, along with their applications:

Table 1.4: List of Industrially (Microbially) Produced Enzymes, Their Sources and Applications

Enzymes	Sources	Applications
α-Amylase	*Aspergillus oryzae* *Aspergillus niger* *Bacillus subtilis* *Bacillus licheniformis*	Production of beer and alcohol. Preparation of glucose syrups. As a digestive aid. Removal of starch sizes.
Amyloglucosidase	*Aspergillus niger* *Rhizopus niveus*	Hydrolysis of starch.
Cellulase	*Aspergillus niger* *Trichoderma koningi*	Production of a lcohol and glucose.
Glucoamylase	*Aspergillus niger* *Bacillus amyloliquefaciens*	Production of beer and alcohol. Hydrolysis of starch.
Glucose Isomerase	*Arthrobacter* sp. *Bacillus* sp.	Manufacture of high fructose syrups.

Glucose Oxidase	*Aspergillus niger*	Antioxidant in prepared foods.
Invertase	*Saccharomyces cerevisiae*	Inversion of sucrose. Preparation of artificia l honey, confectionaries.
Keratinase	*Streptomyces fradiae*	Removal of hair from hides.
Lactase	*Kluyveromyces* sp *Saccharomyces fragilis*	Hydrolysis of lactose. Removal of lactose from whey.
Lipase	*Candida lipolytica* *Asperigillus niger*	Preparation of cheese. Production of flavour.
Pectinase	*Aspergillus* sp *Sclerotina libertina*	Clarification of fruit juices and wines. Production of alcohol and concentration of coffee.
Penicillin Acylase	*Escherichia coli*	Production of 6-aminopenicillanic acid.
Penicillanase	*Bacillus subtilis*	Removal of penicillin.
Protease, Acid	*Aspergillus niger*	As a digestive aid. As a substitute for calf rennet.
Protease, Neutral	*Bacillus amyloliquefaciens*	As a fish and meat tenderiser.
Protease, Alkaline	*Aspergillus oryzae* *Streptomyces griseus* *Bacillus* sp	As a meat tenderiser. As a detergent additive. As a beer stabiliser.
Pullulanase	*Klebsiella aerogenes*	Hydrolysis of starch.
Takadiastase	*Aspergillus oryzae*	As a supplement to bread. As a digestive aid.

General considerations:

Figure 1.8 represents the flow chart for enzyme production by microorganisms:

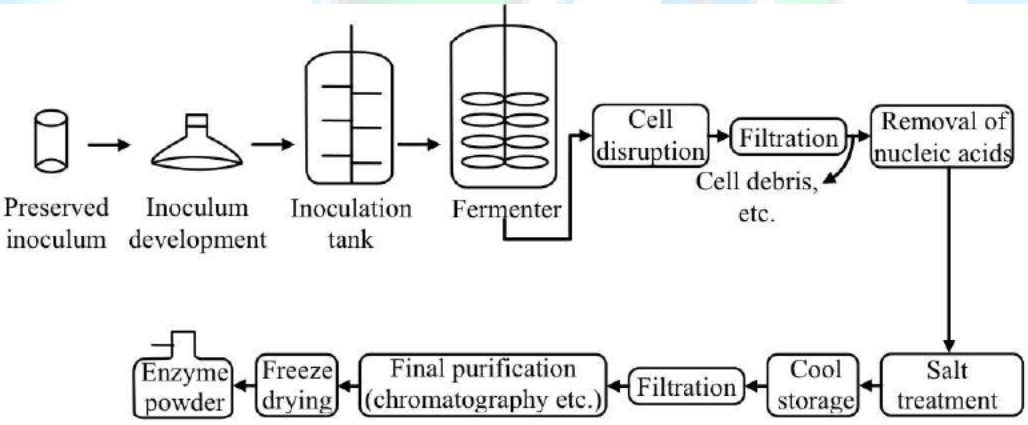


Figure 1.8: Flow Chart for the Production of Enzymes by Microorganisms

The techniques for microbial production of enzymes are comparable to the methods for manufacturing other industrial products. The salient features are discussed below:

1) Selection of Organism: Such microorganisms should be selected that can produce maximum quantities of desired enzymes within a short time period. Also, the metabolites produced by them should be minimal. After selecting the microorganism, strain improvement is done using mutagens and UV rays for optimising the enzyme production. From the selected microorganism, inoculum is prepared in a liquid medium.

2) Formulation of Medium: The selected culture medium should have all the nutrients that provide adequate support to microbial growth so that enzymes can be produced in sufficient quantities. The medium ingredients should be nutritionally safe, easily available, and of low cost. Starch hydrolysate, molasses, corn steep liquor, yeast extract, whey, soy bean meal, some cereals (wheat), and pulses (peanut) are some of the substrates used commonly for the medium. The medium pH should be optimal for adequate microbial growth and enzyme production.

3) Production Process: Industrial production of enzymes is carried out either by submerged cultured liquid or solid-substrate fermentation (to a lesser extent) technique. The former method is preferred as the yields are more and the chances of infection are less. However, the latter method was used traditionally and is stillusedint he production of fungal enzymes, e.g. amylases, cellulases, proteases, and pectinases.

The medium is sterilised by batch or continuous sterilisation techniques. Fermentation is started by inoculating the medium. Optimum levels of growth conditions (e.g., pH, temperature, oxygen supply, and nutrients) should be maintained. Anti-foaming agents are added to minimise froth formation. Production of enzymes is carried out by batch fermentation process (mainly) and by continuous process (to a lesser extent). The bioreactor system should be sterile during fermentation, which is carried out for 2 -7 days in most of the production processes. Apart from the desired enzymes, some other metabolites are also produced. Thus, the enzymes should be recovered and purified.

4) Recovery and Purification of Enzymes: The desired enzymes are either excreted into the culture medium (and are termed extracellular enzymes) or are present within the cells (and are termed __ intracellular enzymes). The commercial enzymes can be either crude o r highly purified or in the solid or liquid form as per the requirement. The steps involved in recovery and purification processes depend on the enzyme nature and the desired degree of purity. An extracellular enzyme present in the broth can be recovered easily in comparison to the recovery of an intracellular enzyme. The intracellular enzymes can be released by disrupting the cell using special techniques.

Microbial cells can be disrupted physically by methods like sonication, and using high pressure and g lass beads. The bacterial cell walls can be lysed by using lysozyme. Yeast cells can be lysed using B-glucanase enzyme. The most important consideration is to minimise the loss of desired enzyme activity.

1.2.5.3. Amylase

Amylase is an enzyme that breaks down the starch into sugar. It is present in human saliva, where it begins the process of chemical digestion. Amylase was the first enzyme to be discovered and isolated. All the amylases are glycoside hydrolases and act on a-1,4-glycosidic bonds. They are specifically used in the industrial starch c onversion process. The g-amylase is obtained from plants (barley and rice), animals, or microbes. Cassava mash wastewater is also a source of a-amylase. This enzyme shows its activity in a wide range of pH and temperature. Table 1 .5 enlists the microbial s ources and methods used for producing a-amylase:

Table 1.5: Microbial Sources and the Mode of Fermentation Used for Production

Bacterial Sources	Methods
Bacillus amyloliquefaciens	SSF
Bacillus licheniformis	SSF
Bacillus coagulans	SSF
Chromohalobacter sp. TVSP-101	
Bacillus polymyxa	SSF
Bacillus mesentericus	SSF
Bacillus vulgaris	SSF
Bacillus megaterium	SSF
Bacillus licheniformis GCB-U8	SmF
Bacillus sp. PS-7	SSF
Bacillus licheniformis M27	SSF
Halobacillus sp. MA-2	SmF
Halomonas meridiana	SmF
Rhodothermus marinus	SmF
Bacillus cereus MTCC 1305	SSF
Fungal Sources	**Methods**
Aspergillus oryzae	SSF
Penicillium fellutanum	SmF
Thermomyces lanuginosus	SSF
Aspergillus niger	SSF, SmF
Penicillium roqueforti	SSF
Streptomyces rimosus	SSF, SmF
Aspergillus kawachii	SSF, SmF
Penicillium chrysogenum	SSF
Penicillium janthinellum (NCIM 4960)	SSF
Aspergillus awamori	SmF
Pycnoporus sanguineus	SSF

SSF-Solid State Fermentation
SmF-Submerged Fermentation

The B-amylase (or a-1,4-glycanmaltohydrolase) invariably belong to the plant origin. This enzyme can also be produced from microorganisms, __e.g., Bacillus polymyxa, Bacillus cereus, Bacillus megaterium, Streptomyces sp., Pseudomonas sp., and Rhizopus japonicus.

Production of Amylase

a-Amylase can be commercially produced by submerged fermentation and solid state fermentation techniques. The former is a traditional method of enzyme production from microbes, while the latter is a new method.

Submerged Fermentation (SmF) utilises free flowing liquid substrates, such as molasses and broths. The products obtained from fermentation are secreted into the fermentation broth. Since the substrates are rapidly utilised, they

need to be constantly replaced. This fermentation technique employs bacteria that require high moisture content for their growth. SmF is used for the extraction of secondary metabolites to be used in liquid form. This method allows the utilisation of genetically modified organisms to a greater extent. The medium can be sterilised and the end products can be purified easily. The process parameters like temperature, pH, aeration, oxygen transfer, and moisture can also be easily controlled. Solid State Fermentation (SSF) employs microbes that require less moisture content for their growth. Bran, bagasse, and paper pulp are the commonly used solid substrates. In this method, the nutrient -rich waste materials can be easily recycled and further used as substrates. The substrates are not utilised rapidly (like the SmF technique); rather they are used very slowly and steadily so that the same substrate can be used for a longer duration, and the need to constantly supply substrate is also eliminated.

Some other advantages of SSF technique over SmF technique are that it requires simpler equipments, has higher volumetric productivity, yields higher concentration of products, and generates lesser effluent. Thus, SSF is considered a promising method for commercial production of enzymes.

Importance of Amylase

1) It is used for the productio n of sweeteners in food industries, e.g., glucose syrup, high mannose sugar, high-fructose syrup, etc.

2) Dextrins (short -chain polymers) are produced by the hy _ drolysis of starch with amylase followed by maltose (disaccharide) and ultimately glucose (monosaccharide).

3) It has a good heat stability.

4) It does not undergo browning reactions.

5) It is used for converting glucose into fructose.

6) It is used commercially for the preparation of sizing agents, starch sizing pastes for use in paper coatings, for the removal of starch sizing from woven cloth, and for the liquefaction of heavy starch pastes.

1.2.5.4. Catalase

Catalase is an enzyme that catalyses the reaction by which hydrogen peroxide decomposes into water and oxygen ($2H_2O_2$ ----$\overset{Catalase}{\longrightarrow}$---> $2H_2O + O_2$). It is found in the organisms living in the presence of oxygen. It is a hemoprotein with 4 ferri-protoporphyrins prosthetic groups and has a molecular weight of 250,000 daltons.

Source: Commercially, catalase is prepared from Aspergillus niger, Penicillium vitale, or Micrococcus lysodekiticus. The optimum pH for the growth of the organisms is in the range of 3 to 9. Substances such as cyanides, phenols, alkali, urea interfere with the growth of the organisms.

Culture medium: The culture medium consists of L-cysteine, DL-methionine, Calcium carbonate along with agar, tryptone, dextrose and sodium chloride. Potassium dihydrogen phosphate is used for adjusting the pH of the medium to promote the growth of bacteria. Concentrated medium yields more catalase when compared to dilute medium. Glucose oxidase is obtained as a byproduct which should be removed during the fermentation along with catalase.

Technique: The inoculum is introduced into the inoculation tank and the culture is allowed to grow for a period of 12-15 hours which is then transferred to fermentation tank. Fermentation is carried out using deep tank cultivation with continuous agitation for a period of 24-36 hours. Aeration is critical for the optimum growth of the culture.

Extraction: The solid biomass is removed by using filtration and centrifugation. Catalase is adsorbed by using adsorbents like alumina or kaolin or charcoal. Finally, it is crystallized using organic solvents such as ethanol (30-40%) enriched with Potassium chloride and sodium chloride.

Uses: The enzyme prevents the accumulation of peroxide (continuously produce d by various metabolic reactions) and also prevents the damage of cellular organelles and tissues by peroxide. In mammals, liver is the rich source of catalase. Catalase is used in industries for various purposes. In food industries, it is used along with other enzymes for preserving foodstuffs and for manufacturing beverages and other food items. Commercial catalases are used for breaking down hydrogen peroxide in waste water.

1.2.5.5. Peroxidase

Peroxidase is an enzyme that decomposes hydrogen peroxide by the oxidation of wide range of phenolic and non -phenolic substrates (RH). They are abundantly found in bacteria, fungi, algae, plants, and animals. Peroxidase enzyme is classified in to heme and non -heme peroxidase based on the presence or absence of heme , respectively . More than 80% of known peroxidase genes can code for heme peroxidase; while the non -heme peroxidases, e.g., thiol peroxidase, alkylhydroperoxidase, and NADH peroxidase constitute only a small proportion.

Heme peroxidase enzyme is further assigned to two superfamilies:

1) The Peroxidase-Cyclooxygenase Superfamily (PCOXS): This superfamily contains animal peroxidase _—_ involved in the innate immunity, defense responses, etc. The Myeloperoxidase (MPO), Eosinophil Peroxidase (EPO), Lactoperoxidase (LPO), and Thyroid Peroxidase (TPO) belong to this family. In this superfamily, the prosthetic heme group is covalently bonded to the apoprotein.

2) The Peroxidase -Catalase Superfamily (PCATS): This is the most intensively studied superfamily of non-animal heme peroxidase.

Source: It is obtained from Klebsiella and Ensifer adherens

Culture media: Agar, peptone, yeast extract, Potassium dihydrogen phosphate, Magnesium sulphate.

Technique: Solid state fermentation at 30 ^{0}C for a period of 48 hours with continuous agitation (140 rpm)

Extraction: The solid residues are removed by filtration and centrifugation. The pH is adjusted to 9 and the enzyme peroxidase is obtained by solid-liquid separation method.

Importance of Peroxidase

Application as Biosensor

1) Horseradish Peroxidase (HRP) has been used extensively in the development of biosensors.

2) HRP biosensors are made by applying various detection methods (such as amperometric immunosensor, mass balance, potentiometric methods, photovoltaic spectroscopy, optical and chemiluminescent methods, etc.).

3) Potentiometric based biosensors are developed by combining peroxidase enzyme with a transducer that can detect variations in protons.

4) Glucose, maltose, or lactate can be detected by peroxidase based biosensor using potentiometric methods.

HRP-based biosensors for antioxidant monitoring are used in the detection of superoxide radical, nitric oxide, glutathione, uric acid , and phenolic compounds.

Analytical Applications

1) Enzyme Linked Immunosorbent Assays (ELISA) technique is used for detecting antigens or antibodies by producing colour changes catalysed by peroxidase enzyme. In ELISA, an enzyme -linked antibody specific t o the antigen is used along with a chromogenic substrate, which gives a coloured product in the presence of the enzyme; the product colour is proportional to the amount of the desired antigen/antibody. The most commonly used enzyme for linking with antibody is HRP.

2) This enzyme is used for screening monoclonal antibodies against dangerous mycotoxins of various fungal species like Aspergillus, Penicillium (ochratoxins), Fusarium (T-2 toxin, trichothecenes), etc.

3) Peroxidase enzyme is also used in the detection of Gonyautoxin, Chlamydia, Fusarium toxin, Dengue virus proteins, and Hepatitis-E virus.

4) HRP-based immunoassays are also used for the detection of undeclared milk proteins (e.g., bovine B-lactoglobulin) in foods.

Application in Analytical and Diagnostic Kits

1) Peroxidase is also used in the development of analytical and diagnostic kits.

2) HRP is most commonly used for analytical purposes. Other plant peroxidase having wide pH and temperature stability are also being used now.

3) Peroxidase enzyme can produce stable chromogenic products, thus are suitable for manufacturing various diagnostic kits based on enzyme conjugated antibody technology.

4) Turnip root peroxidase has been used in uric acid detection kits.

5) Peroxidase enzyme combined with cholesterol oxidase and cholesterol esterase, is used for developing cholesterol detection kits that aid in the quantification and monitoring of human serum cholesterol.

6) Peroxidase enzyme is also usedi _n developing kits for the diagnosis of bladder and prostate cancers through the detection of 8 - hydroxydeoxyguanosine and its analogues in urine.

7) Lactose-containing monitoring strips for determining hypoxia and ischemia have been developed to be used in com bination with immobilized B-galactosidase, galactose oxidase, and HRP enzymes.

8) In biomedical sectors, peroxidase -[AA based Gene-Directed Enzyme/Prodrug Therapy (GDEPT) has been used for cancer treatment. This system of enzyme _ -prodrug is effective against hy poxic and anoxic tumour cells and also has anti-cancer potential.

9) Antibody-Directed Enzyme/ Prodrug Therapy (ADEPT), specific HRP - conjugated antibodies are also used.

1.2.5.6. Lipase

Lipase is an enzyme that breaks down dieta ry fats into fatty acids and glycerol. The cells in stomach produce a small amount of gastric lipase, which helps in the digestion of butter fat in food.

Lipase is extensively found in the pancreas, which produces pancreatic lipase (or steapsin) that acts in the small intestine. The bile made in the liver is released into the intestine and converts dietary fat into small fatty globules. Pancreatic lipase acts on these fat globules and convert s them into fatty acids and glycerol (small, energy-dense molecules used by the cells). Fatty acids and glycerol travel in the blood and lymph vessels to reach all the body parts.

Importance of Lipase

1) Lipase mainly helps in the body processes and absorbs fat.

2) It manages triglycerides (a form of fat required for energy, thus some levels of triglycerides should be present in the body). Lipase helps in maintaining the levels of triglycerides in the body by breaking them down into smaller molecules to be used by the body for energy.

3) Microbial lipase is used to obtain PUFAs from animal and plant lipids (e.g., menhaden oil, tuna oil, and borage oil); and its mono and diacylglycerides are used to produce various pharmaceuticals.

4) PUFAs have metabolic benefits, thus are used as food additives, pharmaceuticals, and nutraceuticals.

5) Some of the PUFAs are required for the normal synthesis of lipid membranes and prostaglandins.

6) Profens is a class of NSAIDs and is synthesised as pure (s) -ibuprofen using lipase-catalysed kinetic resolution via hydrolysis and esterification. Efficient kinetic resolution processes using lipase are available for the preparation of optically active homochiral intermediates to synthesise nikkomycin-B, NSAIDs (naproxen, ibuprofen, suprofen, and ketoprox), lamivudine (anti -viral), and for the enantiospecific synthesis of alkaloids, antibiotics, vitamins, anti-arteriosclerotic, anti-tumour and anti-allergic compounds.

7) Novel biotechnol ogy tools, like enzyme immobilis ation, have also been applied for the isolation and incorporation of food components in ordinary foods. Many nutraceuticals have been synthesised by employing immobilized lipases, such as those from C. Antarctica and Lactobacillus reuteri .

1.2.5.7. Protease

Protease (or proteinase or proteolytic enzymes) is an enzyme whh hydrolyses (i.e., breakdown) the peptide bonds —-CO—-NH—] joining the array of amino acids in a protein. Thus, the protein is cleaved into amino acids (its basic building blocks). It differs in its ability to hydrolyse various peptide bonds. Each type protease breaks a specific kind of peptide bond. Fungal protease, pepsin, trypsin, chymotrypsin, papain, bromelain, and subtilisin are somexamples of protease enzyme. Proteases are produced by various bacteria, for example, species belonging to Bacillus; Pseudomonas, Clostridium, Proteus, and Serrata; and by various fungi, such as Aspergillus niger, Aspergillus oryzae, Aspergillus flavus, and Penicillium roquefortii. However, the enzymes intimately associated with the above microorganisms are mixtures o f proteinases and peptidases; and the former are excreted into the fermentation medium during the growth phenomenon, while the latter are liberated during autolysis of the cells.

Protease enzymes are classified according to the sites at which they catalyse the cleavage of proteins. The two major groups are the exopeptidases targeting the terminal ends of proteins and the endopeptidases having target sites

within the proteins. Endopeptidases, e.g., aspartic endopeptidases, cysteine endopeptidases, glutamic endopeptidases, metalloendopeptidases, serine endopeptidases, and threonine endopeptidases act via different catalytic mechanisms.

Source: Commercially proteases are obtained from Bacillus anthracis, B. thermoprotelyticus, Streptomyces griseus, Aspergillus oryzae, A. saitoi, Mucor pucillus.

Culture medium: The culture medium ideal for obtaining maximum enzyme content is Fish meal-enzose-cerelose medium

Technique: Surface culture technique (48-72 hours)

Extraction: Filtration and solid liquid separation

Importance of Protease

1) Protease enzymes are required in digestion as they breakdown the peptide bonds in the protein foods t o release the amino acids which are utilised by the body.

2) They have also been used in various forms of therapy as they are beneficial in oncology, inflammatory conditions, blood rheology control, and immune regulation.

3) Protease can hydrolyse most of the proteins as long as they are not components of living cells.

4) Heavy metals (such as lead and mercury) bind to ionisable or sulfhydryl groups of proteins and exert their poisoning effect. On binding to an essential functional protein, such as an enzyme, they denature it and/or inhibit their activity. This interaction between the heavy metals and proteins lead to degenerating diseases, nerve damage, or even death. It has been observed clinically that high intake of oral protease has decreased the heavy metal concentrations in the blood. The protease enzyme binds to o-macroglobulin and forms an activated complex which alters the binding affinities and increases the clearance rate by the liver.

1.2.5.8. Penicillinase

Penicillinase is a bacterial enzyme that inactivates most of the penicillins. It is an extracellular enzyme produced by the members of the Coliform group of bacteria, by the Bacillus species, and some of the strains of Staphylococcus. The enzyme hydrolyses the pen icillin into penicilloic acid, i.e., a dicarboxylic acid (figure 1.9). Penicillinase obtained from B. subtilis and B. cereus is industrially produced and exerts action to some extent in the removal of penicillin via specific inactivation.

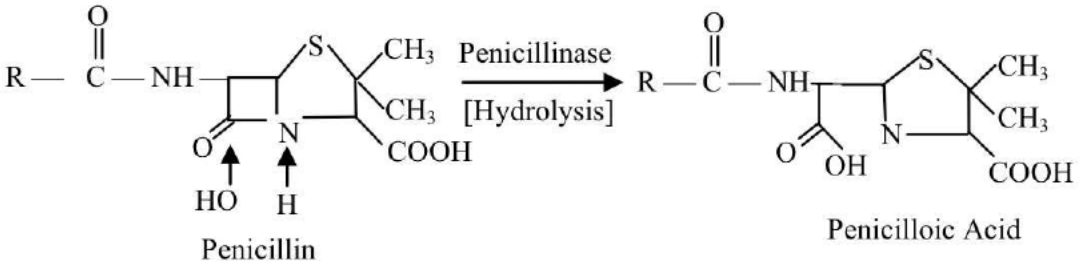


Figure 1.9: Hydrolysis of Penicillin to Penicilloic Acid by Penicillinase

Penicillinase has been categorised into two major classes based on its activity:

1) Penicillin amidase or penicillin acylase, and

2)Beta -lactamase or penicillinase.

Penicillinase inhibits the therapeutic activity of penicillins. Penicillin amidase enzyme specifically attacks the acyl group attached to the basic nucleus, i.e.,6 - aminopenicillanic acid (figure 1.10), thus is also named as penicillin acylase.

This enzyme is more specific against penicillin V and K. The 6-lactamase enzyme acts on the basic nucleus by breaking the Beta-lactam bond and producing penicilloic acid. This enzyme is more specific against penicillin G and X, and to a lesser extent against penicillin V.

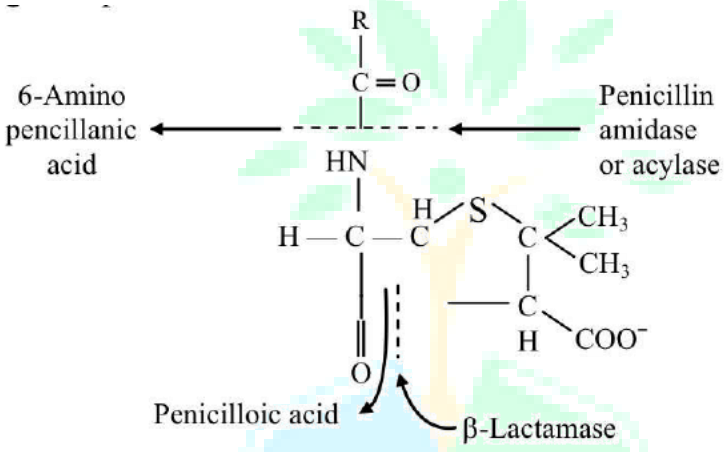


Figure 1.10: Structure of Penicillin and its Enzymatic Conversion to 6-Aminopenicillanic Acid and Penicilloic

Source: Commercially penicillinase is produced from alkalophilic bacteria such as Bacillus cereus, B. megatherium, E.coli.

Culture medium: Glucose, polypentones, yeast, KH_2PO_4, Yeast extract, Magnesium sulphate, Agar. Temperature is maintained at 30^0C. Inducers like cloxacillin, amoxicillin increase the output of penicillinase.

Technique: Fermentation is carried out by using shake flask technique where the starter culture is agitated for 16-20 hours which is then transferred into fermentation tank and allowed to settle for 24 hours. The growth of the bacteria is measured by taking a small sample of the culture and obtaining its optical density at 610 nm using colorimeter.

Extraction: The pH of the final mixture is adjusted to 6-7 to disrupt the bacteria. The obtained biomass is filtered and centrifuged. The amount of penicillinase is estimated by using iodometry.

Importance of Penicillinase

1) Penicillinase is a narrow spectrum f-lactamase which opens the f-lactam ring and inactivates Penicillin G and some closely related congeners.

2) Majority of Staphylococci and some strains of Gonococci, B. subtilis, E. coli, H. influenza, and few other bacteria produce penicillinase. The gram-positive penicillinase produces large quantities of the enzyme which diffuses into the surroundings and protects other inherently sensitive bacteria. The gram - positive penicillinase produces small quantities of the enzyme found between the lipoprotein and peptidoglycan layers of the cell wall.

3) Some resistant bacteria become penicillin tolerant and not penicillin destroying. Their target enzymes are altered to have low affinity for penicillin, e.g., highly resistant Pneumococci isolated in some areas have altered Penicillin Binding Proteins (PBPs).

4) The Methicillin Resistant S$. aureus (MRSA) has acquired a PBP which has a very low affinity for B-lactam antibiotics.

5) The low level penicillin resistant Gonococci are less permeable to the drug, while high degree resistant ones produce penicillinase, as do highly resistant H. influenzae.

6) The gram -negative bacteria have porin cha nnels formed by the proteins in their outer membrane. Various B-lactam antibiotics have different permeability through these channels. Ampicillin and other members active against gram -negative bacteria cross the porin channels much better than Penicillin G .Some gram -negative bacteria become resistant by loss or alteration of porin channels.

GENETIC ENGINEERING

2.3.1. Introduction

Genetic engineering involves deliberate DNA manipulation in organisms to alter their genes. Although the organisms whose genes are _ being altered may not be microbes, but the substances and techniques involved are obtained from microbes and adapted for use in more complex organisms.

2.3.2. Historical Background

The term genetic engineering initially was used for various techniques used for modifying or manipulating organisms through heredity and reproduction processes. Genetic engineering involves artificial selection and also all the interventions of biomedical techniques (artificial insemination), in vitro fertilisation (e.g., test-tube babies), cloning, and gene manipulation. In the 20th century, the term genetic engineering was used to indicate more specific methods of recombinant DNA technology (or gene cloning), in which DNA molecules obtained from two or more sources are combined in vivo (within cells) or in vitro (outside cells) and then inserted into the host organisms forpropagation. The techniques of recombinant DNA technology were developed with the discovery of restriction enzymes by Werner Arber (a Swiss microbiologist) in 1968. The next year Hamilton O. Smith (an American microbiologist) purified type II restriction enzymes having the ability to cleave a specific site within the DNA (in contrast to type I restriction enzymes that cleave DNA at random sites), and thus essential to genetic engineering. Daniel Nathans (an American molecular biologist) helped in modifying the DNA recombination technique in 1970-71 and demonstrated that type II enzymes could be useful in genetic studies. Genetic engineering based on recombination was p ioneered in 1973 by Stanley N. Cohen and Herbert W. Boyer (American biochemists), who were the first to cut DNA into fragments, rejoin different fragments, and insert the new genes into E. coli bacteria, which then reproduced.

2.3.3. Basic Principles of Genetic engineering:

Gene cloning involves inserting a specific piece of “desired DNA’ into a host cell in such a manner that the inserted DNA is replicated and handed onto the daughter cells during cell division. The factors involved in gene cloning are:

1) Isolation of the gene to be cloned.

2) Insertion of the gene into a vector (piece of DNA) which allow it to be taken by bacteria and replicate within them as the cells grow and divide.

3) Transfer of the recombinant vector into bacterial cells by transformation or infection with viruses.

4) Selection of the cells containing the desired recombinant vectors.

5) Growth of the bacteria, that can be continued indefinitely, to give the required cloned DNA.

6) Expression of the gene to get the desired product.

2.3.4. Process

The recombinant DNA technology mainly involves insertion of foreign genes into the plasmids (small rings of DNA) of common laboratory strains of bacteria. Plasmids are not the part of bacterium's chromosome (the main source of genetic information in the organisms). However, they can direct protein synthesis, and are reproduced and passed on to the bacterium's progeny (like chromosomal DNA). Thus, foreign DNA (e.g., a mammalian gene) can be inserted into a bacterium to obtain immeasurable number of copies of the inserted gene. Also if the inserted gene has the ability to direct protein synthesis, the modified bacteritum will produce the protein specified by the foreign DNA.

A subsequent generation of genetic enginee ring techniques that emerged in the early 2is^{t} century centred on gene editing. Gene editing is based on CRISPR-Cas9 technology, and the researchers by using it can customise a living organism's genetic sequence by making specific alterations in its DNA Gene editing can be used for the genetic modification of crop plants and livestock and of laboratory model organisms (e.g., mice). It is also used in gene therapy for humans as it can correct the genetic errors associated with disease in animals.

2.3.5. Applications

Genetic engineering has improvised the understanding of many theoretical and practical aspects of gene function and organisation. Through recombinant DNA techniques, researchers can produce bacteria which can synthesise human insulin, human growth hormone, a-interferon, hepatitis B vaccine, and other medically useful substances. Plants can be genetically altered to enable them to fix nitrogen. Genetic diseases can be corrected by replacing the dysfunctional genes with normally functioning genes.

However, special attention has been paid on such achievements as they might introduce unfavourable and dangerous traits into microorganisms that were earlier free of them, e.g., resistance to antibiotics, production of toxins, or a tendency to cause disease. Genetic engineering in humans has raise ethical concerns regarding its use to alter traits, like intelligence and beauty.

Genetically Modified Organism s (GMOs or transgenic organism s) contain genes from different organisms, and are the sources of:

1) DNA: GMOs can be made such that a DNA piece can be easily replicated, thus providing a large source of that DNA. For example, a gene associated with breast cancer can be cut into the genome of E. coli , allowing the rapid production of the gene so that it may be sequenced, studied, and manipulated, without requiring repeated tissue donations from human volunteers .

2) RNA: Antisense RNA is ssRNA that is complementary to the mRNA that will code for a protein. In cells, itis made to control target genes. The use of antisense RNA for preventing diseases caused by the production of a particular protein is increasing.

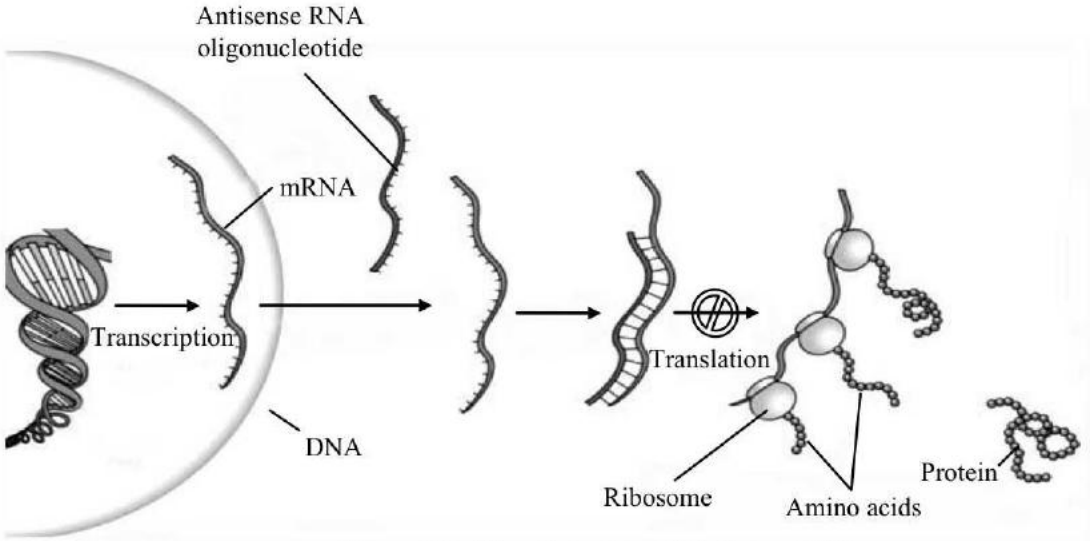


Figure 2.5: Manufacturing RNA Using GMOs

3) Protein: Since microbes replicate rapidly, it can be advantageous to use them for manufacturing the desired proteins. Given the right promoters, bacteria will express genes for proteins not naturally found in bacteria, such as cytokine. Genetically engineered cells are used to make various proteins essential for humans, e.g., insulin or human growth hormone.

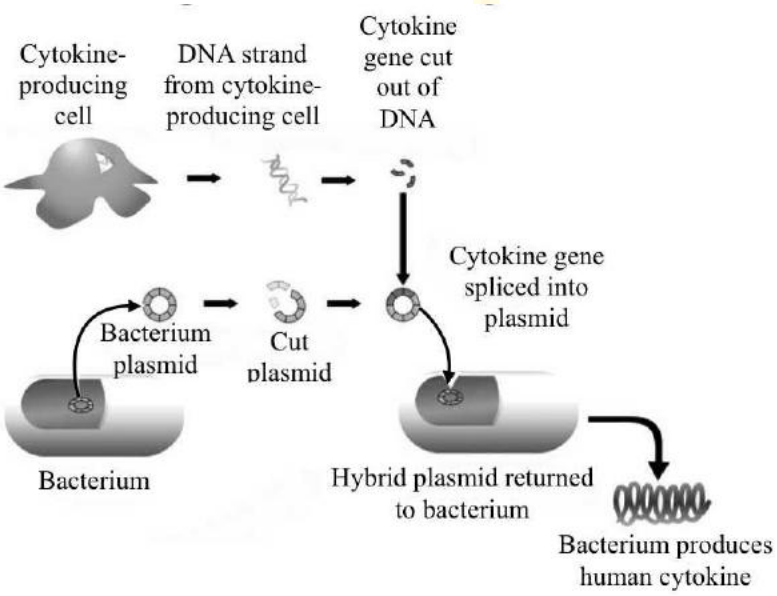


Figure 2.6: Manufacturing Proteins Using GMOs

UNIT 2

Study of Cloning Vectors

Cloning vector is a DNA molecule in which when a foreign DNA is integrated achieves the capability to replicate it within itself to produce many clones of recombinant DNA.

Characteristics of a Cloning Vector

1) Its size should be small.

2) It should bear the capability to self-replicate within the host cell.

3) It should carry a restriction site for the action of restriction endonuclease.

4) Its replication property should not be compromised by the insertion of donor DNA fragment.

5) To ease the identification of recombinant cell it should possess some marker gene.

6) It should bear multiple cloning sites.

General Design Features of an Expression Vector

To simplify manipulation, plasmids are main ly preferred to construct an expression vector. Plasmids have been modified to incorporate control elements [such as strong promoters, efficient ribosome binding site (rbs), multi –restriction insertion sequences, and terminator sequence| intended for high level expression of inserted target gene. The genes for origin of replication (ori) and for selectable markers (antibiotic resistance) were also retained. This expression vector includes multi -cloning short segment (McS) that encodes several restriction sites where foreign gene coding segment s are to be inserted. Initiation codon (ATG) is positioned downstream from rbs. This excludes the inclusion of rbs and initiation codon on insert segment. Prokaryotes do not have these sequences. Downstream McS is a strong transcription terminator signals sequences.

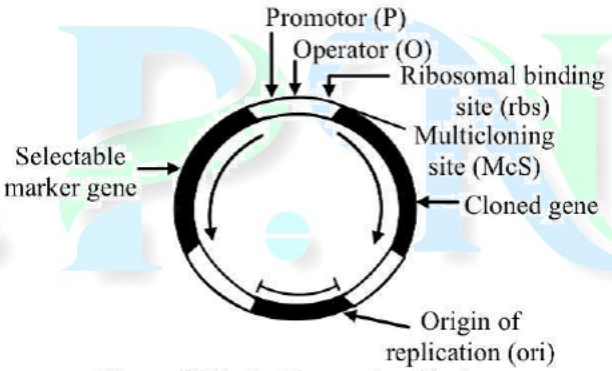


Figure 3.17: An Expression Vector

Different types of cloning vectors:

a. Conventional vectors:

i. Plasmids,

ii. Bacteriophages,

iii. Cosmids,

iv. Phagemids,

b. Vectors for cloning larger DNA fragments

v. Bacterial Artificial chromosome vectors (BAC)

vi. Bacteriophage P1-Derived Chromosome vectors (PAC)

vii. Yeast Artificial Chromosome (YAC) vectors.

c. Vectors for preparing single stranded DNA fragments:

viii. M13 bacteriophage

ix. M13 vectors

d. Vectors for special purposes

x. Shuttle vectors

i. Plasmids

Plasmids are naturally derived, extra chromosomal, double -stranded DNA molecules that replicate autonomously in bacterial cells. Their size ranges across a few thousand base pairs to more than 100 kilo-bases (kb). Plasmid DNA forms replica prior to each cell division just as the host –cell chromosomal DNA does. One copy of the plasmid DNA is divided into individual daughter cell during the cell division. This daughter cell propagates the plasmid through successive generations of the host cell. Plasmids mostly are circular; however, Streptomyces and Borrelia burgdorferi bacteria provide linear plasmids.

The naturally occurring plasmids bear genes that aid the host cells. For example, certain bacterial plasmids encode enzymes responsible for inactivating antibiotics; hence, a bacterial cell with such plasmid develops resistance to the antibiotic and is capable of replicating in antibiotic-rich environment; and if the same bacterium lacks the drug-resistant plasmid, it cannot survive. This property of plasmid favors them to act as cloning vectors.

Properties required in plasmids to serve as cloning vectors are:

1) Their isolation from the host cells should be easy.

2) They should possess a restriction site for one or more restriction enzyme(s).

3) Their replication properties should not be changed upon insertion of a linear foreign DNA at one end of the restriction sites.

4) They can be reintroduced into the host bacterial cells. Cells bearing plasmid with or without the insert should be identifiable.

Given below are some of the examples of plasmids:

1) pBR Vector Plasmids: The name pBR was derived from the name of its discoverer, i.e., Bolivar and Rodriguez. Modification of the former plasmids of E. coli (i.e., pBR318 and pBR320) produced pBR322 plasmid of 4362 bp length. Its origin of replication (ori) is derived from ColEI (a naturally occurring plasmid). The pBR322 plasmid contains genes conferring resistance to antibiotics, like ampicillin (amp^r) and tetracycline (tet^r). More than 10 enzymes are present with distinctive cleavage sites onthe pBR322 genome. The tetracycline resistant gene (tet^r) carry the target sites of these enzymes and the promoter of that gene carry sites for further t wo (Clal and HindIII). In the amp^r gene, six specific restriction sites are present. With the help of these enzymes cloning in pBR322 causes insertional inactivation of either the amp^r or the tet^r markers. The host cells with recombinant plasmid grow in the medium either containing ampicillin or tetracycline but not both. Easy selection of recombinants is not favoured by cloning in other unique sites, since none of the antibiotic resistance determinants are inactivated. The 3’ tetra-nucleotide extensions formed won digestion are ideal for terminal transferase; therefore the PstI site in the amp ‘ gene in pBR322

plasmid is important for cloning by the homopolymer tailing method. When cloning occurs at HindIII site of pBR322 plasmid, tetracycline resistance is lost . In certain recombinants however, tet is retained or increased as the HindIII site is present in the promoter rather than the coding sequence. Thus, the occurrence of insertional inactivation depends on whether or not the cloned DNA carries a promoter like sequence that may initiate transcription of the tet gene.

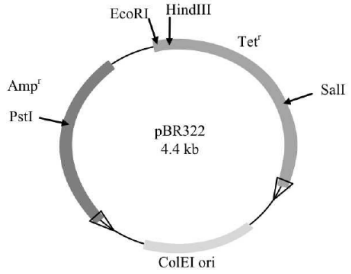


Figure 3.8: pBR322 Vector Plasmid

2) pUC Vector Plasmids: The name pUC stands for Plasmid University of California as these genetically modified plasmids were first developed in the University of California. Advantages of these plasmids as a vector for cloning are:

i) They are smaller in size but still can hold relatively large DNA inserts.

ii) In a host cell, pUC (like pUC18) can replicate to form 500 copies per cell, producing numerous clones or copies of inserted DNA fragments.

iii) They bear an ampicillin resistance gene, and an origin of replication (derived from E. coli plasmid pBR322).

iv) They bear a lac Z gene obtained from E. coli. In the lac region , many restriction enzyme sites, known as multiple cloning poly-linker sites have been engineered.

v) They have a selection system to differentiate between recombinant (plasmid with inserted DNA fragment) and non-recombinant plasmids.

2. Bacteriophages

Bacteriophages (or phages) are viruses infecting bacterial cells and are used as cloning vectors. Since plasmids are small in size, they are used for single gene insertion with inserts not larger than about 2 kb. However, plasmids are avoided for cloning large DNA pieces (e.g., for genomic library preparation), since larger inserts make the plasmids unsuitable for transformation by expanding their size. The bacteriophage has a lin ear DNA molecule that forms two fragments on a single break. A foreign DNA is used to join these fragments. The so produced chimeric phages can be isolated after allowing them to infect bacteria and collecting progeny particles after a lytic cycle. To optimise the insertion capacity, phage DNA has to be modified as per the purpose.

Bacteriophage λ as Vector

Bacteriophage λ (Lambda) is the broadly explored bacterial virus employed in DNA cloning. The A phage virion (virus particle) bears a head containing viral DNA genome, and a tail that infects its host, the LE. coli (figure 3.9). The genome ofa_ wild-type A virion is a 50 kb line are double-stranded DNA packed within a protein coat. As the virion attaches to the host bacterial cell , the protein coat degrades and the DNA enters the cell. The extreme end of the A DNA has overhanging 5’ ends that are 12 nucleotides long. These large 5’ overhangs are sticky ends and form base pair. They are although similar to the small sticky ends formed by restriction nucleases but are more cohesive in nature, thus are named as **cos sequence**. A double-stranded circular DNA is formed when the **cos sequences** enter the bacterial cell, forms base-pair and seal the nicks with cellular ligases. Thereafter, the λ DNA choose either of the lytic cycle or lysogenic cycle.

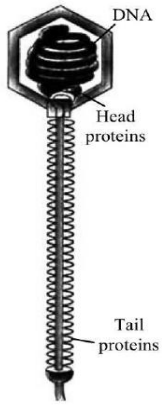
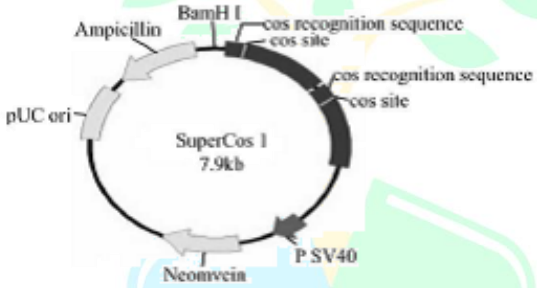


Figure 3.9: Structure of Bacteriophage λ

3. Cosmids

Insertion of the cos sequence of a phage into a small plasmid vector produces cosmid vectors. The cos-sites allow the DNA to be packed in J particle, thus the cosmids enable in vitro packaging of DNA in phage particle. This is useful in their selection and purification.



Similar to plasmids, the cosmids propagate in bacteria. The genes for lytic development are not carried bythem . Use of cosmids for cloning is advantageous as they efficiently produce a complete gen omic library of 10^{6} - 10^{7} clones from just 1 pg of insert DNA. Cosmids have a disadvantage of unable to accept more than 40-50 kbp of DNA.

4. Phagemids

Like cosmids, phagemid vectors are prepared artificially by combining the features of phages with plasmids (thus the name phagemids). The characteristics of phagemids are:

1) They contain multiple cloning sites.

2) They have an inducible lac promoter.

3) They bear an origin of replication (obtained from both phage and plasmid).

pBluescript IIKS

It is 2961 bp long and is obtained from plasmid pUC19. The KS indicates the orientation of poly -linker to facilitate transcrip tion of lac Z gene from the restriction site for KpnI to SacI . It is an artificial vector with multiple cloning sites flanked by T 3 and T7 promoters. It has an inducible lac promoter (Lac J), upstream of lac Z site complementary with EF. coli ($lac\ Z^{-}$).

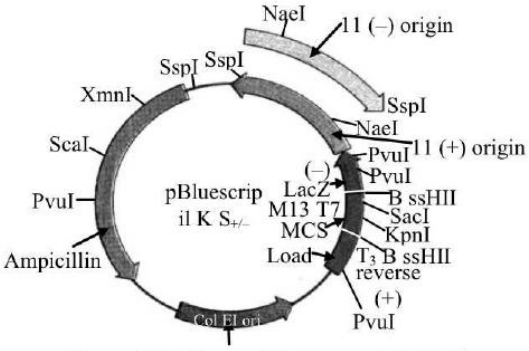


Figure 3.13: Phagemid pBluescript IIKS$^{+/-}$

pBluescript IIKS is useful to select chimeric vector through criterion of white colonies formed against blue colonies if no foreign DNA is ins erted. This vector has fl(+) and fl(-) origin of replication derived from a filamentous phage responsible for sense (+) and antisense (-) strands of lac Z gene, upon co-infection of host with a helper phage; an origin of replication (ColEI ori) originated from plasmid used when helper phage is not present; an ampicillin-resistant gene for antibiotic selection of chimeric phagemid vectors (figure 3.13).

Vectors for Cloning Larger DNA Fragments

Human and other mammalian genomes are very large, therefore, vectors are needed to clone large DNA fragments. Many vectors have been prepared that aid in the characterisation and expression of large genes or gene complexes. These vectors exhibit varied cloning capacity (table 3.2).

Table 3.2: Maximum DNA Insert Possible with Different Cloning Vectors

Vectors	Hosts	Insert Size
λ phage	*E. coli*	5-15kb
λ cosmids	*E. coli*	35-45kb
P1 phage	*E. coli*	70-100kb
PACs	*E. coli*	100-300kb
BACs	*E. coli*	≤ 300kb
YACs	*Saccharomyces cerevisiae*	200-2000kb

Bacterial Artificial Chromosome (BAC) Vectors

Vectors used for DNA cloning in bacterial cells have high to moderate number of replicons. Greater number of DNA clones is formed by these vectors depending on the replication efficiency of its replicon.

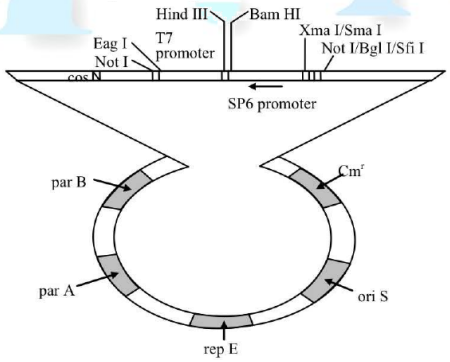


Figure 3.14: Structure of a BAC Vector Derived from A mini-F Plasmid. Its oriS and repE genes mediate the unidirectional replication of the F factor, while par A and par B maintain the copy number at a level of one or two per genome. Cm^r is the chloramphenicol resitance gene, cosN and loxP are the cleavage sites for λ terminase and P1 cre protein. HindIII and BamHI are cleavage sites for inserting foreign DNA.

Disadvantage regarding these vec tors is structural instability of the inserts, causing deletion or rearrangement of a segment of cloned DNA. This is observed in DNA inserts of eukaryotic origin where repetitive sequences frequently occur. Hence, cloning and maintaining large DNA in bacterial cells is not easy. This limitation is however overcome by vectors with low copy number of replicons. An example is a vector prepared from the E. coli fertility plasmid or the F-factor. It has two genes (par A and B) to sust ain the copy number for F -factor at 1-2 per E. coli cell. This vector accepts large foreign DNA fragment (>300kb). Thus, the recombinant vector can be transferred into bacterial cells through electroporation method (in which cells are exposed to high voltage to relax selective permeability of the plasma membrane). Such vectors provide a low yield of recombinant DNA from the host cells (figure 3.14).

Bacteriophage P1 and P1-derived Artificial Chromosome (PAC) Vectors

Certain bacteriophages carry large genomes and hold larger DNA fragments. An example is bacteriophage P 1 that packages linear DNA (110 -115kb) in Pl protein coat. The P1 cloning vectors are prepared from components of P1 phase provided in a circular plasmid. The plasmid vector when cleaved generates two vector arms that allow _ in vitro ligation and packaging of ~100kb of foreign DNA into a P1 protein coat. This recombinant P1 phage is then adsorbed o n a suitable host, and then the recombinant PI DNA is injected into the cell, circu larised and amplified. Employing bacteriophage Ty, in vitro packaging system with P1 vectors aid in the recovery of inserts (of around 122kb). The features of Pl and F –factor systems when assembled together deve lop P1 -derived Artificial Chro mosome (PAC) cloning system.

Yeast Artificial Chromosome (YAC) Vectors

Yeast artificial chromosomes are advantageous in cloning of bacterial cells as it can clone very large DNA fragments.

A YAC has the following features:

1) At each end of YAC a yeast telomere (TEL) is present.

2) Inorder to facilitate regulated segregation during mitosis, a yeast centromere sequence (CEN) is present.

3) Each arm has a selectable mark er to detect the YAC in yeast (for example, TRPI and URAII for tryptophan and uracil independence in trpI and uralll mutant strains respectively).

4) To allow the replication of vector in a yeast cell, an origin of replication ARS (Autonomously Replicating Sequence) is present.

5) For inserting foreign DNA, restriction sites unique to the YAC are present (figure 3.16). To have a successful cloning, a restriction enzyme is used to cut a circular YAC in multiple cloning sites and another restriction enzyme is used to cut between the two TELs; thus, the left and right arms are formed. The DNA with high molecular weight is ligated to the two arms.

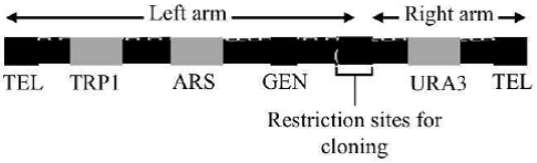


Figure 3.16: Structure of Yeast Artificial Chromosome (YAC) Cloning Vector. It contains a telomere (TEL) at each end, a yeast centromere sequence (CEN), a yeast selectable marker for each arm (TRP and URA3), a sequence that allows autonomous replication in yeast (ARS) and restriction sites for cloning

The transfection of so produced YAC into yeast cells cannot be achieved directly. So yeast cells are treated appropriately to eradicate the external cell wall. The yeast spheroplasts obtained accept the exogenous fragments, and are yet osmotically unstable and thus embedded in agar.

6. Vectors for Preparing Single-Stranded DNA

For conventional DNA sequencing, templates of single-stranded DNAs are used. PCR-based methods can be employed to manufacture them. Also vectors based on certain bacteriophages whose genomes assume a single -stranded DNA at some stages in their life-cycle can be used.

M13 Bacteriophage

M13 is a filamentous bacteriophage. Its genomes bear single—stranded circular DNA (~6.4kb long), associated to each other in DNA sequence. A protein coat covers the genomes forming a long filamentous form. The single -stranded form of genome of the phage converts into a double stranded or replicative form in the host cell after getting adsorbed on the host E. coli. Later a phage genome product triggers DNA synthesis to obtain single-stranded DNA, which is surrounded by a protein coat and migrates towards the cell membrane. Likewise many mature phage particles are extended from the host cell without lysis.

M3 Vectors

M3 vectors are synthesized by double-stranded replicative form with multiple cloning sites that can produce double -stranded recombinant DNA circles. These vectors transfect the host, E. coli. Later the phage particles are harvested, and their protein coat is removed to liberate the single -stranded recombinant DNA. These DNA serves as templates for DNA sequencing.

Vectors for special purposes:

Shuttle vector:

Specific recombinant plasmids incorporate multiple replication origins and other elements that allow them to be used in more than one species (E. coli or yeast). Plasmids which are propagated in cells of two or more different species are called shuttle vectors. Such vectors (Fig. 4.10) possess two origin for replication (ori', orifuk). The ori functions in Escherichia coli and oriEuk functions in eukaryotic cells like yeast. The important gene are ori (origin for replication in E. coli), amp (ampicillin resistance), ars (autonomously replicating sequence), cen (centromere of yeast) and leu-2 (complements of a defective gene encoding for leucin)

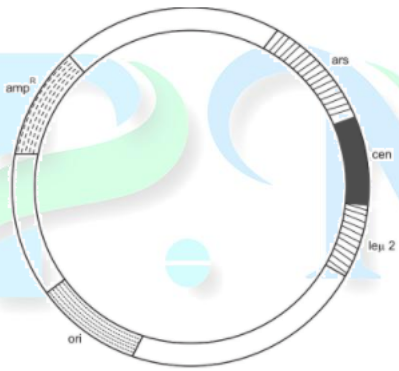


Fig. 4.10: A typical shuttle vector constructed for yeast and Escherichia coli

Restriction Endonucleases

Restriction endonuclease enzymes aid in cutting the DNA molecules at specific sites. These enzymes form internal cuts (cleavage) in DNA strands, only within or near some specific sites called recognition sites or recognition sequences or restriction sites or target sites Each restriction enzyme work on specific recognition sequences. Restriction endonuclease enzymes are important foDNA technology. **W. Arber in 1962** first reported the presence of restriction enzymes when he observed that on inserti ng the phage DNA into a host bacterium, it fragmented into small pieces. In **1970s, Meselson** and **Yuan** isolated the first true restriction endonuclease enzyme from the bacterium E. coli.

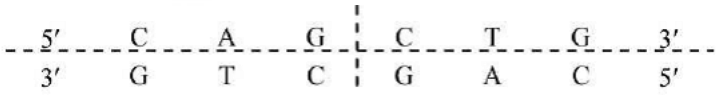
The three main categories of restriction endonuclease enzymes are:

1) Type-I Restriction Endonucleases: This type includes complex endonucleases, and cut one strand of DNA only. The recognition sequence for these enzymes is about 15 bp length (table 1). They function properly by utilising Mg' ions and ATP. Thes e types of restriction endonucleases cut DNA about 1000 bp away from the 5' end of the sequence *TCA' within the recognition site. EcoK, EcoB, etc. are some examples of type-I restriction endonuclease enzyme.

Table 3.1: Recognition Sequences of Several Restriction Endonucleases

Enzymes	Source Organisms	Recognition Sequences 5′ … 3′	Blunt or Sticky Ends
EcoRI	*Escherichia coli*	GAATCC	Sticky
BgIII	*Bacillus globigii*	AGATCT	Sticky
HindII	*Haemophilus influenzae*	GTPyPuAC	Blunt
HindIII	*Haemophilus influenzae*	AAGCTT	Sticky
HinfI	*Haemophilus influenzae*	GANTC	Sticky
HpaI	*H. parainfluenzae*	GTTAAC	Blunt
HaeIII	*H. aegyptius*	GGCC	Blunt
Sau 3A	*Staphylococcus aureus*	GATC	Sticky
PvuI	*Proteus vulgaris*	CGATCG	Sticky
BamHI	*Bacillus amyloliquefaciens*	GGATCC	Sticky
TaqI	*Thermus aquaticus*	TCGA	Sticky
SmaI	*Serratia marcescens*	CCCGGG	Blunt
SfiI	*Streptomyces funbriatus*	GGCCNNNNNGGCC	Sticky
SalI	*Streptomyces albus*	GTCGAC	Sticky

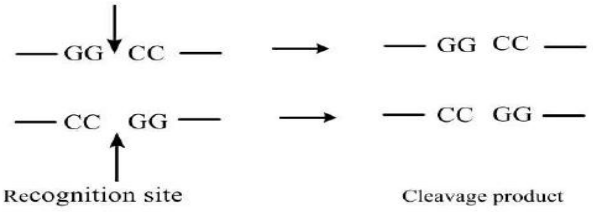
2) Type-II Restriction Endonucleases: This type is most stable and is significant for gene cloning and rDNA technology. They cleave specific sites and produce DNA fragments of definite lengths. They can cleave at each strands of DNA, immediately outside the re cognition sequences. They utilize Mg^{++} ions for working. These enzymes do not require ATP for cleavage and cleaves both the strands of DNA, thus are advantageous. Only these enzymes are used for gene cloning due to their appropriateness. Type-II restriction endonuclease has recognition sequences in the form of palindromic sequences with rotational symmetry. This means that the base sequence in the first half of one DNA strand is the mirror image of the second half of other strand of DNA double helix (figure 3.2). HinfI, EcoRI, PvuII, AluI, HaeIII , etc. aresome examples of type -II restriction endonuclease enzyme.



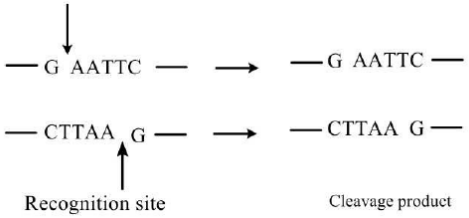
Type III Restriction Endonucleases: This type is not employed in gene cloning. They areintermediate enzymes between TypeI and Type-II restriction endonuclease. They need Mg' ions and ATP for cleavage and cleave DNA at specific sites in the immediate vicinity of recognition sequences. Hinf III is a common example of typeIII restriction endonuclease enzyme.

The DNA is cut in two patterns by the restriction enzyme:

1) Blunt End Pattern: Some restriction endonuclease enzymes cleave both the DNA strands at the same position. This pattern helps during the joining of DNA fragments. Any pair of ends can be joined together regardless of sequence. Two defined sequences can be linked without introducing any material between them.



2) Sticky or Cohesive End Pattern: Restriction endonuclease enzymes cut some nucleotide pairs apart in the opposite strands of DNA, forming fragments with protruding ends.



These DNA fragments either join through base pairing with hydrogen bond between the overlapping ends, or the fragments circularise by intermolecular reaction, and hence the fragments are termed sticky or cohesive ends.

cDNA Clones

The cDNA clones are preferably used when the target DNA to be isolated has been obtained from eukaryotic organisms and the aim is to express it in a prokaryotic cell. Expressing the DNA of a eukaryotic cell into a prokaryotic cell is difficult since both the cells vary in gene organisation and regulation. Introns (segments of non-coding DNA) in eukaryotic gene (figure 3.3) disrupt the exons (coding sequence) of the gene.

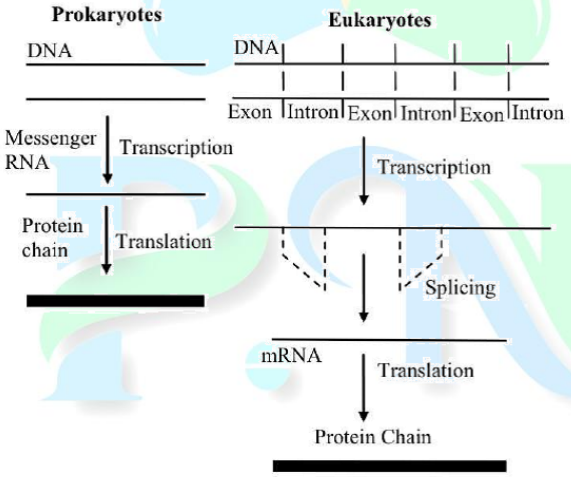


Figure 3.3: Expression of DNA in Prokaryotes and Eukaryotes

Transcription of introns into mRNA takes place. The eukaryotic cell machinery process (splicing off introns) these mRNAs before reaching the cytoplasm for translation. Since prokaryotic cell is devoid of introns, it cannot process the precursor mRNA. Hence, a complementary DNA copy (cDNA) is suitable for genomic DNA as the target gene is in eukaryotic cell. For the enzymatic synthesis of complementary DNA (cDNA), mRNA is used as the template along with reverse transcriptase acting as a ca talyst. The primer DNA is needed in this process. Poly A tail is present in mRNA at its 3' end (figure 3.3). Poly T is added to mRNA, and it base pairs with poly A tail of the mRNA. This base pair now acts as a primer for reverse transcriptase and transcribes the mRNA utilising a mixture of dATP, dTTP; dGTP and dCTP to make a complementary strand of cDNA (figure 3. 4). Alkaline hydrolysis separates mRNA from mRNA -cDNA hybrid. The single strand

cDNA left behind is replicated by DNA p olymerase I, producing a 'hairpin' double stranded DNA. Cleavage of hairpin yields a synthetic double stranded cDNA, coding for specific gene for desired protein. The prepared cDNA is then inserted into a plasmid or viral vector, and thus should bear appropriate tails or cohesive ends.

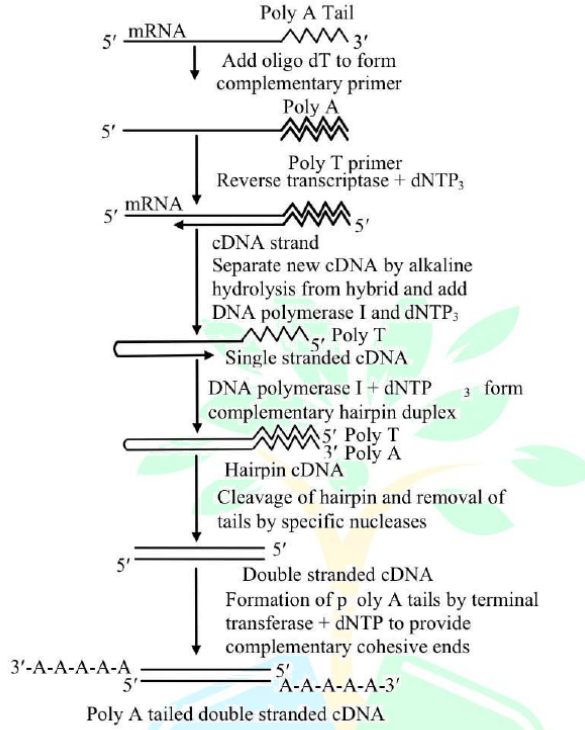


Figure 3.4: Construction of a Double Stranded cDNA from a mRNA

2.3. Mechanical Shearing

Mechanical shearing forms random fragments of DNA. Sonication or high speed mixing also generates the target fragments. By varying the intensity of the treatment, the average size of the fragments (with 5 ' and 3' overhangs, and blunt ends) can be managed. The produced fragments have no cohesive ends. Therefore, the process of homopolymer tailing is required to facilitate ligation with the vector.

2.4. Chemical Synthesis

The gene of interest can be synthesised chemically if the amino acid sequence of the concerned protein is known for which the nucleotide sequences in mRNA and DNA can be determined. Short nucleotides produced are spliced into larger fragments. This process is more practicable with the existing triester method and automatic synthesizers.

Insertion of Target DNA Into a Vector

The target DNA or the DNA insert isolated and enzymatically cleaved by the selective restriction endonuclease , are ligated (joined) through 'the action of **DNA ligase enzyme** to the vector DNA. As a result, an rDNA molecule known as cloning-vector-insert DNA construct is produced.

DNA Ligase- For direct ligation of cohesive ends

DNA ligase (or molecular glue) forms phosphodiester bond to join two DNA fragments. They repair the single stranded nicks in DNA double helix. In rDNA technology, they seal the nicks between adjacent nucleotides.

The target gene can be inserted into a vector when both are cleaved with the same restriction enzyme , producing identical cohesive ends. A staggered two stranded cut ends complementary to each other in sequence, are produced (figure 3.5). When performed annealing, the joint linking two DNAs _ bear one pair of staggered nicks on each strand. The two DNAs are connected with DNA ligase under suitable conditions to create a phosphodiester linkage among the adjacent nucleotide. Generally, ligation is performed for 12 -24 hours at 4 -15°C temperature and is catalysed by either T, DNA ligase or E. coli DNA ligase. The former uses ATP as a cofactor, while the latter uses NAD^+. The ligated fragments thus produced have a mixture of DNA fragments and linear vector, undesirable circularized vector with no inserts, length of DNA formed from two or more fragments, circularised fragments, two or more vector molecules connected together, etc.

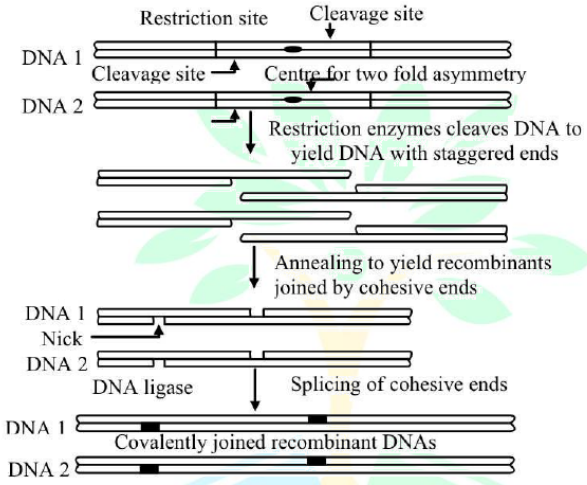


Figure 3.5: Ligation of Cohesive Termini

Blunt End Ligation

It is possible to transform any cohesive ends into blunt ends, by eliminating the protruding nucleotides using SI nuclease that will break the single stranded protruding DNA. The cohesiv e ends can also be transformed by filling in the single stranded ends by DNA polymerase. The blunt end ligation has a disadvantage that once ligated (using T4 DNA ligase), the DNA cannot be detached and becomes less efficient.

Linkers and Adapters

The blunt end molecules canbe ligated through single strands of synthetic oligonucleotides with previously formed restriction sequences. The ligation conditions remain the same as required by blunt ended ligation. Upon annealing to each other these single stranded linkers become ligated to the DNA ends. After ligation each end of DNA molecules carries several linkers. When treated with restriction endonuclease, the pieces of restricted linker are separated, and the linker terminated DNA is ready for ligation.

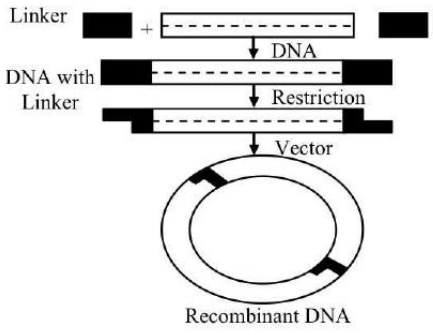


Figure 3.6: Use of Linkers

Adapters

Linkers and adapters resolve the problem of compatibility. Adapters are synthetic oligonucleotides of double stranded molecules. They have a blunt end on one side and a cohesive single strand tail on the other side.

RECOMBINANT DNA TECHNOLOGY

In recombinant DNA (rDNA/rec DNA) technology , DNA molecules from two different sources are joined and inserted into a host organism to get —products useful for human use (figure 3.1). A clone refers to a group of cells or organisms obtained from one progenitor, thus indicating that the members of a clone are genetically same. This is because each time identical daughter cells are formed by cell replication. The word clone associated with recombinant DNA technology provides the ability to produce several copies of a single DNA fragment, like a gene, creating identical copies producing a DNA clone.

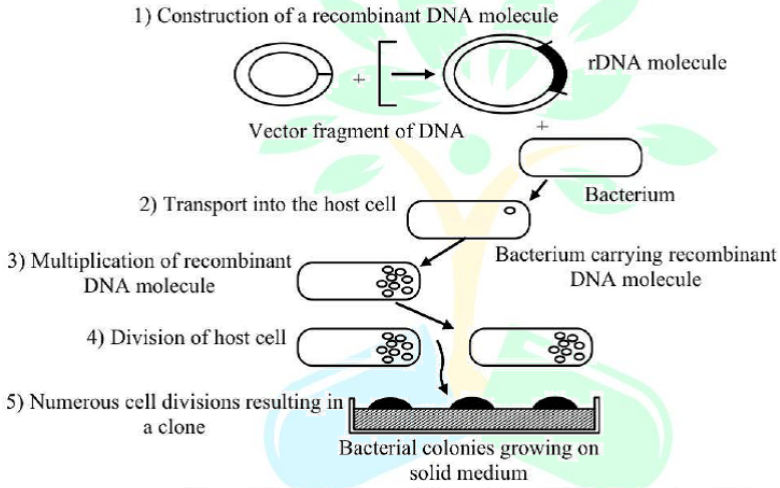


Figure 3.1: Basic Steps of Recombinant DNA Technology Using the Bacterial Plasmid as Cloning Vector

Steps Involved in rDNA technology

Strategies for gene cloning along with the techniques commonly employed are mentioned below:

1) Isolation of target DNA by:

i) Restriction endonuclease digestion,

ii) cDNA clones,

iii) Mechanical shearing, and

iv) Chemical synthesis.

2) Insertion of target DNA into a vector by:

i) Direct ligation of cohesive termini,

ii) Blunt end ligation,

iii) Linkers and adapters, and

iv) Homopolymer tailing.

3) Cloning vectors:

i) Plasmids,

ii) Bacteriophages,

iii) Cosmids,

iv) Phagemids,

v) Vectors for cloning larger DNA fragments,

vi) Vectors for preparing single-stranded DNA, and

vil) Expression vectors.

4) Identification and isolation of recombinant genes.

5) Expression of cloned genes within the host.

Application of genetic engineering in medicine:

Genetic engineering has an important role to play in the production of medicines. Microorganisms and plant -based substances are used for producing various drugs, vaccines, enzymes, and hormones at low costs. Genetic engineering involves the study of inheritance pattern of diseases in man and collection of human genes that provide a complete map for inheritance of healthy individuals.

1) Vaccines

Recombinant DNA technology is used for producing vaccines against diseases by isolating antigen or protein present on the surface of viral particles. Vaccines contain a form of an infectious organism that does not cause disease but allow the body immune system to form antibodies against the infective organism. When an individual receives vaccination against any viral disease, the antigens produce antibodies to act against and inactivate the viral proteins. The scientists with the help of recombinant DNA technology have transferred the genes for some viral sheath proteins to vaccinia vir us which was used against small pox. Vaccines produced by gene cloning are non-contaminated and safe as they contain only coat proteins against which antibodies are produced. Vaccines against viral hepatitis influenza, herpes simplex virus, virus-induced foot and mouth disease in animals are being produced by gene cloning.

2) Hormones

Insulin was commercially produced in 1982 through biogenetic or recombinant DNA technology. Its medicinal use was approved by Food and Drug Administration (FDA) of the USA in the same year. The human insulin gene has been cloned in large quantities in £. coli bacterium that can be used for synthesising insulin. Humulin is the commercially available genetically engineered insulin.

3) Lymphokines

Lymphokines are proteins regulating the immune system inhuman body. — a- Interferon is an example of lymphokines that is used to fight viral diseases (such as hepatitis, herpes, and common cold) and cancer. Such drugs can be manufactured in large quantities in bacterial cell. Lymphokines are also helpful in AIDS. Interleukin-II is a commercially available genetically engineered substance that stimulates the multiplication of lymphocytes.

5) Somatostatin

Somatostatin is used in some of the growth related abnormalities. This drug appears to be species specific and the polypeptide obtained from other mammals has no effect on human, hence it is extracted from the hypothalamus of cadavers. Genetic engineering has helped in the chemical synthesis of gene which is joined to the pBR 322 plasmid DNA and cloned into a bacterium. This transformed bacterium is converted into somatostatin synthesising factory.

6) Production of Blood Clotting Factors

Blockage of coronary arteries by cholesterol or blood clots causes heart attack. Plasminogen is a substance found in blood clots. Genetically engi neered tissue Plasminogen Activator (tPA) enzyme is used to dissolve these clots in individuals who have suffered heart attacks.

7) Cancer

Antibodies cloned from a single source and targeted for a specific antigen (monoclonal antibodies) have proved useful in the treatment of cancer. Monoclonal antibodies have been targeted with radioactive elements or cytotoxins (e.g., Ricin from castor seed) to make them more deadly. Such antibodies seek cancer cells and kill them with their radioactivity or toxin.

Application of rDNA Technology and Genetic Engineering in the Production of Interferon

Interferons are low molecular weight proteins produced by virus infected cells, which in noninfected cells can induce the formation of a second protein. This protein has the ability to prevent transcription of any viral mRNA produced in that cell. Interferons exhibit antiviral activity, antproliferative activity, and many effects on the immune system. Interferons on the basis of their source, biological, physicochemical and antigenic properties are grouped into the followifigree classes

1) IFN-α (leukocytes),

2) IFN- β (fibroblasts), and

3) IFN-γ (immune).

IFN- α can be effectively used in the treatme nt of some melanomas and renal cell cancers, while IFN -B can be used in solid tumours. Trials of IFN -y have proved it a potential stimulator of the immune system with enhanced anti-tumour properties. IFN - α with 865 nucleotide (166 amino acids) sequences and IFN -β with 836 nucleotide sequences can be expressed in E. coli. The genomic DNA sequences of both IFN -a and IFN-f lack introns; while IFN –γ codes for 146 amino acids and include introns. Production of interferons encoded by DNA sources involves the synthesis of cDNA to the mRNA isolated from a cell line, tissue or organ followed by screening and selection of cDNA clone. Synthesis of interferon gene can also be done chemically if the sequence of the gene encoding that particular interferon is known. Thus, a variety of interferon molecules, i.e., IFN - α (IFN- α 1, IFN - α 2, IFN - α 4, IFN - α β, IFN -ac), IFN - β and IFN-y, can be produced. Synthetic genes have the advantage over the natural gene sequence for high level expression in microorganisms. IFN synthesis in ba cteria depends on the transcription of genes to mRNA and translation of mRNA to produce the protein. Transcription requires suitable promoters, adjacent operator region, ribosome binding site, and a start or initiation codon. In additional processes, the primary translated IFN is manipulated to functionally active IFN, i.e., the signal sequence is removed and glycosylated in case of IFN -B and IFN -y. Intramolecular disulphide bridges are essential for the biological activity of IFN-a and IFN-f.

Human IFN- α

Synthesis of IFN - α; gene involves assembly of 67 oligonucleotide fragments to give a double stranded molecule, in which each strand contains 514 base pairs. Natural gene sequence obtained by cDNA prepared from 125 fractions of mRNA from IFN producing human leukocytes can be cloned in E. coli using pBR322 vector. The cDNA inserted in the [-lactamase gene at the PstI site expresses a fusion protein. An mRNA hybridisation translation assay is used for identifying the cDNA clone containing an IFN- α. The colonies are screened using 32p-labelled 320 bp PstI fragments as probe. Constructed plasmids can be used for synthesizing IFN molecules fused to beta-lactamase. Biologically active material hybrid with the IFN gene in three different reading frames can be produced. The various molecules of human IFN - α are expressed in E. coli, utilising different promoter or construct expression systems (i.e., B-lactamase, trip, lac UV5, lac,

etc.). Human myeloblastoid line KG-1 also serve as a source of mRNA for IFN coding sequences. A gene coding fora complete IFN -aA can be isolated after the formation of double stranded cDNA and cloning in E. coli. Inserting this gene in a plasmid vector (containing a part of the trp operon) results in a biologically active fusion product of amino acid and IFN- α a.

Human IFN- β

In an approach to form mRNA from cDNA, production of human IFN-B involves insertion of cDNA into pBR 322, followed by transformation into E. coli. Identification follows hybridisation to cDNA, enriched for IFN –containing sequences. The cDNAs hybridizing to total mRNA (containing sequence for human IFN-P) are isolated. Various molecules of human IFN -B in E. coli cloned from cDNA are expressed by incorporating various promoters or expression systems (i.e., lac or trp).

Human IFN-y

The DNA fragment encoding IFN -y with a sequence coded for a polypeptide of 166 amino acids (of which 20 constitutes signal sequence) can be produced from mRNA to cDNA approach. Bacterial clones can be screened from cDNA library by using 32 -labelled cDNA probe prepared from either induced or unstimulated peripheral blood lymphocytes. cDNA can be expressed by transforming the fragment containing vector to E. coli. A restriction site at codon -4 of the mature coding sequence is used for cleaving the sin gle peptide. Two synthetic oligonucleotides are induced for restoring the codons or amino acids 1 -4, incorporating an initiation codon ATG, with a restriction site cohesive terminus for joining a trp promoter. The product contains only a single gene, cross hybridising with IFN-y-cDNA sequence.

Since the genomic DNA contains three introns, it cannot be expressed directly in prokaryotes; rather can be expressed from cDNA sequence in yeast cells and higher eukaryotic cells [like Ap , monkey cells, Chinese Hamster Ovary (CHO cell line)]. Chemically synthesised gene for IFN -y can be expressed in E. coli from a lac UV; promoter. Anti IFN-y serum can be used for neutralising antiviral cell lysate. IFNs can be purified by High Performance Liquid Chromatography (HPLC) or reverse phase liquid chromatography.

Application of rDNA Technology and Genetic Engineering in the Production of Hepatitis Vaccine

The Recombivax HB (Merck)isa __ hepatitis B vaccine and one of the most significant developments in recombinant DNA technology field. This vaccine comprises of highly specific antibodies. Hepatitis causes a severe acute infection that ultimately results in chronic infection and permanent liver damage. It is caused by Hepatitis B Virus (HBV) which is an enveloped and double –stranded DNA virus. Hepatitis B infection can be prevented with a vaccine made using recombinant DNA technology.

In figure 3.22, the steps followed in the production of Hepatitis B vaccine using recombinant DNA technology are enlisted:

1) The DNA (genetic material) of the hepatitis virus is extracted. At this stage the surface proteins incite an immune response.

2) The individual genes are analysed and identified.

3) The specific gene which directs production of surface protein is located.

4) The gene is then removed from the viral DNA and inserted into the plasmid.

5) The plasmids are accurately inserted into the corresponding yeast cells.

6) This cell is then allowed to grow via fermentation. Thus, the cells reproduce and generate more quanta of surface proteins.

7) After 2 days the yeast cells are ruptured to release the surface proteins.

8) The resulting mixture is processed to extract and purify the surface proteins.

9) A large amount of pure surface proteins are obtained which provoke an immune response.

10) The resulting surface proteins are mixed with other ingredients under optimum conditions to obtain the vaccine.

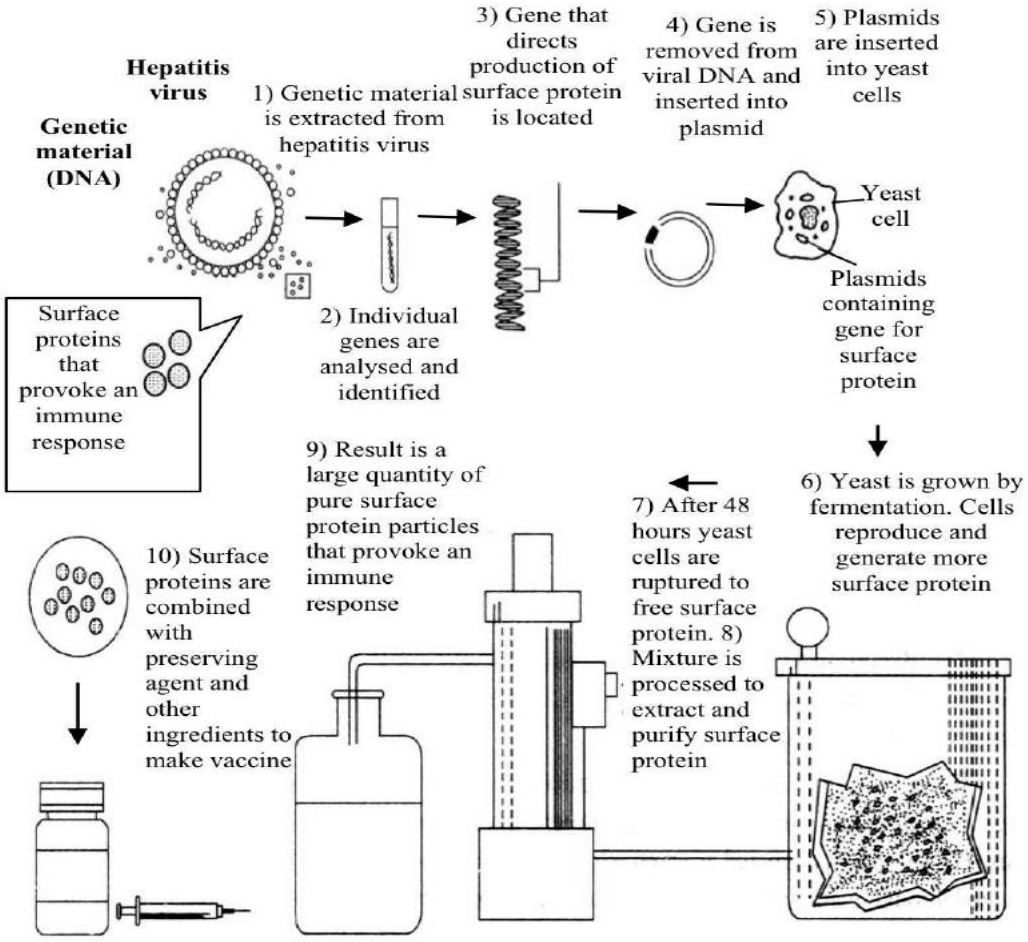


Figure 3.21: Production of Hepatitis B Vaccine
Source: Adapted from *Remington: The Science and Practice of Pharmacy* Volume I (20th ed.), by Alfonso G. (2000), (Lippincott Williams & Wilkins).

Antigenic Markers for HBV-Infection

The following three antigenic markers have been identified for the HBV infection:

1) HBsAg: It is a surface antigen found on the viral envelope. It represents one of the earliest marks and appears in the blood during incubation.

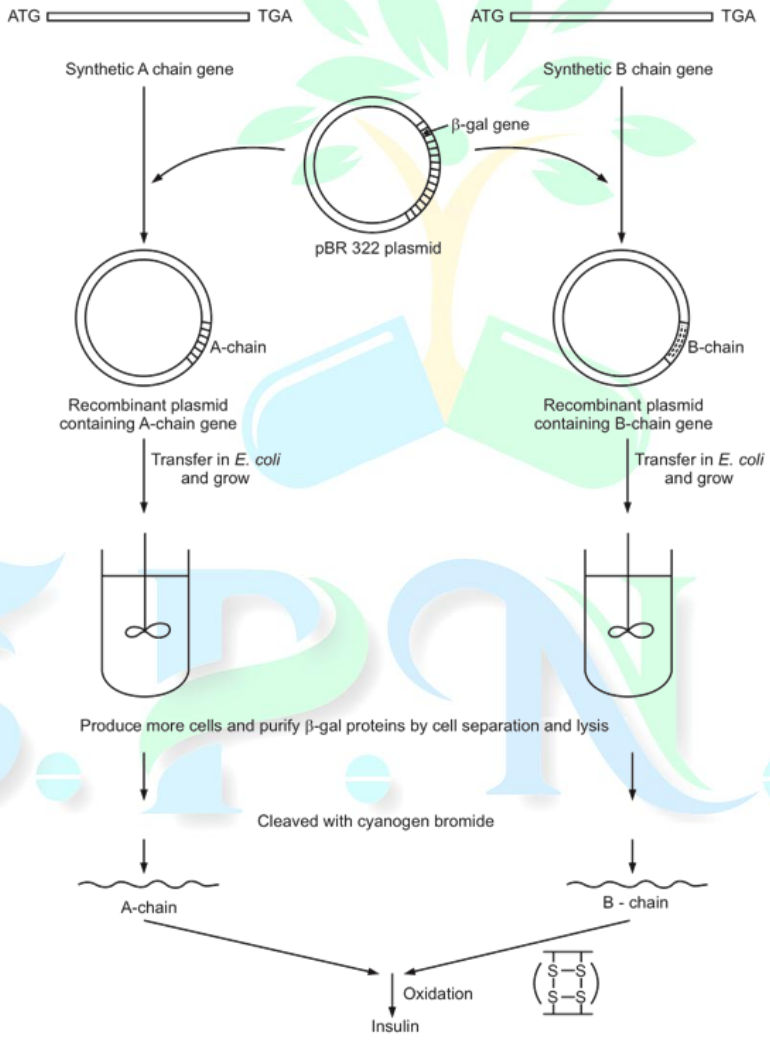
2) HBeAg: It is obtained from the prote in capsid surrounding the DNA. It is acmarker for causing active infection.

3) HBc: It is a core antigen which does not circulate in the blood, and stimulates the production of the primary antibodies against HBV. These antibodies are not protective, and hence, do not provide immunity.

Application of rDNA Technology and Genetic Engineering in the Production of Insulin

Human insulin was the first recombinant -derived product. It is used for the treatment of diabetes. Fredrick Sanger and his co-worker identified the insulin structure stating that it is made up of two polypeptide chains of A and B with a total 51 amino acid units (1.e., 21 in A -chain and 30 in B-chain) held by disulphide cross bridges.

Insulin was produced by the chemical synthesis of genes, i.e., chains A and B. Both the chains were separately cloned and attached to B-galactosidase, resulting in the synthesis of a fusion polypeptide that is relatively stable in E. coli. Two bacterial strains were created, each producing a fused protein with A and B chains. The genes synthesized lacked promoters. To express the cloned genes into functional protein, a gene is cloned into a plasmid vector close to the bacterial promoter. A synthetic gene lacks ribosomal binding site, therefore, the gene should be inserted downstream from a promoter and ribosomal binding site of the vector. During the production of synthetic genes, a signal sequences are to be cloned with additional 15 -30 amino acids at the N-terminus. These sequences have a central core of hydrophobic amino acids lined with polar or hydrophilic residues. While passing through the membrane, the signal sequence is cleaved off. The synthetic gene does not contain any methionine residue (initiation codon), thus it is constructed by incorporating methionine residue at the junction of fusion peptide. The accurate insulin can be obtained by cleaving the methionine residue with cyanogen bromide, purifying, and then linking the two chains chemically in vitro.



In another approach to obtain insulin, the gene for precursor molecule was constructed synthetically. The proinsulin gene is cloned to produce a B-galactosidase proinsulin hybrid protein. The proinsuli nis chemically cleaved from B-galactosidase with cyanogen bromide. Then in vitro proteolytic digestion with trypsin cleaves out amino acids (c -chain) in the middle of the molecule to obtain insulin. The quality assurance of the recombinant insulin involves purity and identity tests. The insulin obtained from bacterial cell should be identical to human insulin. By sequencing the cloned cDNA, the accuracy of the nucleotide sequence can be established. The primary structure of insulin can be determined from the quantitative amino acid data, and its purity can be established by HPLC.

PCR (Polymerase Chain Reaction)

PCR technique discovered by Kary Mullis in 1985 is important in molecular biology (figure 3.20). It is employed for the amplification (or cloning) of a target DNA sequence. Sometimes it is also referred to as in vitro gene cloning (without expression of that gene) as it is carried out in vitro. Around billion copies of the target DNA sequence can be obtained from a single copy within few hours only using PCR.

PCR results in selective amplification of a selected DNA molecule. It has a limitation that the border region sequences of the DNA to be amplified should be known so that the appropriate primers can be selected to be annealed (attached) at its 3' ends. Primer annealing is important as the DNA polymerase enzyme requires double stranded (ds) primer regions for initiating DNA synthesis.

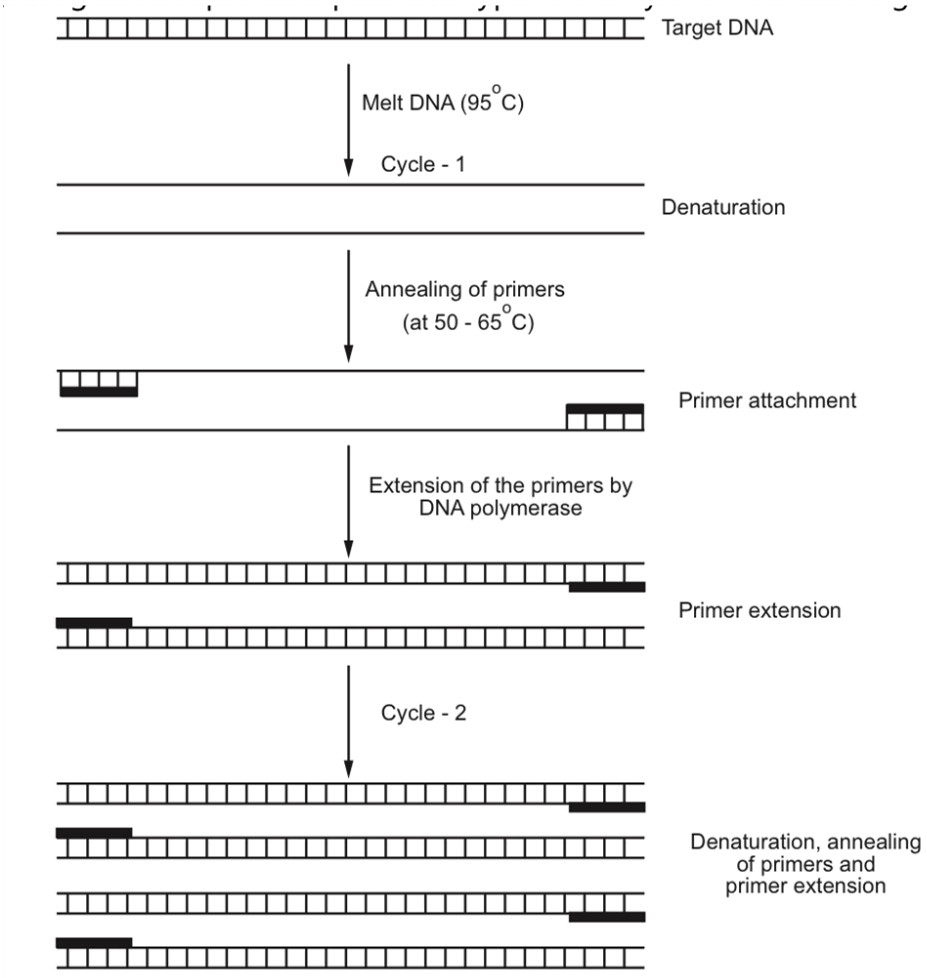


Figure: Steps involved in PCR

The PCR reaction takes place in **eppendorf tube.** The technique is used by the scientists in various disciplines since it is a quick, simple and extremely accurate technique. The major limitation of PCR is that being highly sensitive it may produce inaccurate results caused by several inhibitors or contaminating DNA segments that may be present in the sample DNA preparation.

Main Requirements of PCR

1) **Two nucleotide primers complementary to 3' ends of target DNA strands:** Each PCR requires a pair of oligonucleotide primers. These are short single- stranded DNA molecules obtained by chemical synthesis. These

primers are designed to anneal on opposite strands of target sequence so that they will be extended towards each other by addition of nucleotides.

2) **Target DNA sequence:** DNA Template: Any source that contains one or more target DNA molecules to be amplified can be taken as template. RNA can also be used for PCR by first making a DNA copy using the enzyme reverse transcriptase.

3) A heat stable DNA polymerase: The most commonly used enzyme in PCR is Taq DNA polymerase isolated from a thermostable bacterium called Thermus aquaticus. It survives at 95°C for 1 to 2 minutes and has a half life for more than 2 hours at same temperature. The DNA polymerase binds to a single-stranded DNA and synthesizes a new strand complementary to the origin strand. The role of this enzyme in PCR is to copy DNA molecules.

4) Deoxynucleotide triphosphates: PCR requires four deoxynucleotide triphosphates, dNTPs (Deoxy Adenosine Triphosphate (dATP), Deoxy Thymidine Triphosphate (dTTP), Deoxy Cytidine Triphosphate (dCTP), Deoxy Guanosine Triphosphate (dGTP),) which are used by the DNA polymerase as building blocks to synthesize new DNA and

8) A thermal cycler in which PCR is carried out.

Steps in PCR

1) The target DNA sequence, excess of primers, dATP, dTTP, dCTP, dGTP, and Taq polymerase are mixed together in eppendorf tube, and the tube is placed in thermal cycler.

2) The reaction mixture is provided with high temperature (95°C) for 1 minute for denaturing the DNA. Consequently, the double stranded DNA becomes single stranded.

3) Temperature is lowered to 55°C for 1 minute so that the primers are annealed at 3' ends of DNA.

4) Temperature is now maintained at 72°C for 1 minuyr to facilitate the functioning of Taq polymerase and to synthesise the complementary strand of DNA.

5) One cycle of PCR completes resulting in the formation of two ds DNA molecules from one ds DNA.

6) The same cycle is repeated to obtain the required number of DNA copies.

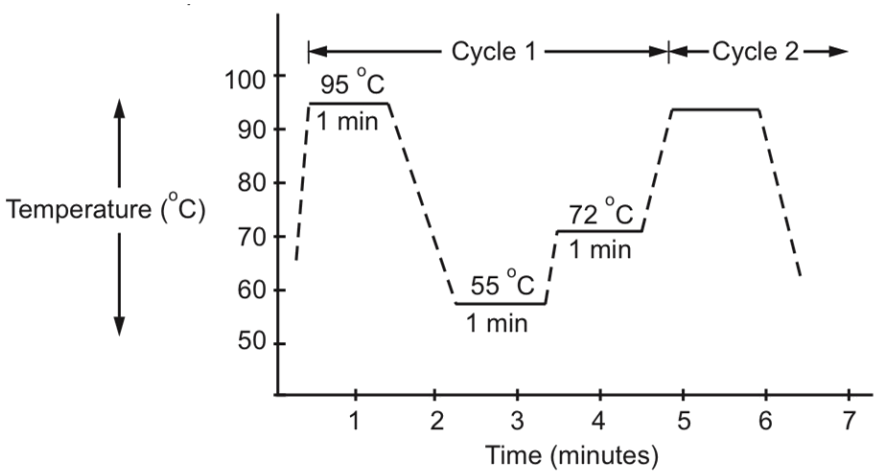


Fig. 3.15: Temperature profile of a typical PCR cycle

Variants of PCR:

Inverse PCR: PCR can be used to the amplification of those DNA sequences which are away from the primer and not of those which are flanked by the primer. The sequences to be amplified may be cloned in a vector and border sequence of the vector may be used as a primer in such a way that the polymerization proceeds in reverse direction.

Anchored PCR: In this method only one primer is used. One strand is copied first and then poly G tail is attached at the end of the newly synthesized strand. This allows the use of complementary homopolymer, poly-C, to be used as primer for copying the DNA single strands generated by PCR. It gives rise to the complete DNA duplex that can be amplified normally.

RT-PCR: Reverse transcription-mediated PCR includes a single application combining the process of cDNA synthesis (by reverse transcription) and PCR amplification. It can also be applied to double stranded cDNA also which is synthesized from mRNA using the enzyme reverse transcriptase. Thermostable enzyme rith uses RNA templates from cDNA synthesis and thus allows single enzyme RT-PCR viral reverse transcriptase from avian murine virus (AMV RTase).

Asymmetric PCR: It is used to generate single-strand copies of a DNA sequence which can be directly used for DNA sequencing. The two primers (100 : 1 ratio) are such adjusted in the reaction mixture that one of them is exhausted about 10 or more cycles. After these cycles, only a single strand of DNA segment is copied and these copies are the ideal starting materials for DNA sequencing. This variation is known as asymmetric PCR.

AP-PCR: Arbitrary primed PCR (AP-PCR) is a type of random amplified polymorphic DNA (RAPD) where single primers of 10 to 50 bases are used to amplify genomic DNA in PCR. Welsh and McCleland (1990) developed the arbitrary primed – PCR and carried out finger printing of genomes with arbitrary primers.

Applications/ Uses of PCR

1) **Diagnosis of pathogens:** PCR is commonly used for diagnosis of infections caused by viruses (e.g. HIV-1, HIV-2, Herpes simplex virus, Hepatitis B virus etc.), bacteria (Mycobacterium tuberculosis, Helicobacter pylori, Mycoplasma pneumoniae etc), fungi (Candida albicans) and protozoa (Toxoplasma gondii, Trypanosoma cruzi etc).

2) **Diagnosis of plant pathogens:** Various plant pathogens are detected by using PCR such as viruses (plum pox virus, cauliflower mosaic virus), fungi (Verticillium spp., Laccaria spp., Phytophthora spp. etc), mycoplasms bacteria (Agrobacterium tumifaciens, Rhizobium leguminosarum, Xanthomonas compestris etc) and nematodes (Meloidogyne incoginta) etc.

3) **Inherited diseases:** Inherited disorders are caused by gene mutations passed on from parents to their children e.g. hemophilia, cystic fibrosis etc. PCR is used to amplify gene sequences which can then be screened for disease causing mutations.

4) **Research:** PCR is used extensively as a research tool for identification of new species. Many bioactive microbial species are isolated from various extreme environment such as soil, water, air, sediments etc. DNA fingerprinting of new microorganisms is carried out to confirm their identity by comparing with the DNA sequences of known microorganisms.

5) **Cancer research:** Polymerase chain reaction has been widely used in studies for the role of genes in cancer. Tumour-supressor genes and mutations in oncogenes have been identified in DNA from tumors using PCR-based strategies.

5) **Biotechnology:** PCR has played major role in the production of recombinant proteins. Insulin and growth hormones are recombinant proteins, widely used as drugs and recombinant vaccines are developed for hepatitis B virus. It is an important tool in the biotechnology industries of research institutes.

6) **Forensic science:** PCR is most applicable in forensic science where it is being used in search of criminals through DNA fingerprinting technology. PCR allows amplification of DNA from individual hairs, stains of blood or seminal fluid having partially degraded DNA. Analysis of variable sequences is also used in tissue typing to match organ donors with recipients.

7) **DNA polymorphism:** PCR is used to study DNA polymorphism in the genome using known sequences as primers. PCR can be used to study RFLPs (restriction fragment length polymorphisms) as well as RAPDs (random amplified polymorphic DNA).

8) **Gene therapy:** PCR proves to be immense help in monitoring a gene in gene therapy experiments. This PCR technology provides shortcuts for many cloning and sequencing applications.

IMMUNITY

4.1.1 Introduction:

The word immunity (Latin *immunis* meaning exempt) describes the protection against diseases. It indicates that an individual has developed lifelong resistance to a certain disease after being infected with it only once. Thus, immunity is an individual's ability to respond to foreign substances and microbes.

Immune system includes cells and molecules responsible for immunity of the body; and a collective and coordinated response of cells and molecules to foreign substances is known as immune response.

4.1.2. Types of Immunity:

The principle function of the immune system is to protect the body from any microbial infection or pathological conditions. During the immune response, the first step is recognition of the foreign substance (e.g., bacteria, viruses, parasites, and fungi). The defence mechanism of the body may be divided into two forms (figure 4.1):

1) Non-specific/innate/neutral immunity, and

2) Specific/acquired/adaptive immunity.

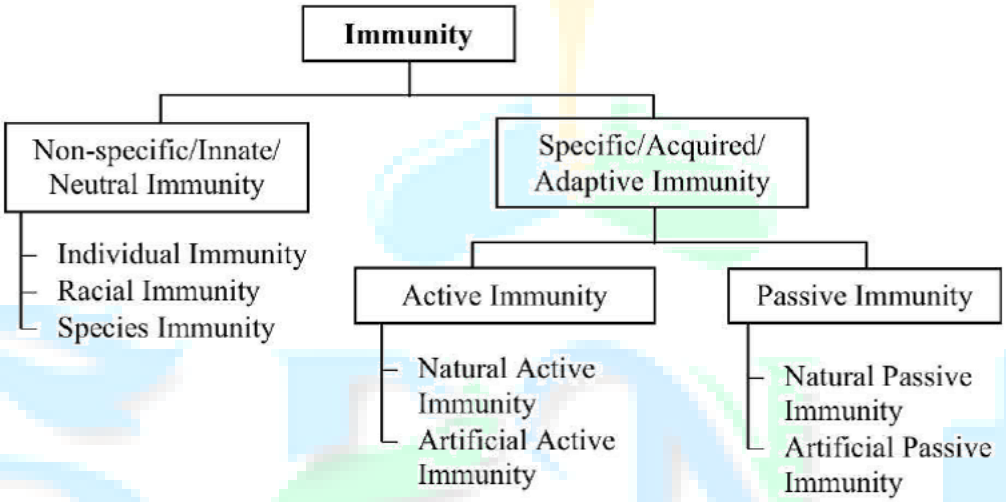


Figure 4.1: Classification of Immunity

4.1.3. Non-Specific/Innate/Neutral Immunity

Non-specific immunity is present in an individual since birth. This immunity is affected by the genetic and molecular structure of the genes; and is independent of previous contact with foreign antigen molecules. It may be non-specific when there is resistance to infections in general or may be specific when resistance to a particular pathogen is involved.

4.1.3.1. Types

Various types of non-specific immunity are as follows:

1) Individual Immunity: Different individuals of same race and species possess variable resistance against infection known as individual immunity. The genetic basis of individual immunity is evident from the observation that the

homozygous twins show similar degrees of vulnerability or resistance to tuberculosis. However, this kind of correlation is not observed in heterozygous twins.

2) Racial Immunity: Different races within the same species show marked variation in the degree of resistance for different infections. This type of immunity that is exhibited by different races of same species is known as racial immunity, e.g., Algerian sheep is highly resistant to anthrax which is a common disease of other races of sheep. It is noticed that American Negroes are less resistant to tuberculosis, than the white race. In many instances, it is observed that the racial immunity is based on the genetic features.

Human races with sickle cell anaemia dominant in Mediterranean coast have developed immunity for P. falciparum infection. A genet ic abnormality of RBCs (sickling) confers immunity to P. falciparum because such RBCs cannot be parasitised by these parasites. Individuals with hereditary glucose-6-phosphate dehydrogenase deficiency are less susceptible to P. falciparum.

Species Immunity: This immunity is found in all members of a particular species, such as many microbes are pathogenic for human but does not infect animals, e.g., B. anthracis infects human but not chickens. The mechanism behind species immunity is not clearly understood. Probably, the physiological and the biochemical differences between tissues of different host species are responsible for the species specific resistance or immunity.

4.1.3.2. Mechanisms

Some of the common mechanisms of non-specific immunity are as follows:

1) Epithelial Surfaces

i) Skin: Skin is a mechanical barrier to microorganisms and also provides a bactericidal secretion that kills the pathogens. The bactericidal properties are due to the presence of high content of salt in drying sweat, the sebaceous secretions, and long chain fatty acids. The skin itself may be freed of any bacterial depositions on it (transient flora), but sometimes bacterial flora (resident flora) is found on skin. Such bacterial flora is not easily removable even by washing and using disinfectants. The resident bacterial flora of skin and mucous surfaces help to prevent colonisation by pathogens. Alteration of normal resident flora may lead to invasion by extraneous microbes and thus cause serious diseases, e.g., invasion of Clostridial enterocolitis due to oral antibiotics.

ii) Respiratory Tract: The moist mucous secretion in the nasal passages traps the inhaled foreign particles. These particles are passed towards the pharynx by the hair-like cilia present on the mucous membrane surface. In the pharynx, the foreign particles are either swallowed or coughed out (cough reflexes are important defence mechanism that expels out the foreign particles). However, some particles that reach the alveoli are phagocytised by the macrophages present there.

iii) Intestinal Tract: The saliva has an inhibitory effect on many microorganisms. The acidic pH of the stomach kills many bacteria. Also, the presence of normal bacterial flora in the intestine prevents colonisation by pathogenic bacteria.

iv) Conjunctiva: Lysozyme present in the tears has a bactericidal action that plays a major role in antibacterial activity by flushing away bacteria and other dust particles.

v) Genitourinary Tract: Urine flushes away the bacteria from the urethra. The acidic pH of the adult vagina (due to the fermentation of glycogen by Lactobacillus) makes it unsuitable for many pathogens. On the other hand, the semen in males contains some antibacterial substances.

2) Antibacterial Substances: Several non-specific antibacterial substances have been found in blood and tissues. These substances include lysozyme, properdin, betalysin, interferon (show s_ antiviral activity), complement, and basic polypeptides (leukins from leukocytes, plakins from platelets). The pathogenic microorganisms attacking the blood and tissues are killed by complement system.

3) Cellular Factors: If the epithelial surface is crossed by a pathogen, the tissue factors play a role of next line of defence. Due to the attack of the infective agents, exudative inflammatory reactions occur by the accumulation of phagocytes at the infection site. Fibrin molecules are deposited at this site and entangle the pathogenic organisms to check the spreading of infection. The pathogenic organisms are killed and ingested by the phagocytic cells that are classified as:

i) **Microphages,** e.g., polymorphonuclear leukocytes (neutrophils).

ii) **Macrophages,** e.g., mononuclear phagocytic cells.

Phagocytosis involves four stages:

a) Chemotaxis: Chemotactic substances attract phagocyte s to reach the infection site.

b) Attachment: Pathogenic agents get attached to phagocytic membrane.

c) Ingestion: Foreign particles or infective agent s present in the vacuole (phagosome) are engulfed by the phagocytic cells. The membrane of phagosome fuses with a lysosome to form a phagolysosome.

d) Intracellular Killing: By the hydrolytic enzymes of lysosomes, most bacteria are destroyed in the phagolysosome. But, a few bacteria (M. tuberculosis and M. leprae) resist such type of killing and multiply within the phagolysosome, thus causing disease. In such cases phagocytosis actually helps in spreading of infection to other body parts.

A class of lymphocytes called Natural Killer (NK) cells play a major role in non-specific defence against tumours and viral infections.

4) Inflammation: It is an important mechanism of non-specific defence system. It results from irritation and tissue injury initiated by the entry of pathogens or other irritants. Inflammation results in increased vascular permeability, cellular infiltration, and vasodilation.

Microorganisms are killed and phagocytised due to the increased vascular permeability. There is an outpouring of plasma which helps to dilute the toxic products present. A fibrin barrier is laid to wall off the site of infection.

5) Fever: Rise in temperature following infection is a protective natural defence mechanism. It kills the harmful infectious microorganisms. It stimulates the interferon production, helping in the recovery from viral infections.

6) Acute Phase Proteins: After any infection or injury, a sudden increase in plasma con centration of certain proteins [e.g., C Reactive Protein (CRP), mannose binding proteins, etc.] is observed; phase proteins. CRP and some other acute phase proteins active this phase is known as activate the alternative pathway of complement, and prevent tissue injury and enhance the repairing of inflammatory lesions.

4.1.4. Specific/Acquired/Adaptive Immunity

Acquired immunity is also known as adaptive immunity because the potency of immune response is adapted by experience only. The difference between nonspecific and specific immunity is summarised in table 4.1:

Table 4.1: Differences between Non-Specific and Specific Immunity

Features	Non-Specific Immunity	Specific Immunity
Definition	The resistance to infection that an individual possesses by virtue of genetic and constitutional structure.	The resistance that an individual acquires during life.
Types	Individual, racial, and species.	Active and passive.
Time taken to Develop	Hours	Days
Specificity	For structures shared by groups of related microbes.	For antigens of microbes and for non-microbial antigens.
Memory	None; repeated exposure brings primary response.	Yes; secondary response much faster than primary response.
Physical and Chemical Barriers	Skin, mucosal epithelia, and antimicrobial chemicals.	Lymphocytes in epithelia and antibodies secreted at epithelial surfaces.
Blood and Tissue Antimicrobial Substances	Complement; leukins from leukocytes, plakins from platelets, lactic acid in muscle tissue, lactoperoxidase in milk, and interferons.	Antibodies
Cells	Phagocytes (macrophages and neutrophils) and natural killer cells.	Lymphocytes

4.1.4.1. Active Immunity

Active immunity is the resistance acquired or developed by an individual after effective contact with an antigen. This contact may be in the form of either natural infection or by vaccination. This involves the active functioning of the immune apparatus leading to the synthesis of antibodies and/or the generation o f immunologically active cells.

Immune system requires specific time -period known as latent period for the development of active immunity. During the latent period the immunity of the host is stimulated to act against the microorganism. Therefore, once the active immunity develops, it persists for a long time; this is the most important characteristic of the active immunity.

Active immunity is of two types:

1) Natural Active Immunity: This results from either clinical or natural sub -clinical infections. Such immunity is usually long -lasting. For example, individuals recovering from chicken pox infection acquire natural active immunity against it.

2) Artificial Active Immunity: This results from vaccination. Vaccines are preparations of live, attenuated or killed microorganisms, or their antigens or toxoids. For preparing vaccines, the organisms are killed by the application of formalin, phenol, alcohol, or by heat. They are persevered in alcohols, phenols, or N-merthiolate. Toxoids are immunogenic but not toxigenic. For preparing toxoids, bacterial exotoxins are inactivated by using formalin (formol toxoid) or alum (Alum Precipitated Toxoid - APT).

4.1.4.2. Passive Immunity

Passive immunity occurs in an individual by readymade antibodies (usually in the form of antiserum) against infective agent or toxin s. This antiserum is prepared by injecting infective agent or toxin in another host. In passive immunity, the immune system of the individual or host does not play any active role. As the immune serum is introduced into the host, it starts working and provides protection against the infection or injury. In passive immunity, there is no latent period; and is effective for a very short duration. Passive immunity is important when immediate immune response is required, e.g., anti-venom is used for snake bite.

Passive immunity is of two types:

1) Natural Passive Immunity: This is seen when antibodies are transferred from mother to the foetus (by placenta or milk) to protect it till its own immune system develops to function.

2) Artificial Passive Immunity: Convalescent sera, hyperimmune sera of animal or human origin and pooled humany-globulin are used for inciting artificial passive immunity. These chemicals or agents are introduced in the body through parenteral route. One of the oldest methods of developing these agents is to employ horse hyperimmune sera. Antitetanus Serum (ATS) is prepared by introducing a series of tetanus toxoid to horses, and bleeding them to separate the serum.

Comparison between Active and Passive Immunity

Table 4.2: Comparison between Active and Passive Immunity

Active Immunity	Passive Immunity
Developed immunity.	Produced immunity.
Develops slowly and is long-lasting.	Relatively fast and short-lived.
A booster dose, if required can be given to give lifelong immunity.	A booster dose also does not help in maintaining it for long.
Prevents a disease and is administered before infection.	Develops after the subject has been exposed to an infection.
Given in long-term prophylaxis.	Given in short -term prophylaxis and therapeutically.
Antigens are administered.	Antibodies are administered.

4.1.5. Cellular/Cell-Mediated/T-Cell Immunity

Cell-mediated immunity is an immune response that does not involve antibodies or complement, but rather involves the activation of macrophages, natural killer cells, antigen-specific cytotoxic T -lymphocytes, and the release of various cytokines in response to an antigen.

Historically, the immune system was separated into two branches, i.e., cellular immunity, for which the protective function of immunisation was associated with cells. CD4 cells or helper T cells provide protection against different pathogens and humoral immunity, for which the protective function of immunisation could be found in the humour (cell-free body fluid or serum).

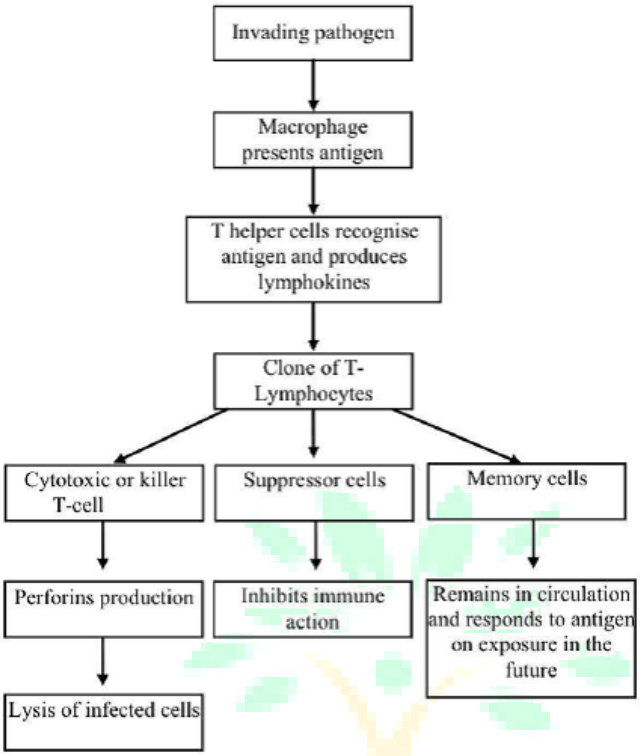


Figure 4.2: Flow Chart of Cell Mediated Immune Response

4.1.5.1. Mechanism

Cellular immune response is also called T-cell immunity as it occurs by the T - cells or T -lymphocytes (figure 4.2). This immune response defends against pathogens invading the host cells. The surface of T-cells has receptor molecule that binds with antigens. A single T-cell has about 100,000 receptor sites. An antigen on entering the body is first attacked by the macrophages and gets fragmented into pieces. It then presents a piece of antigen to the T-helper cells, which recognise the antigen and trigger a series of cellular response. A clone of T-lymphocytes is first formed after getting activated by the T -helper cells. There are different kinds of T-cells, which are morphologically similar but functionally different.

The principal cells involved in cellular immunity and their functions are given in table 4.3:

Table 4.3: Different Cells Involved in Cell-Mediated Immunity and their Functions

Cell	Functions
Helper T cells (T_H)	Necessary for B-cells activation by T-dependent antigens.
Suppressor T cells (T_S)	Regulates immune response and helps in maintaining immune tolerance.
Delayed hypersensitivity T cells (T_D)	Provides protection against infectious agents; causes inflammation in association to tissue transplant rejection.
Cytotoxic T cells (T_C)	Destroys target cells on contact.
Killer cells (K)	Attacks antibody-coated target cells.
Natural Killer cells (NK)	Attacks and destroys target cells.

4.1.5.2. Importance

Cellular immunity protects the body by:

1) Activating antigen -specific cytotoxic T -lymphocytes that can induce apoptosis in body cells displaying epitopes of foreign antigen on their surface, such as virus -infected cells, cells with intracellular bacteria, and

cancer cells displaying tumour antigens;

2) Activating macrophages and natural killer cells, enabling them to destroy intracellular pathogens; and

3) Stimulating cells to secrete a variety of cytokines that influence the function of other cells involved in adaptive and innate immune responses.

Cellular immunity is directed primarily at microbes that survive in phagocytes and microbes that infect non-phagocytic cells. It is most effective in removing virus - infected cells, but also participates in defending against fungi, protozoans, cancers, and intracellular bacteria. It also plays a major role in transplant rejection.

4.1.6. Humoral/Antibody-Mediated/B-Cell Immunity

Antibody-mediated immunity refers to the destruction of antigens by producing antibodies, and as the antibodies are present in body fluids (humours), this type of immunity is also called humoral immunity.

B-cells cannot bring about humoral immune response but needs the cooperation of other cells such as macrophages, T -helper cells (T_H), and dendritic cells possessing high levels of surface MHC class I] molecules that process and present antigens to T-cells. Antibodies are immunoglobulin (Ig) molecules (e.g.,serum proteins) and are usually comprised of several categories designated as IgM, IgA, IgE, and IgG.

4.1.6.1. Immunoglobulins Participating in Humoral Immunity

The various immunoglobulins participating in humoral immunity are:

1) IgM: It is the first class antibody generated invariably in most humoral responses but normally gets switched over to the corresponding IgA, IgE, or IgG at a very early stage in the immune response.

2) IgG: Itis the m ost versatile, important and abundantly available class of antibodies taking part in largest humoral immune reactions. It can cross the placenta, thereby providing a new born baby absolute temporary immunity against the immunogens the mother had earlier against IgG.

3) IgA: It is invariably found ina plethora of secretions like tears, saliva, and mucous membranes. This immunoglobulin is frequently termed as the first line-of-defence mechanism due to the fact that most bacteria, viruses and fungi that eventually gain entry into the body do cross a mucous membrane.

4) IgE: It is also important in the body's defence against the parasitic worm infections. Prominently and predominantly several allergic manifestations give rise to the release of histamine e.g., allergy due to pollens, house dust, dust mite, human hair, food allergens etc.), which in turn afford the apparent discomforts resulting into extrinsic asthma, hay fever, hives, or excessive sneezing (during seasonal change and due tthe presence of pollen grains in the air).

Immunoglobulins normally serve as surface receptors strategically located on certain immunologically active cells so as to enable them to bind immunogen.

4.1.6.2. Mechanism

Humoral immunity s also called B-cell mediated immunity since B-lymphocytes are involved in this response. This immune response defends the body against pathogens invading body fluids or human B-cells are antigen specific. During the immune response, B -cells specific for specific antigens enlarge to become lymphoblasts that differentiate into plasma cells. These mature plasma cells rapidly produce y-globulins or immunoglobulins or antibodies (at a rate

of about 2000 molecules/second) for each plasma cell. These immunoglobulins are secreted into the lymph and they eventually enter the blood.

The different types of cells or entities that are held responsible for contributing to the humoral immunity are:

1) B-Lymphocytes (or B-cells),

2) Immunodominant Peptides (IDPs),

3) Antigen-Presenting Cells (APCss),

4) T-cell subsets, and

5) Class II MHC (Major Histocompatibility Complex) proteins.

4.2. IMMUNOGLOBULINS

4.2.1. Introduction

Immunoglobulins are glycoprotein molecules. They are produced by plasma cells in response to an antigen or immunogen, and they function as antibodies. Antigens present in the foreign bodies (microorganisms or parasites) are protein molecules responsible for producing specific antibodies or immunoglobins in the host. Some low molecular weight antigen molecules, called haptens do not bind to antibodies, but stimulate the formation of antibodies. Specific regions of antigen molecules having 5 -8 amino acids are called epitopes that are required for antibody production.

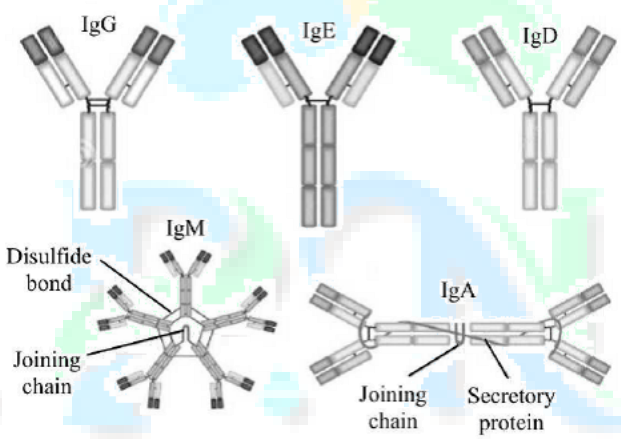


Figure 4.3: Types of Immunoglobulins

4.2.2. Classification

Immunoglobulins are classified on the basis of _ the nature of the heavy chain. Around 10,000 different antibodies formed by plasma cells have been found in a human body. These antibodies are categorised into IgG, IgM, IgA, IgD and IgE:

Name of Ig	Nature of Heavy Chain
IgG Immunoglobulin	G – Gamma (γ) H-chain
IgA Immunoglobulin	A – Alpha (α) H-chain
IgM Immunoglobulin	M – Mu (μ) H-chain
IgD Immunoglobulin	D – Delta (δ) H-chain
IgE Immunoglobulin	E – Epsilon (ε) H-chain

The five main types of immunoglobulins have been discussed below:

1) IgG: It is the most versatile immunoglobulin that is capable of carrying out all the functions of immunoglobulin molecules. In a normal human adult, approximately 75% of the total serum immunoglobulin is constituted by IgG.

The IgG immunoglobulin is further sub -divided into IgG1, IgG2, lgG3 , and IgG4. This is the only immunoglobulin which is capable of crossing placenta. Its transfer is mediated by the receptors present on the placental cells. Serum complements (e.g., found in serum and lymph) can also be fixed by IgG.

Properties

i) It constitutes about 75% of serum immunoglobulin, thus is the major immunoglobulin in serum.

ii) It is the major immunoglobulin in extravascular spaces.

iii) It is the only immunoglobulin that can cross the placenta.

iv) It fixes complement, but all sub -classes do not fix equally well, as IgG4 class does not fix the complement.

2) IgA: It constitutes approximately 15% of the total immunoglobulin, and is the major immunoglobulin in body secretions. The secretory IgA is responsible for the primary defence mechanism, against some local infections as these are present in abundance in saliva, tears, bronchial secretions, and mucous secretions of small intestine. The IgA is present as dimer in secretions, while in serum secretion it exists as monomer. However, when IgA exits as a dimer, a J chain is aligned with it. The IgA present in secretions has another protein associated with it known as secretory or T-component; however sometimes IgA is referred to as 1is immunoglobulin. Opposite to the remainder of the IgA (which is formed in plasma cell), the secretory component is formed in the epithelial cells and can be added to the IgA as it passes into the secretions. The secretory component supports the IgA to be carried across the mucosa and also protects it against degradation in the secretions.

Properties

i) It is the second most common immunoglobulin of serum.

ii) It is mainly present in body secretion § s, e.g., saliva, tears, colostrum, mucus; therefore is responsible for local (mucosal) immunity.

iii) It is not involved in the fixation of complement, unless and until it is aggregated.

3) IgM: It is the most effective complement fixing immunoglobulin, present in serum and lymph. It is prominent in early immune responses to most antigens and predominates in certain antibody responses such as natural blood group antibodies.

IgM is found in pentamer (19S immunoglobulin) form, but also exists in the form of monomer. In the pentameric form, all — the light as well as heavy chains are identical.

Properties

i) It is the third most common serum immunoglobulin, and is the first immunoglobulin to be made by the foetus.

ii) It is a good complement fixing immunoglobulin due to its pentameric structure; therefore, efficiently leads to the lysis of microorganisms.

iii) It binds to the cells by Fc receptors.

iv) Due to its pentameric structure, it is also a good agglutinating agent. It is very good in clumping microorganisms for elimination from the body.

4) IgD: It exists in monomeric form. Its main function has not been known. It is found in serum and on lymphocytes. However, in isolated cases IgD shows activity against certain antigens like milk proteins, diphtheria toxoids, penicillin, thyroid antigens, and insulin.

Properties

i) It is present in serum in low concentration.

ii) It does not bind to the complement.

iii) It is present on the surface of B-cells where it functions as a receptor for antigen. On the surface of B_ -cells, IgD has extra amino acids at C -terminal end for attachment to the membrane. It is also attached to the Ig-alpha and Ig-beta chains.

5) IgE: It actively takes part in allergic reactions and is found in serum. It is found in monomeric form and has an extra domain in the constant region.

Properties

i) It is found in serum in very low concentration, as it binds very firmly to Fe receptors on basophils and mast cells even before interacting with antigen.

ii) It does not fix the complement.

iii) It binds to basophil mast cells, therefore actively participates in allergic reactions. Binding of the a llergen to the IgE onthe cells releases many pharmacological mediators that result in many allergic symptoms.

iv) It is actively involved in the control of helminthic diseases. The parasitic infection is diagnosed on the basis of IgE concentration in the serum, as its serum concentration rises when the infection occurs.

4.2.3. Structure

Structurally immunoglobulin of each class differs but all of them are formed of same basic unit (figure 4.4):

I) Heavy and Light Chains: Immunoglobulin is a tetramer made up of two similar high molecular weight or heavy chains and two low molecular weight or light chains. Heavy chain consists of 500 amino acids (having molecular weight of 50,000 -70,000 Daltons). The 5 types of heavy chains in IgA, IgD, IgE, IgG, andIgM are designated as a α (alpha), $\triangle$ (delta), ε (epsilon), y (gamma), and μ(mu) respectively.

Both the chains of antibody molecule have a variable (V) and a constant (C) portion. Half of each light chain consists of a variable region (V_L), one-quarter of each heavy chain consists of a variable (V_H) in different patients, and the remaining three-quarters of the heavy chain (G;) are constant for all IgGs. This constant portion of heavy chain is sub -divided into three sections of

equal length. Each of these sub-sections of C part of IgG heavy chain originated during evolution by the duplication of an ancestral gene that encoded for an Ig unit of around 110 amino acids. The variable regions (V_H or V_L) have originated by evolution from the same ancestral Ig units.

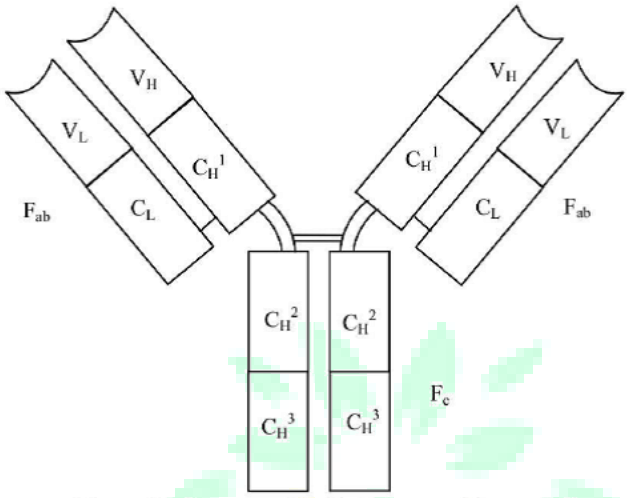


Figure 4.4: Immunoglobulin Structure. Heavy chains determine the isotype; light chains are identical in each molecule but may be kappa or lambda

Light chains are made up of two low molecular weight chains designated κ (kappa) and λ (lambda) present in five different antibodies. Each light chain consists of 200-250 aminoacids (having molecular weight of 23,000 Daltons). Both the light chains have the following domains:

i) Antigen-Binding Domains: They are made up of Fv, and Fab segments. The former is a heterodimer having variable domains of heavy chains (V_H) and light chains (V_L). The latter is also a heterodimer of entire light chain + V_H and C_{H1} parts of heavy chains.

ii) Effector Domains: They are also called Fc segment which helps in the elimination of antigen. The F,. region isa homodimer having CH) and CH; domains of heavy chains. The terminal regions of both heavy and light chains have three small hyper variable regions that form the antigen binding sites or **Complementarity-Determining Regions** (CDR). These hyper variable regions in the chains contain deletions and insertions of amino acids and also substitutions of one amino acid for another.

Each antigen binding site in the hyper variable regions is made up of about 5-10 amino acids. Therefore, an antibody molecule's combining site specificity is determined by the variable domains of the molecule. The proximal remaining parts of the chains are constant (i.e., not variable) and are termed as framework regions . These constant domains facilitate the anchorage of antibody on the plasma membrane and activation of the complement system by which bacterial cells are punctured and destroyed.

2) Variable (V) and Constant (C) Regions: On examining and comparing the amino acid sequences of many different heavy and light chains, it becomes clear that both the chains are divided into two regions, on the basis of the variability in their amino acid sequences:

i) Light Chain: V_L (110 amino acids) and C; (110 amino acids).

ii) Heavy Chain: V_H(110 amino acids) and Cy (330-440 amino acids).

3) Disulfide Bonds: Two types of disulphide bonds are present:

i) Intra-Chain Disulphide Bonds: These disulphide bonds are present within the polypeptide chains.

ii) Inter-Chain Disulphide Bonds: Two heavy chains and two light chains are held to each other by inter -chain disulphide bonds and by non -covalent interactions. Different immunoglobulin molecules have different number of inter-chain disulphide bonds.

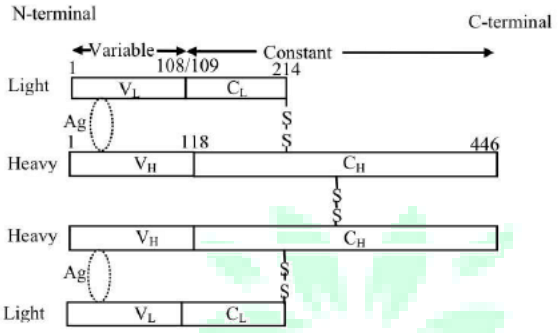


Figure 4.5: Basic 4 Polypeptide Chain lgG molecule. Two Heavy and Two Light Chains Joins together by Disulphide (S-S) Bonds

4) Hinge Region: At hinge region, the arm of the antibody molecule forms a Y shaped structure. The name hinge is given due to the flexibility of molecule at this point.

5) Oligosaccharides: In most immunoglobulins, carbohydrate molecules are linked at CH2, domain. But in few cases, carbohydrate molecules may be attached at other locations.

4.3. MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

4.3.1. Introduction

Major Histocompatibility Complex (MHC) encodes the MHC proteins present on the plasma membrane of all human tissue/cells. MHC is a tightly linked group of genes located on chromosome 6 in humans. MHC proteins are involved in intercellular recognition of foreign substance (antigen) to T-lymphocytes. Haplotypes is the term used for a group of linked MHC genes inherited as a unit from parents. Human MHC molecules are termed as Human Leucocyte Antigens (HLA). MHC genes are polymorphic, i.e., many alleles are present for each gene and polygenic, i.e., many different MHC genes are present.

4.3.2. Classes

The classes I, II, and III of MHC genes express for molecules of classes I, I] and III, respectively (table 4.4). Class I MHC genes have A, B and C gene loci. Glycoproteins (or class I MHC molecule) secreted by these genes are present on surface of nucleated cells. Class I MHC molecules present the peptide antigens to Tc cells.

The length of class I MHC gene in humans is ~2,000kb (about 20 genes) and is present at the telomeric terminus of the HLA complex. The length of class II MHC gene range is ~1,000kb and is present at the centromeric terminus of HLA. The length of class III genes is-10,000kb and is present between the class I and II genes.

Table 4.4: Organisation of Major Histocompatibility Complex (MHC) HLA Genes in Chromosome 6 in Humans

Classes	Class II			Class III		Class I		
Regions	DP	DQ	DR	C_4, C_2, and BDF		B	C	A
Genes	DP	DQ	DR	C Proteins	TNF-α	HLA-B	HLA-C	HLA-C
Products	αβ	αβ	αβ		TNF-β			

In humans, the class IT MHC genes have the DP, DQ and DR regions encoding the class II MHC molecules (glycoproteins) expressed on antigen presenting cells like macrophages, dendritic cells and B -cells. Class II genes present the processed antigenic peptides to Ty cells, and class II molecules provide immune response.

Class I and II molecules share the same _ structural features, and both function in antigen processing. Class III MHC gene is flanked by the Class I and II regions and encodes molecules important for immune function. The complement components of Class III MHC molecules are C4, C2, BF, inflammatory cytokines, Tumour Necrosis Factor (TNF), and heat shock proteins.

4.3.3. Structure

The Class I molecule acts as a trans -membrane glycoprotein. It has two chains, i.e., alpha-chain (or heavy chain) of molecular weight 42KD, associated non - covalently with a B2-microglobulin (or light chain) of molecular weight 12KD. The alpha-chain has three extracellular domains (alpha1, alpha2, and alpha3), a hydrophobic transmembrane segment, and a short hydrophilic cytoplasmic t ail (figure 4.6A). These are encoded by the A, B, and C regions of HLA complex, and are expressed on the plasma membrane of all cells (excluding erythrocytes).

Different chromosomes express the B2-microglobulin molecule. The a-chain together with B.-microglobulin is required for expressing class I molecules on the cell membrane. The alpha1 and alpha2 form the antigenic-binding cleft situated on the top of molecule surface. Class II MHC molecules also serve as trans membrane glycoprotein. Separate MHC genes encode them. They have two different alpha- (33KD) and beta-chains (28KD) associated through non-covalent bond (figure 4.6B).

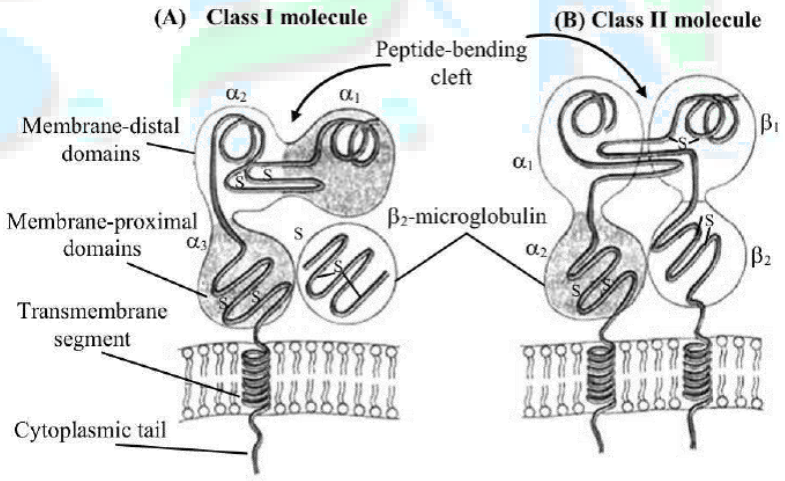


Figure 4.6: Structure of MHC Molecules

Two domains (Beta1 and Beta2 domains in other domain) are formed upon folding of the alpha- and Beta-chains. One domain is the membrane-proximal domain, and the other is membrane-distal domain. Similar to class I MHC molecules, trans-segment and a cytoplasmic anchor segment are present in class II molecules also. The chains of

class II molecule bear two external domains (alpha1 and alpha2 domains in one chain, and Beta1; and Beta2 domains in the other chain).

4.3.4. Functions

MHC provides cell mediated as well as humoral immune responses. The antibodies selectively bind with antigens. T-cells recognise only those antigens combined with MHC molecules therefore, MHC molecules are antigen -presenting structure.

MHC partially ascertains how an individual responds to antigens of infectious microorganisms. Hence, MHC is associated to disease susceptibility and autoimmunity. The natural killer cells also express receptors for MHC c lass I antigens. The interaction of receptor with MHC may cause inhibition or activation.

Class I and II MHC molecules provide processed endogenous antigen to CD8 T cells. Class I] molecules provide exogenous antigen to CD4T -cells. Class | molecules identifies all the body cells as 'shelf' and induce antibodies synthesis that enter the host having different class I molecules. This forms the base for MHC typing in patients undergoing antigen transplantation.

Class II molecules have D group of MH C responsible for stimulating antibody formation. They also participate inT-cell communication with macrophage and B cells. An important step in immune response is that prior to cytokine secretion by F cells, the class II molecules on the adjacent cell and identified by the T-cells receptor.

Microorganisms are identified by both class I and II molecules. These molecules engage in the susceptibility of an individual to specific non-infectious diseases, e.g., multiple sclerosis, acute glomerulonephritis, tuberculoid leprosy, paralytic poliomyelitis, etc. During immune responses the _ class III molecules (e.g., C2, C4a, and C4b) involve in the classical pathway and factor B (alternate pathway).

HYPERSENSITIVITY REACTIONS

4.4.1. Introduction

An elevated activity of normal immune system that damages the body tissues is known as_ hypersensitivity. Hypersensitivity, also termed as _ hypersensitivity reaction refers to inappropriate immune responses (like damaging, discomforting, and sometimes fatal). A pre-sensitised (immune) state of the host initiates hypersensitivity reactions.

4.4.2. Types

Different hypersensitive reactions are studied and differences in the effector molecules generated during the reaction are identified. Four types of hyper -sensitivity reactions (Types I, II, III and IV) were illustrated by Gell and Coomb. The initial three types are antibody -mediated and the last type is mediated by T-cell and macrophages (table 4.5).

Table 4.5: Gell and Coomb's Classification of Hypersensitivity Reactions

Types	Descriptive Names	Initiation Time	Mechanisms	Typical Manifestations
I	IgE-mediated or anaphylactic hypersensitivity	2-30 min	Antigen (Ag) induces cross-linkage of IgE bound to mast cells and basophils, along with the release of vasoactive mediators.	Systemic and localised anaphylaxis, hay fever , asthma, hives, food allergies, and eczema.
II	Antibody-dependent cytotoxic hypersensitivity	5-8 hrs	Antibody (Ab) directed against cell surface ; antigens mediate cell destruction by complement activation or ADCC	Blood-transfusion reactions, erythroblastosis foetalis, and autoimmune haemolytic anaemia.
III	Immune complex-mediated hypersensitivity	2-8 hrs	Ag-Ab complexes deposited in various tissues induce complement activation and an inf lammatory response.	Localised arthus reaction, generalised reactions, serum sickness, glomerulonephritis, rheumatoid arthritis, and s ystemic lupus erythematosus
IV	Cell-mediated or delayed type hypersensitivity	24-72 hrs	Sensitised T_{DTH} cells release cytokines that activate macrophages or T_C cells that mediate direct cellular damage.	Contact dermatitis, tubercular lesions, and graft rejection.
V	Stimulatory hypersensitivity	-	-	Thyroid hyperreactivity in Grave's disease

4.4.2.1. Type I Hypersensitivity

The most common hypersensitivity reaction is the type I hypersensitivity. These are anaphylactic reactions induced by antigens (allergens). Anaphylaxis (opposite of protection) occurs when IgE antibodies interact with an allergen.

Mode of Action

Upon exposure of the individual to an allergen, the B-cells are activated and IgE secreting plasma cells are formed. These cells attach with high affinity to Fc (Fragment crystalised) receptors present on the constant domains of mast cells (tissue) and basophils (blood). These mast cells and basophils are coated with IgE and are sensitis ed. Upon subsequent exposure to the same allergen, cross -linking of the bound IgE occurs. This results in the degranulation of the mast cells and basophils and release of pharmacologically active mediators from these cells (figure 4.7). The released mediators cause smooth -muscle contraction, increased vascular permeability, and vasodilation.

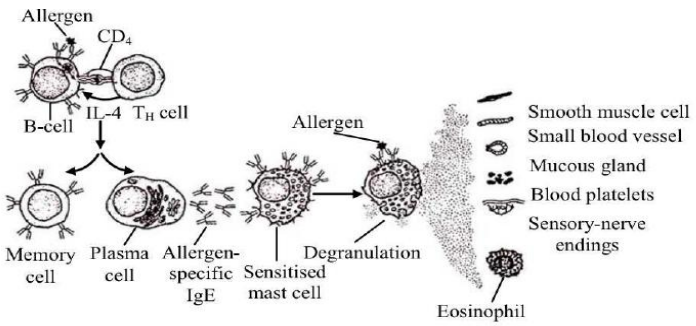


Figure 4.7: Mechanism of Type-I Hypersensitive Reaction

Biological Effects

1) Anaphylactic responses give rise to mild symptoms, like hay-fever, running nose, skin eruptions (hives) or breathing difficulties.

2) The granules release certain pharmacologically active mediators that impart biological effects on the surrounding tissues.

3) Sometimes anaphylactic shock may develop within 2-30 minutes which may lead to death if the individual is not attended with medical help.

4) The main effects of vasodilation and smooth muscle contract ion can be systematic or localised.

Examples: Allergic asthma, allergic conjunctivitis, allergic rhinitis (hay fever), anaphylaxis, angioedema, atopic dermatitis (eczema), urticaria (hives), and eosinophilia.

4.4.2.2. Type II Hypersensitivity

The type II hypersensitivity reactions cause tissue or cell damage as a direct result of the actions of antibody and complement.

Mode of Action: The reaction during blood transfusion is an example of type II hypersensitivity reactions. In blood transfusion, reaction occurs between the host antibodies and foreign antigens present on the incompatible transfused blood cells. This reaction mediates cell destruction.

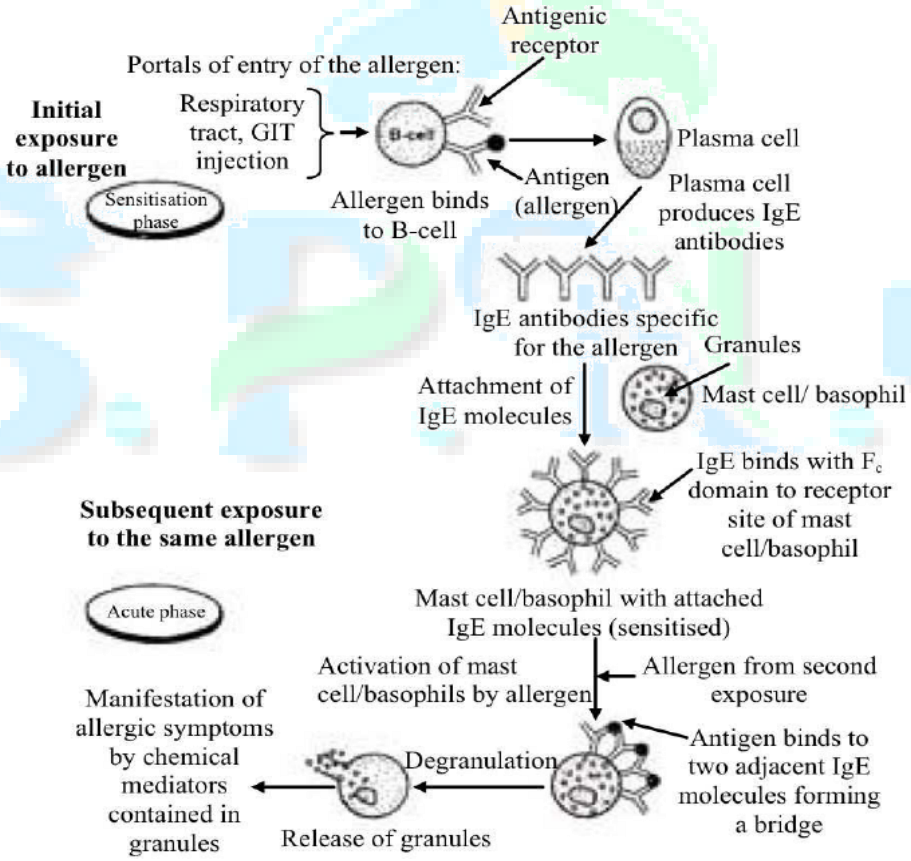


Figure 4.8: Events Following Initial and Subsequent Exposures to an Allergen Resulting in Sensitisation and Manifestation of Allergic Responses

Antibody-mediated cell destruction occurs through activation of complement system. This increases membrane porosity in foreign cell by forming Membrane Attack Complex (MAC). Cell destruction can also be mediated through Antibody Dependent Cell-Mediated Cytotoxicity (ADCC).

Haemolysis of the donor's erythrocytes occurs in the recipient's blood vessels as a result of faulty cross -matching in which the alloantigen of the donor's erythrocytes react with the serum antibodies of the recipient al = ong with the activated complement (figure 4.8).

Biological Effects

1) When the maternal IgG antibodies specific for antigens of foetal blood-group cross the placenta and destroy the erythrocytes of foetus, haemolytic disease occurs in new-born.

2) A haemolytic medical condition affecting the new -borns is erythroblastosis foetalis in which the Rh' foetus expresses an Rh antigen on its blood cells that the Rh mother does not express.

3) Drug-induced haemolytic anaemia occurs when some antibiotics (e.g., penicillin, cephalosporin and streptomycin) get non -specifically absorbed to proteins on erythrocyte membranes and form a complex (like hapten –carrier complex) that induces anaemia.

Examples

Autoimmune haemolytic anaemia Goodpasture syndrome, erythroblastosis foetalis pemphigus, pernicious anaemia (if autoimmune) , immune thrombocytopenia, transfusion reactions, Hashimoto's thyroiditis Graves' disease, myasthenia gravis, rheumatic fever, and haemolytic disease of the newborn.

4.4.2.3. Type II Hypersensitivity

Upon invasion of an antigen in an individual, the antibody reacts with it to form an immune complex which aids in the removal of antigen through phagocytosis. Type III hypersensitivity reaction occurs as a result of tissue -damage caused by large number of immune complexes. Hence, type III hypersensitivity reaction is also called immune complex hypersensitivity.

Mode of Action

When the complement system's array of immune effector molecules is activated by the immune complexes, type ILI hypersensitivity reactions develop. Splitting of complement components (C3a C4a and C5a) produce anaphylatoxins leading to degranulation of localised mast cell and rise in local vascular permeability. The so formed bulky antigen-antibody complexes aggregate and combine with the activated complement. This chemotactically attracts the polymorphonuclear leukocytes that release large quantities of lysosomal enzymes causing tissue damage.

Biological Effects

1) Antibodies are developed by the recipient of a foreign antiserum. These antibodies are specific for foreign serum proteins from circulating immune complexes. An individual develops serum sickness within days or weeks of exposure to foreign serum antigens. Symptoms of serum sickness include fever, weakness, vasculitis (rashes) with oedema, erythema, lymphadenopathy, arthritis and glomerulonephritis.

2) The IgG antigen complexes deposit in the blood vessels and cause local damage. When they deposit on blood vessels of kidney glomeruli, they cause Arthus Reaction.

3) Farmer's lung is a disease in which immune complexes are formed in the epithelial layers of the respiratory traatpon inhalation of bacterial and fungal spores.

4) Systemic lupus erythematosus isan autoimmune hypersensitive reaction (autoimmune) that occurs when IgG and the nucleoproteins of the disintegrated leukocytes (auto-antigens) interact.

Examples

Immune complex glomerulonephritis, rheumatoid arthritis, serum sickness, sub-acute bacterial endocarditis, symptoms of malaria, systemic lupus erythematosus, Arthus reaction, and Farmer's lung (Arthus-type reaction).

4.4.2.4. Type IV Hypersensitivity

Type IV hypersensitivity reaction is a type of delayed hypersensitivity controlled by T-cells, macrophages, and dendritic cells. It does not occur as an instant response but after the second exposure to an allergen. The allergic symptoms appear after sometime.

Mode of Action

The T- lymphocytes play a major role in maintaiming delayed hypersensitivity. They are categorised into CD4~ and CD8" cells. The former cells are required in type IV hypersensitivity reactions. The spe cial group o f CD4" cells called T –D cells (delayed), participate in this reaction. The T-helper cell (T_H cell) includes TD cells that make the bulk of CD4_~ T-cells. The T 4 cells are categorised into Ty and T yp type. The latter cells acti vate B-cells to produce immunoglobulins and the former cells initiate inflammatory responses like delayed hypersensitivity reactions (figure 4.9).

Biological Effects

1) Tuberculin is a purified protein derivative (PPD) of tubercle bacilli (Mycobacterium tuberculosis). It is a microbi al agent that triggers delayed hypersensitivity. The microbial agents obtained from Mycobacterium leprae also stimulate delayed hypersensitivity.

2) The tuberculin skin test (or Mantoux test) determines whether a person presents T-cell mediated reactivity for tubercle bacilli (Koch's bacilli).

Examples

Contact dermatitis (poison ivy rash) , temporal arteritis, symptoms of leprosy and tuberculosis, transplant rejection, and celiac disease.

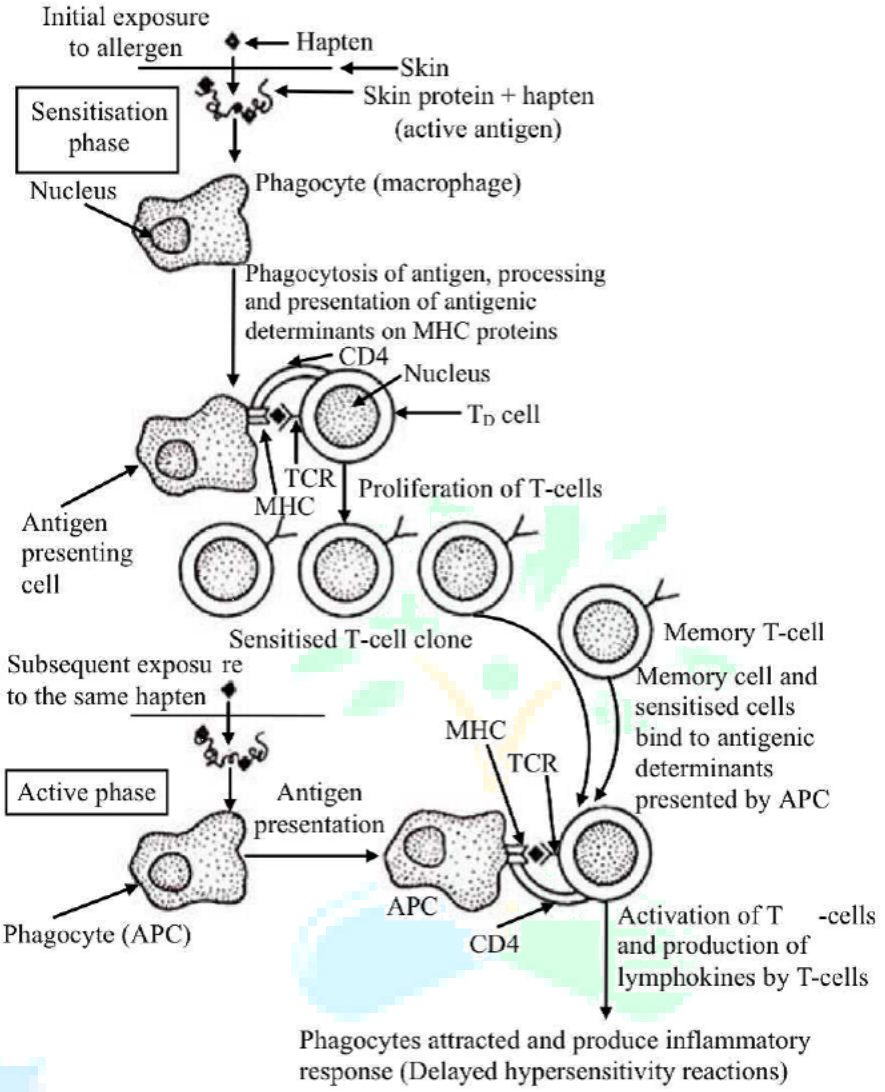


Figure 4.9: Delayed hypersensitivity reactions like skin -contact sensitivity to small molecules acting as haptens. During the sensitisation phase, exposure leads to formation of sensitised T -cells. A subsequent exposure to the same hapten stimulates the sensitised T -cells to produce lymphokines which attract phagocytic cells and cause an inflammatory skin response (allergic dermatitis).

4.4.2.5. Type V Hypersensitivity

The type V-stimulatory hypersensitivity reactions occur when the antibody undergoes specific reactions with a key surface component, e.g., hormone receptor, and switches on the cell.

Examples

A typical example of type V-stimulatory hypersensitivity is thyroid hyperreactivity in Grave's disease due to a thyroid-stimulating autoantibody.

4.4.3. Immune Stimulation

Stimulation of the immune system by an external source is termed immune stimulation or immunostimulation. This stimulation builds up a protective mechanism against microorganisms. Substances that induce or elevate the activity of one or more of the immune system components are termed immunostimulants.

Immunostimulants stimulate the immune system. These agents enhance the body's resistance against infections They stimulate the mmune system by fighting against immunodeficiencies (like AIDS), infections and cancers. Immunostimulants enhance the basal levels of immune response. Immunotherapeutic agent are given to individuals with impaired immune response.

Some commonly used immunostimulating agents are:

1) Levamisole: It is an anthelmintic drug thatrestores the functions of B - lymphocytes, T-lymphocytes, monocytes, and macrophages. Thus, it is used in combination with 5-FU in colon cancer.

2) Thalidomide: It produces different effects that are utilised in the following conditions:

i) Its anti-inflammatory effect is used in erythema nodosum leprosum.

ii) Its anti-angiogenesis effect is used in multiple myeloma.

iii) Its anti-TNF effect is used in rheumatoid arthritis.

3) BCG: It is used in bladder cancer.

4) Recombinant Cytokines

i) Interferons: They are used in tumours and chronic hepatitis B and C.

ii) Interleukin 2 (Aldeslukin): It is used in renal cell carcinoma and melanoma.

4.4.4. Immune Suppression

A phenomenon in which an organism's ability to form antibodies in response to an antigenic stimulus is reduced or suppressed is termed immune suppression or immunosuppression.

There are many available immunosupp ressive drugs that are used for prolonging the life -expectancy of a transplanted organ, e.g., heart, kidney, eyes, etc. When small doses of immunosuppressive drugs are administered to an individual, his/her immune system overcomes the drug and rejects it. This rejection is indicated by the gradual functional loss of an organ giving rise to the following symptoms:

1) Fibrous thickening of the inmost small arteries of the transplant.

2) Subsequent administration of larger doses of immunosuppressive drugs as an alternative measure, and

3) Scheduling a different immunosuppressive therapy programme for each individual.

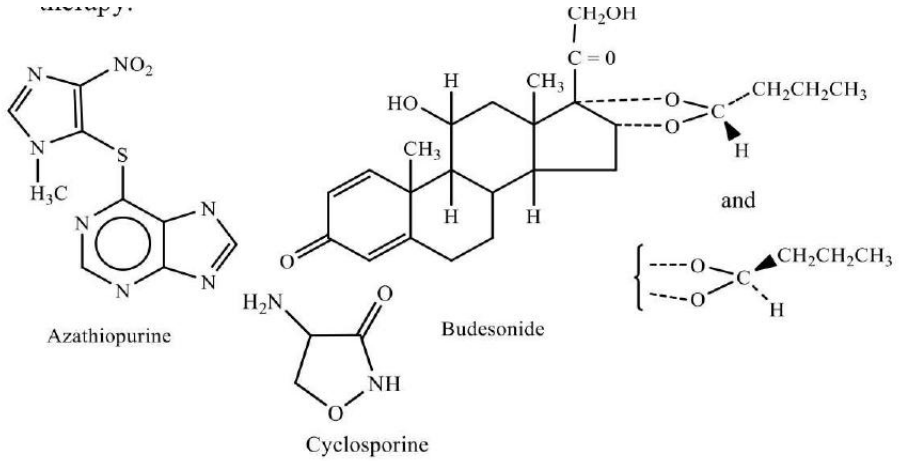
However, another perception of immunosuppression promulgates the resultant immune response as a coordinated mechanism involving antigen recognition by the immunocompetent cells , termed as Anti-T-lymphocyte Serum (ATS); besides, macrophages found to serve as an Antigen-Presenting Cells (APC). Immunosuppression is of the following two types:

1) Non-Specific Immunosuppression: It occurs in the natural cases of immunodeficiency disorders, uremia, etc. It may also be inducted by gradual depletion of lymphoid tissue, or by administering immunosuppressive drugs. A significant depletion in lymphocytes is observed on excessive exposure to radiation; thus the antigens present on the macrophages become impaired and give rise to immunosuppression. Non-specific immunosuppression can also be induced by Anti -Lymphocyte Globulin (ALG) that affects the T -cells by

inhibiting their normal functions or by depleting T -cell dependent areas in the lymphoid tissue.

2) Specific Immunosuppression: It is induced by antigen (immunogen) or antibody. It has been proved experimentally that it is practicable and reasonable to induce the following specific immunosuppression measures:

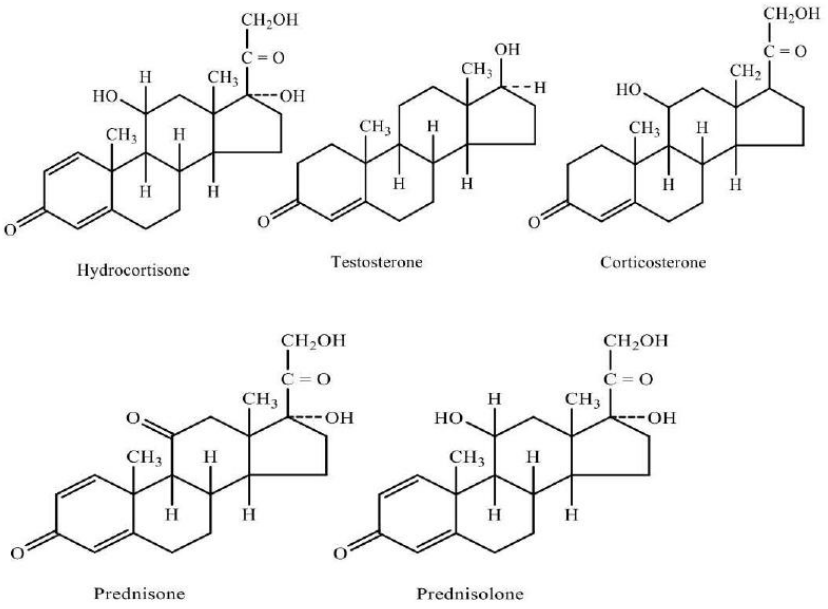
i) Some drug combinations, e.g., azathioprine and corticosteroid (Budesonide) are used in tissue transplantation to inhibit Cell –Mediated Immunity (CMI). Cyclosporine is widely used in immunosuppressive therapy.



ii. In some cases, in order to achieve tolerance the response of a host to a specific antigen is eradicated. Therefore, it is a particular case of immunosuppression where an antigen (immunogen) behaves as an immunosuppresive agent.

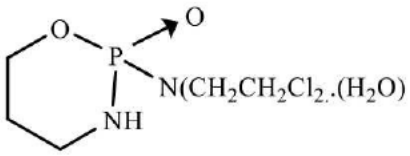
iii. The body on being exposed ionising radiations suppresses the lymphocyte proliferation inthe haemopo _ietic system. The overall effect is non -specific immature, causes impairment of haemopoiesis _, and periodical inoculation of the syngeneic bone marrow cells in the animal.

iv. A specific class of endogenous corticosteroids, e.g., hydrocortisone, testosterone, corticosterone, prednisone, and prednisolone are used to impair the prevailing immune response of the host, i.e., CMI.

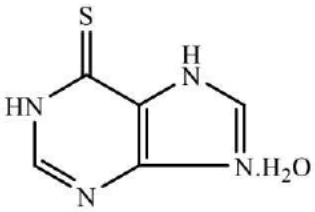


Some other drug substances obtained from natural or synthetic sources that can be used as immunosuppressive agents are:

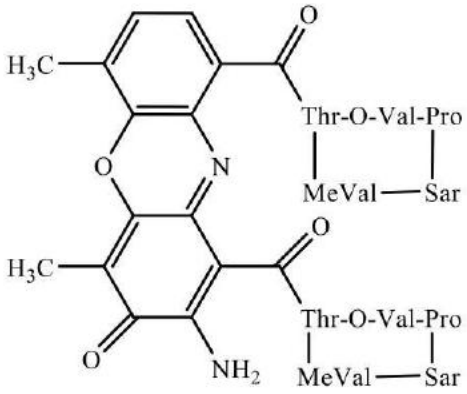
a) Antiproliferative Agent: Cyclophosphamide.



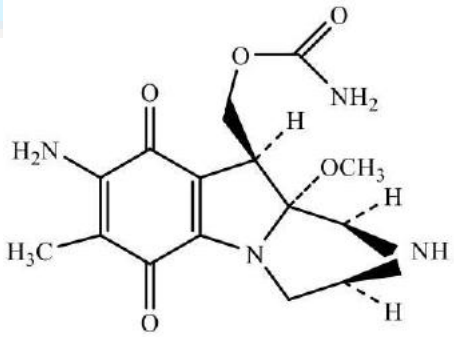
b) DNA-Base Analogue: 6-Mercaptopurine and its derivatives.



c) Antibiotic: Actinomycin-D.



d) Mitotic Poison: Mitomycin C.



vi) Anti-T-lymphocyte Serum (ATS) is also used to battle specific immunosuppression. It is a T-cell inhibitor restricted to the re-circulating peripheral lymphocytes; however, the lymphocytes within the lymphoid organs remain unaffected.

vii) The procedure of inhibiting CMI with the help of homologous humoral antibody in tissue/organ transplantation is termed as enhancement. The survival period of transplants can be enhanced only by infusing a particular antibody to weaken the phenomenon of CMI, whereas the formation of the humoral antibodies is protected.

IMMUNIZATION- VACCINES

4.1.1. Introduction

The term vaccine has been derived from the Latin word **vacc** which means **cow**. But according to medical definition, vaccines are pharmaceutical suspensions or solutions of immunogenic substances intended to induce active immunity.

Previously, the term vaccine had limited use only for specialised compounds which necessarily consist of whole microorganisms; however, nowadays the term vaccine is applied to all the agents of active immunisation. The process of active immunisation is known as vaccination.

According to British Pharmacopoeia , 'vaccines are preparations containing antigenic substances capable of inducing a specific and active immunity in man', Vaccines may also be considered as curative agents , which are predominantly derived either by direct or indirect means from disease-causing microorganisms.

5.1.2. Classification

Vaccines are classified as follows based on the type of preparation involved in their manufacture:

1) Inactivated-Killed Vaccines: These vaccines are prepared either by killing or inactivating the pathogen using heat or some chemical, thus altering its antigenicity. These vaccines are first administered as a primary dose and then as booster dose(s).

The inactivated-killed vaccines may be:

i) Bacterial Vaccines: Vaccination against typhoid, cholera, plague, haemophilus influenza, whooping cough, and meningococcal infections. The patients show a delayed antibody response which may appear after a week or several weeks. These vaccines are ineffective when introduced during the incubation period or when the disease is in active stage.

ii) Viral Vaccines: Rabies, influenza, hepatitis and polio vaccines.

2) Live Attenuated Vaccines: In these vaccines, a non-virulent microorganism is used that retained its antigenicity. | These vaccines make an individual permanently immune (i.e., for a lifetime) against a disease. These vaccines should not be administered to immune-compromised patients (with a weak immune system).

The live-attenuated vaccines may be:

i) Bacterial Vaccines: BCG.

ii) Viral Vaccines: MMR and polio sabin.

3) Toxoids: These preparations are prepared by the treatment of bacterial toxins with formalin. Thus , evenif the toxicity of bacterial toxins is lost, — their antigenicity is retained. Tetanus and diphtheria toxoids are the examples of commonly used ones.

5.1.3. General Method of Preparation

Bacterial vaccines are either sterile suspensions of live or killed bacteria or are sterile extracts of bacterial derivatives. Toxoids are modified toxins which have been detoxified by moderate heat and chemical treatment in order to retain their antigenic property. Thus, they are the toxins whose toxicity has been removed. Toxoids are used for the development of active immunity.

Antisera (singular antiserum) are blood serum containing antibodies and renders passive immunity to an individual against several diseases. Antiserum is also known as_ antitoxin. Injection of antisera provides immunoglobulins or antibodies. Injection of an antigen in animals causes diseases and the resultant antibodies are obtained in the form of antisera. However, probabilities of occurrence of allergic reactions are high because the antibodies are acquired from other animals. Besides being curative, antisera also provide immediate immunity.

5.1.3.1. Bacterial Vaccines

Bacterial vaccines are prepared using the selected strains of bacteria; thus, the bacteria are first tested for purity and identity. The steps involved are:

1) The selected strains of bacteria are cultivated on solid medium for 1 -2 days and then washed with sterile normal saline.

2) The suspension obtained is shaken for uniform distribution.

3) The fragments on the medium (if present) are removed by centrifugation or sedimentation.

4) The suspension is then sterilised by heat treatment or by using alcohol or other bactericides.

5) Vaccines prepared from cultures of non -sporing bacteria are sterilised in a vaccine bath for an hour at 56-60°C temperature to kill all the bacteria.

6) The heat applied should kill the microorganisms but should also retain their antigenic properties.

7) After sterilisation of the suspension, the bacterial concentration in it is determined, desired dilutions are prepared, and a preservative (bacteriostatic agent) is added.

8) The product is filled into previously sterilised containers and sealed under aseptic conditions.

Vaccines are standardised by determining the number or the dry weight of microorganisms in 1ml of the product. As a safety measure against deterioration, such products are not used after 18 -30 months of preparation. The vaccines should be stored at a low temperature (above 0 °C and not exceeding 20 °C). The containers should bear a label with the following contents:

1) Preparation name,

2) Number or the dry weight of microorganisms in 1ml, and

3) Nature and strength of the substance (other than the diluent) with which the vaccine is combined.

The label on the container or the wrapper on the pack should bear:

1) The name, address and licence number of the manufacturer,

2) The preparation date,

3) The date up to which the potency can be retained under proper storage conditions,

4) The name and percentage of antiseptic added,

5) The precautionary measures for storage, and

6) The batch number.

5.1.3.2. Toxoids

Formal Toxoid (FT) is prepared by selecting a suitable strain of bacteria, cultivating on a liquid medium, and incubating under optimum conditions until a satisfactory level of toxin is produced. Microorganisms are retained on the paper pulp and by filtration the filtrate is sterilised. Formaldehyde solution is added and the mixture incubated at 37^0C to remove toxicity. This complete process takes 2 months. Alum Precipitated Toxoid (APT) is a high quality formal toxoid which has been treated with charcoal to remove the colouring matter and other impurities. The charcoal is then removed by filtration. Alum is added in a suitable concentration to react with the bicarbonate, phosphate and protein impurities. As a result, a precipitate (containing aluminium hydroxide and phosphate) is formed on which the toxoid is adsorbed. Then the precipitate is washed and suspended in a bactericide-containing saline. The product is obtained in the form of a depot.

Purified Toxoid Aluminium Phosphate (PTAT) is a purer form of toxoid in comparison to APT. It is prepared using a serssynthetic medium excluding the non specific material. It can also be prepared by purifying the toxoid with magnesium hydroxide to precipitate colour, phosphate, and some proteins. PTAT like APT also has a depot effect (slow release of antigen in the tissues to provide a prolonged effect).

5.1.3.3. Viral Vaccines

Viruses grow on a living medium (contrary to bacteria which can grow on non - living media). They cause infectious diseases in humans, animals, and plants. Some common viral diseases are influenza, common cold, measles, mumps, poliomyelitis, smallpox, yellow fever, rabies, etc. Viral vaccines are prepared using free living animals, fertil e eggs, and tissue cultures. Viruses are grown in the chorioallantoic membrane of incubated fertile hen eggs or in whole animals.

5.1.3.4. Antitoxins

Passive immunisation is needed when the immune system of the host is not capable of producing enough number of antibodies or when the host is already infected. Thus, passive immunisation is a method for curative measurements, and not preventive. The term antiserum (antisera) is used because the antibodies remain present in the serum part of the blood of an animal. Normally, y-globulins are present in the antiserum that is produced in the serum by an artificially inoculated animal. Antisera can also be collected from humans recovering from viral infections (e.g., mumps). For commercial production mainly horses are used. A small dose of tetanus toxins is introduced into the blood of the horse that makes the horse to produce antibodies against tetanus. The antibodies developed against specific toxin are known as antitoxins. Commercially antitoxins are produced for the control of diphtheria, botulism, and tetanus.

The method for the production of stable and concentrated antiserum was first given by **Gracia** in 1976. Normal serum is collected from the previously immunised animals and kept for fractionation into two fractions. First part contains high molecular weight globulins and albumins, whereas the second part contains low molecular weight globulins and albumins.

The first fraction of high molecular weight protein and antibody is treated and the lipid part of itisremoved . The serumisre -precipitated to obtaina highly concentrated antibody paste. The second part of serum that has a low molecular weight fraction is further treated to give a stable protein solution having albumins, and a- and B-globulins. This part is used as diluent for antibodies and other biological matters. The antisera obtained by such procedure have many advantages over the starting material, i.e., it has greater antibody potency, absence of any particulate or foreign infectious agent, as well as remains stable over a wide range of temperature (5-40°C).

5.1.3.5. Serum

Serum is the liquid fraction of whole blood collected after the blood is allowed to clot. The clot is removed by centrifugation and the supernatant (called as serum) obtained is removed with a Pasteur pipette.

Whole blood is collected in a covered test tube and left undisturbed at room temperature to allow clotting. Clot is formed in 15 -30 minutes, and it is then removed by centrifugation for 10 minutes in a refrigerated centrifuge. The supernatant (liquid component) obtained after centrifugation is the serum, which is quickly transferred to a clean polypropylene tube with the help of a Pasteur pipette. The samples should be maintained at 2 -8°C while handling. If the serum is not analysed instantly, it should be distributed into 0.5ml aliquots, stored, and transported at —20°C or lower temperature. Freeze-thaw cycles should be avoided because this is detrimental to many serum components. Samples which are haemolysed, icteric, or lipemic can invalidate certain tests.

5.1.4. Storage Conditions and Stability of Official Vaccines

Many biological preparations become unstable during storage and thus their safety and efficacy are reduced. Proteins and other macromolecules may be sensitive to heat, light, radiation, and environmental changes, or they may interact with the container materials or other components of the vaccine mixture.

Determining these relationships and optimising stability from the time of production to administration is an important part of vaccine development. The potency of vaccine may reduce as a function of the time elapsed since production even if optimal conditions are maintained.

The stability of vaccines may undergo significant changes after being exposed to temperature fluctuations during handling or storage. Thus, their stability characteristics should be determined through testing, and the storage and handling conditions should be defined to ensure that minimum standards of potency, identity, and purity continue to be met throughout the shelf-life.

5.1.4.1. Bacillus Calmette-Guerin or BCG Vaccine

Bacillus Calmette -Guerin (BCG) vaccine provides immunisation against tuberculosis. It is prepared using live attenu ated (weakened) bovine tuberculosis bacillus strain of Mycobacterium bovis, which has lost its virulence in human.

Preparation

A pure culture of an authentic strain of attenuated Mycobacterium tuberculosis (of Calmette and Guerin) is grown for 2 weeks on a suitable media. The attenuated strains are collected and suspended in a liquid medium designed to

preserve the antigenicity and viability of vaccine. It is dispensed either in the freeze dried form or in liquid form.

Method of Administration

Before administering the BCG vaccine, a tuberculin skin test should be done in the patients (except in neonates). If a patient with a positive tuberculin reaction is administered with the vaccine, he/she is at a high risk of severe local inflammation and scarring. It is a false belief that people showing positive tuberculin reaction do not require BCG vaccine as they already possess immunity against it; however, these patients should further undergo a screening for the presence of active tuberculosis. BCG is administered as a single injection given via intradermal route at the point of insertion of the deltoid muscle. Accidental subcutaneous administration develops a localised abscess (known as a BCG -oma), which further leads to ulcerations and is treated with antibiotics.

Storage Conditions and Stability

BCG vaccine is freeze-dried and should be stored in dark places at 2 -8°C temperature. It is more stable if stored at temperatures as low as —20°C. It should be protected from light. The diluent should be stored in a cool place. The diluents should not be frozen or else the vaccine will denature due to freezing and thawing. During transportation, the vaccine should be kept on ice in a cool bag.

BCG is very sensitive to heat, light, and neon light. Under suitable storage conditions, the effectiveness of the vaccine can be maintained even after two years of manufacture. The recommended maximum time for which BCG vaccine can be stored and consumed (i.e., its expiry) is two years from the date when the last potency test performed yielded satisfactory results. However, the vaccine remains effective for 2 years only when stored in dark under the required temperature.

5.1.4.2. Pertussis Vaccine (Whooping Cough Vaccine)

Pertussis (or whooping co ugh) is an acute, communicable disease, caused by Bordetella pertussis (asmall,non -motile, and gram -negative bacillus). This bacterium takes 7-10 days for incubation; a catarrhal stage follows the peculiar paroxysmal cough that ends in a whooping inspiration. Pertussis can be prevented if the infants are immunised against it from the time they are of three months. The patients of whooping cough are provided symptomatic and supportive treatment.

Pertussis vaccine is a whole cell preparation obtained from the killed Bordetella pertussis. It is also the third constituent of the DTP-triple vaccine.

Preparation

1) B. pertussis isolated from an infected patient is dried and cultured under specific culture conditions. This phase of collecting a sterile suspension of B. pertussis is the Phase-I.

2) The **cup-plate method** is used for isolating the microorganisms from the patient, which are then collected once they are in a smooth and virulent form, generally referred to as Phase -I. It has been observed that microorganisms in Phase-I appear smooth, but when grown on an unfavourable media, a S → R variation takes place with the loss of important antigens. Phase –IV corresponds to a fully developed rough form.

3) **Bordet-Gengou blood agar media** is used for growing the microorganis ms and then incubating them for 24-74 hours.

4) Then it is suspended in NaCl -containing injection, and the obtained suspension is centrifuged. The bacterial mass is then shifted to NaCl injection consisting of 0.01 -0.02% thimerosal to prevent the growth of bacteria, without altering the antigenic properties of the prepared vaccine.

Standardisation

Each dose of 0.5ml should have 7.5µg of pertussis antigens, which are expressed as protein nitrogen. The obtained vaccine constitutes inactivated PT and FHA proteins each in 23.4 µg amounts.

Storage Conditions and Stability

The concentrated preparation obtained is stored for 3 months in a refrigerator to reduce its toxicity. After 3 months, the preparation is diluted with adequate amounts of NaCl injection so that 1 ts opacity is greater than twice to that of the standard preparation in a final concentration of 0.01% of thiomersal. Thus, thiomersal kills the present microorganisms. This vaccine does not require heat treatment.

5.1.4.3. Cholera Vaccine

Cholera vaccine is the homogeneous suspension of strains of Vibrio cholera. This vaccine is either dispensed in liquid form or prepared instantly before use by reconstituting the dried vaccine with an appropriate sterile liquid.

Preparation

Equal portion of the strain Inaba and Ogawa of Vibrio cholera are used for preparing cholera vaccine. These strains are selected to provide high proficiency against antigens. Initially, each strain of Vibrio cholerae is grown individually on a solid medium for 1-2 days (seed-lot system). Then the bacteria are washed with a normal saline solution. Thereafter the bacterial suspension is either heated at 56°C temperature for one hour or treated with a bactericide(e.g., phenol or formaldehyde). The vaccine can also be added with a preservative. This process kills the suspended bacteria.

Standardisation

Two variants of cholera vaccine are available. The first one is evaluated in mice and consists of inactivated strains of V. cholera (similar to cellular typhoid vaccine). The effectiveness of the cellular and LPS vaccines depends on their ability to derive serum vibriocidal antibodies. The second type of vaccine consists of an attenuated orally administered strain of V. cholera.

Storage Conditions and Stability

Cholera vaccine should be stored at 2-8°C temperature, and should not be frozen.

5.1.4.4. Tetanus Toxoid

Tetanus vaccine is a clear liquid which may appear either colourless or brownish yellow. It may also be slightly turbid in appearance and is free of visible particles or lumps. It has a characteristic odour of formaldehyde.

Preparation

Tetanus toxins produced in the growing phase of the bacterium Clostridium tetani are used for preparing tetanus vaccine. The tetanus formol toxoid is prepared by the treatment of tetanus toxoid with formaldehyde solution.

Tetanus toxoid adsorbed or adsorbed tetanus vaccines are obtained by adsorbing the tetanus antigens on aluminium phosphate or aluminium potassium sulphate (acting as a mineral adjuvant).

Standardisation

The end-products should meet the established strength of 5 -7.5 Lf units in each 0.5ml of the dose.

Storage Conditions and Stability

Tetanus toxoid should be stored at 2-8°C (35-46°F) temperature, and should not be frozen. If the product has been frozen, it should not be used.

5.1.4.5. Rabies Vaccine

Rabies vaccine is a fluid or a freeze -dried preparation consisting of rabies virus (a neurotropic virus), which has been cultivated either in the neural tissue of sheep, goats, rabbits, mice, or rats or in the cell cultures.

Preparation

Method-I (Seed-Lot System)

1) The virus used in the final vaccine represents not more than five cultures from the seed lot used for producing vaccine on which laboratory and clinical tests were carried out to determine the suitable strain.

2) For initial cell growth, animal serum (in concentration not more than one part per million) may be used in the medium; however, no protein is present in the medium used for maintaining the cell cultures during virus multiplication.

3) The cell culture medium contains a suitable pH indicator (e.g., phenol red) and antibiotics in smallest effective concentrations.

4) Virus suspension is harvested one or more times during incubation. Multiple harvests from a single cell lot are regarded as a single virus suspension.

5) The suspension obtained is tested for identity, bacterial sterility, and freedom from extraneous viruses. If the suspension complies with these three vital tests, it is inactivated, purified, and concentrated.

6) The amplification test should be carried out for the residual infectious rabies virus in cell cultures derived from the species employed in vaccine production to confirm inactivation of the rabies virus. The quantum of virus used is equivalent to not less than 25 human-doses of the vaccine.

7) **Test for Live Virus:** The samples of cell -culture fluids are inoculated into mice, and no live virus is detected.

8) The resulting rabies vaccine is aseptically transferred into sterile containers (e.g., ampoules) and sealed hermetically for freeze drying. The inherent residual moisture content is low enough to ensure vaccine stability.

9) **Potency Test:** The maintenance of potency is verified in an accelerated degradation test in which the vaccine is store at 37°C temperature for 4 weeks.

Method-II (From Chick and Duck Embryos)

Rabies vaccine should be prepared from tissues lacking essential encephalitogenic material. Therefore, the rabies virus strains are grown in chick or duck embryos. The viruses grown in duck-embryo are inactivated by Beta-propiolactone.

Method-III (From Brains of Suckling Mice, Rats, Rabbits, and Sheep)

The rabies vaccines prepared using brains of suckling mice, rats, rabbits, and sheep should follow the vital potential standards. The Ph armacopoeial method involves the following steps:

1) The rabbits and sheep are infected intracerebrally with fixed rabies virus.

2) After 24 hours the expected symptoms appear and the rabbits or sheep become completely paralysed.

3) Thereafter they are killed and t heir brains are harvested and homogenised in NaCl injection.

4) The resulting viruses are inactivated using phenol (the chemical of choice) or other chemical sub _ stances like formaldehyde solution (formalin), Beta-propiolactone or UV-light.

5) The preparation is diluted to contain appropriate amount of brain material.

Standardisation

According to the WHO standards, the rabies vaccine should possess a potency of 16 International Units of Rabies Vaccine (based on the immunogenicity assays) per ampoule. Also, 10 International Units (IU) of the Rabies Virus Glycoprotein and 135 IU of the Rabies Virus Ribonucleoprotein has been assigned to the contents of each ampoule.

Storage Conditions and Stability

Freeze-dried rabies vaccine should be stored at 2-8°C (35-46°F) temperature, and should not be frozen. The vaccine remains stable only when the storage conditions are strictly followed.

5.1.4.6. Polio Vaccine

Polio is an acute infectious inflammation of the anterior horns of the grey matter of the spinal cord, which results in acute systemic infectious disease and paralysis. If the disease is mild, it is limited to respiratory and gastrointestinal symptoms lasting from a few days to weeks; however in case of a major infection, muscle paralysis or weakening along with loss of superficial and deep reflexes occurs. Polio is caused by poliovirus, which initially inhabits the gastrointestinal tract, specifically the oropharynx and the intestine, and infects the mucosal cells of the oropharynx and the int estine. In most of the patients, the virus settles in the gastrointestinal tract, and is thus completely replaced by the cells.

Variants of Polio Vaccine

1) Oral Polio Vaccine (OPV): This variant was initially developed by Sabin (an American researcher), and is a mixture of live attenuated strains of the three polio viruses, i.e., Type 1, 2, and 3:

i) OPV is given orally to the children when they are of 3 months, 4.5 to 5 months, and finally at 8.5 to 11 months. These doses are administered at the same time when the initial course of triple DTP vaccination is given.

ii) A booster dose is given orally when the children are of 4 to 5 years and also when of 15 to 18 years.

iii) The polio virus grows in the lymphoid tissue associated with the gut epithelium; thereby generate local and humoral immunity.

iv) The faecal excretion of the vaccine strains persist for a few weeks, thus the family members of the children given OPV are advised on hygienic handling and proper disposal of napkins/diapers, etc. Any non –immune family member should also be immunised.

v) OPV should not be given to hypogammaglobulinaemic children.

2) Salk Type Polio or Salk Filled Vaccine: This vaccine is a formaldehyde inactivated mixture of the strains of the three types of polio viruses (i.e., Type 1, 2, and 3), and is highly effective. In several countries, this vaccine was later replaced with Sabin type polio vaccine.

Preparation

i) The type 1, 2, and 3 polio viruses are separately grown in suspended or fixed cell cultures of monkey (Rhesus monkeys) kidney tissue.

ii) Rhesus monkeys are quarantined on arrival and checked for TB and other communicable diseases before as well as after death.

iii) The monkey kidney cell should not have been propagated in series and are obtained from a continuous line of cell.

iv) For both types of vaccines, the inclusion of serum in the culture media used for maintaining cell growth during virus propagation is prohibited; however, the serum may be included in the media used to initiate the growth of tissue cells. This prevents the serum reactions on administration of such preparations. This condition is more important in the inactivated vaccine administered parenterally; and thus a prescribed limit of 1ppm of serum is added in the final product.

v) Healthy Rhesus mon keys are anaesthetised and their kidneys are removed and decapsulated. Their cort ical tissues are coarsely disintegrated and suspended in Number 199 substrate.

vi) The chopped tissues are treated for 20 minutes with several lots of dilute, warm trypsin soluti on (each of 0.5% concentration and 7.6 pH). As a result of this treatment, the tissue framework undergoes partial hydrolysis, and helps in separating the cells into a free suspension while maintaining their viability and efficacy.

vii) The cells are then centri fuged, washed, and re -suspended in a complex medium (of a density of 3 x 10^5ml), which is either an admixture of No. 199 substrate plus calf -serum or an admixture of lactoalbumin hydrolysate plus serum.

viii) The suspension obtained is inoculated into a largeressel and incubated for 5 days so that the cells properly form a monolayer on the glass surface.

ix) When optimal growth has been achieved, the liquor is poured off and the cells are washed with BSS -medium. After adding fresh medium, a small inoculation withone specific strain on the ‘virulent stock’ virus is made.

x) Separate batches for each type are made and incubated for 3 days till the full effect of the virus degeneration takes place. By this time, the medium contains free virus particles in high concentration.

xi) The debris is removed by centrifugation and the supernatant layer is cooled. If these are not required immediately, the deep frozen strains are kept separately which can now be batched to larger volume(s) as per the requirement.

xii) After the completion of this harvesting step, the virus suspension obtained is tested to confirm the presence of the correct strain of polio virus, and also to confirm that the virus litter is above the specified bare minimum level. It is also checked whether or not the suspension is free from viral, bacterial and fungal contaminants.

xiii) Then the suspension is passed through the filters having increased fineness so that the remnants of tissue cells (viruses from the inactivating agent) and bacteria can be removed.

xiv)Liquors contai ning the virus particles are treated with dilute formaldehyde (0.01% v/v) under optimum conditions of pH and temperature using a magnetic stirrer. The tissue cells and bacteria can be removed in 6 days, however in actual practice 12 days are required to ascertain 100% absence of any active virus.

xv) The suspension may be refiltered at the half -way stage (i.e., after a week). The rate at which inactivation takes its normal course is followed for several days on regular intervals; and subsequently between the 9" and 12" day larger samples are tested for the total and absolute absence of the infecting virus.

xvi) The formalised and sterile viral solution is subjected to dialysis and checked properly. The univalent vaccines of the three strains are blended to form the desired trivalent product. At this stage, large numbers of samples are again re -examined to confirm that they are free from the infective virus. Finally, the added formaldehyde solution is neutralised by adding sodium metabisulphite.

xvii) A requisite quantum to thimerosal is added as a bactericide. An aliquot of soluble disodium edetate is also added to sequester heavy metals (as chelates) that wo uld otherwise decompose the thiomersal into products toxic to the virus.

xviii) The entire procedure from the beginning to the final stage completes in 120 days. Most significantly, the toxoid is prepared for intramuscular injection by emulsifying it with mineral oil containing 3% highly purified mannide monooleate and 0.01% thimerosal (added as a preservative).

3) Sabin Type Polio Vaccine: The vigorous activity in this direction has revived with the development and broad - scale usage of Oral Polio Vaccine (OPV), comprising of living attenuated strains of the virus. However, the principle of its action is that these virus particles proliferate in the gut and release their modified toxins, which get absorbed directly into the blood stream and induce the formation of specific antibodies.

Preparation

i) The manufacturing procedure of this vaccine is the same as that of the Salk vaccine, except in one aspect that the attenuated strains prepared by rapid passages through tissue cultures of monkey kidney cells are employed.

ii) The virus in the final vaccine should not represent more than three sub - cultures from a strain that has been proved satisfactory by laboratory and clinical tests. This, however, decreases the chance of using a vaccine which has been rendered either more virulent or lost antigenicity.

iii) No activation stage exists in this specific vaccine.

iv) This vaccine is tested to be free fr om the extraneous bacteria, moulds, and viruses. Also special tests are required to confirm the absence of virulent polio virus.

4) Inactivated Poliomyelitis or Inactivated Polio Vaccine (BP -1993): This vaccine is an aqueous suspension of appropriate strains of poliomyelitis virus (types 1, 2, and 3) grown in suitable cell cultures and inactivated by a suitable method. This vaccine is obtained as a clean liquid.

Preparation

i) The inactivated polio vaccine is prepared based on a seed-lot system. The virus used in the final vaccine represents 10 sub -cultures (from the seed lots) used for producing the vaccine on which the laboratory and clinical tests to prove the strains to be suitable were carried out.

ii) Animal serum is used in the medium for the initial cell growth but no protein is present in the medium used for maintaining cell culture during virus multiplication. The concentration of serum in the vaccine is not more than 1 ppm.

iii) A pH indicator (e.g., phenol red) and suitable antibodies (in smallest effective concentrations) are also added in the medium.

iv) Each virus suspension is tested for its identity and bacterial sterility. After neutralisation they are also tested with specific antiserum to get rid of extraneous viruses.

v) The virus suspension is then filtered, concentrated, and purified.

vi) The suspension for each type of virus should contain not less than 7.0 $\log_{10}$ CCID $50mL^{-1}$.

vii) Within a suitable time period of the last filtration (within 24 hours), suitable chemical substances are added which inactivate the virus filtrate without affecting its antigenicity. During inactivation, filtration is performed, and the inactivating substance is later neutralised.

viii) Each monovalent suspension is tested in cell cultures to prove to be free from infective poliomyelitis virus and other human and simian (i.e., monkey like) viruses.

ix) The trivalent vaccine is prepared by mixing suspensions of each type.

x) Before any antimicrobial preservative is added, the trivalent suspension is tested to be free from infective poliomyelitis virus and other human and simian viruses.

5) Poliomyelitis Vaccine, Live (Oral) or Polio Vaccine Live (Oral) (BP -1993):

This vaccine is an aqueous clear suspension of live, attenuated strains of poliomyelitis virus (types 1, 2, or 3), grown in suitable cell cultures. The vaccine either contains any one of the three types of virus or a mixture of two or three of them.

Preparation

i) This vaccine is prepared based on a seed -lot system. The final vaccine represents not more than three sub -cultures (from the vaccine) made by carrying out the laboratory and clinical tests to show the strains to be suitable.

ii) Each type of polio virus is grown in cultures that do not contain any extraneous microorganism.

iii) Animal serum is used in the medium for the initial cell growth but no protein is present in the medium used for maintaining cell culture during virus multiplication.

iv) A pH indicator (e.g., phenol red) and suitable antibodies (in smallest effective concentrations) are also added in the medium.

v) Each virus suspension is tested for its identity, bacterial sterility, and freedom from extraneous viruses.

vi) Then the virus harvests that pass these tests are filtered through a bacteria-retentive filter.

vii) The filtered virus harvest is tested in cell cultures for its identity, growth capacity at different temperatures, and virus concentration.

viii) A test for neurovirulence is performed by intraspinal injection into *Macacairus* (cynomolgus monkey) or equally susceptible animals. The vaccine and a reference homotypic vaccine are simultaneously examined in monkeys of the same quarantine batch.

Storage Conditions and Stability

Polio vaccine is potent if stored at temperature below -20°C until the expiry date indicated on the vial. It can be stored for six months at 5 $\pm$ 3°C temperature.

5.1.4.7. Typhoid Vaccine

Typhoid vaccine is a mixture of suspension of killed Salmonella typhi and tetanus formol (i.e., formaldehyde solution) toxoid. In 1ml of this vaccine, either 1000 or 2000 million typhoid bacilli, viz., S. typhi.

Preparation

1) Strain of Salmonella typhi: The Ty2 strain of S. typhi is used for preparing vaccine as it is considered safe and effective in humans. The culture used should be identified by a record of its history, including the source from which it was obtained, an d particulars of all tests made periodically for verification of strain characters. The culture used should exhibit the following features:

i) Stained smears made from a culture should be of S. typhi.

ii) The colonies of a culture should show the opalescence of a Vi-rich strain of S. typhi.

iii) When a live suspension of a culture in saline is titrated with potent anti -Vi, anti -H and anti -O sera of known agglutinating titres, it should agglutinate to titre with the anti -H and anti-Vi sera, but show little or no agglutination with the anti -O serum. The suspension after boiling, however, should agglutinate to titre with anti-O serum.

iv)The use of calibrated agglutinating sera for these tests and the ranges of titres cannot be specified as relevant international reference sera are not yet established and different laboratories have different testing methods.

v) A saline suspension of a young culture (in a dose of not more than 50 x 10^6 organisms) on intraperitoneal injection into 15 -20gm mice of a susceptible strain should kill at least 50% of the animals.

2) Seed-Lot System: Typhoid vaccine should be prepared based on a seed -lot system. A large seed-lot should be set aside as the basic material from which the manufacturer can prepare vaccine and further seed lots can also be prepared easily. Cultures of a seed-lot should have the same characteristics as required of cultures of the strains from which the seed-lot was derived.

Standardisation

1) Identity Test: This test should be performed on a labelled container from each filling lot. The antigenicity test on this labelled container is performed by immune precipitation method.

2) Sterility Test: The sterility test on each filling lot should be performed as per the requirements.

3) Innocuity Test: Each filling lot should be tested for abnormal toxicity by tests (approved by the National Control Authority) involving parenteral injections into guinea pigs and mice. In the tests, 0.5ml of subcutaneous or intramuscular injection is given to atleast two mice (of 20gm each). 5ml dose is injected in to two guinea pigs (of 350gm each). The animals should be observed for a week. The injection should cause neither significant symptoms nor death during this period.

4) Antigenicity Test: Each filling lot should be tested for specific antigenic characteristics by two tests; one for the protection of mice against challenge with a virulent strain of S. typhi, and the other for anti -O, anti-H and anti-Vi agglutinin-production in rabbits.

Storage Conditions and Stability

The dried typhoid vaccine should be stored at below 25°C temperature, whereas the liquid typhoid vaccine should be stored between 2-10°C.

5.1.4.8. Hepatitis Vaccine

Hepatitis A and B are two members of a family of closely related diseases; while the others are hepatitis C, D, and E caused by a viral infection. The viruses causing each type of hepatitis are different, but their transmission modes for the diseases are the same. Hepatitis is characterised by inflammation in liver. The consequences of the disease are potentially serious, and even fatal. There are no vaccines available for hepatitis C, D, or E; however, hepatitis A and B can be prevented by the available effective vaccines.

Preparation

Hepatitis vaccine in laboratories are prepared by a series of steps, which include initial concentration of surface antigen by ammonium sulphate precipitation, and then isopycnic banding and rate zonal centrifugation in a K-II centrifuge. The partially purified antigen concentrate is digested with pepsin at pH 2 and the antigen is unfolded in 8M urea solution followed by denaturation. After gel filtration, the antigen is treated with formalin (in 1:4000

dilution), adsorbed on alum, and preserved with thimerosal. The final product obtained is pure hepatitis B surface antigen. The process relies on physical elimination of infectious virus particles and treatment with highly viral -destructive reagents in the pepsin (pH 2), urea and formalin steps.

Storage Conditions and Stability

Hepatitis vaccine should be stored and maintained at 5 -8°C temperature. The vaccine should not be frozen.

5.1.4.9. AIDS Vaccine

An HIV vaccine is currently not available, but efforts are being made to develop such vaccine. Research on vaccine begins with basic laboratory research, product development, and animal experiments (performed in academic laboratories and by the pharmaceutical industries).

Then the so obtained products (candidate vaccines) are tested on healthy human volunteers through sequential phases. Phase I and II trials provide data on the safety of candidate vaccines and on their ability to induce immune responses specific to HIV. These trials are performed on small group of volunteers (50 -200 per trial). If the results obtained are satisfactory, candidate vaccines proceed to large –scale Phase III trials so that complete information on their efficacy in inducing protection against HIV infection or AIDS can be determined. For scientific reasons, Phase III tri als are carried out in populations with a high incidence of HIV infection, involving thousands of volunteers.

Development of AIDS Vaccine

Since HIV was identified as the causative agent of AIDS, many efforts were made to develop a safe and effective vacci ne against it. Some of the vaccine types designed includes:

1) Inactivated Whole Viruses: This is prepared following the same procedure as that for the Salk polio vaccine. HIV -I and SIV preparations are produced by irradiating the virus and then treating with formaldehyde to inactivate the retroviral genome. The resultant non -infectious SIV or HIV preparations are used as vaccines. The initial trials conducted on animals proved to be promising; however, the results were challenged as the SIV vaccine was prepared by growing SIV in human T-cell cultures.

2) Attenuated Viruses: This is prepared following the same procedure as that for the Sabin polio vaccine, in which the live polio virus is grown in monkey kidneys. On growing a live virus under scarce culture conditions, the viruses are forced to mutate to survive in the new growing conditions. The viral vaccines used today are mostly attenuated vaccines. Since the attenuated vaccine is live, it can infect cells and grow for a limited time before it gets eliminated by the immune response. However during this time, the attenuated virus induces a potent immune response, and also generates cell-mediated Cytotoxic T -Lymphocytes (CTLs) specific for the endogenously produced viral antigens. The attenuated viral vaccines also have the advantage of inducing a good memory cell response, which is responsible for the life-long immunity developed by these vaccines.

3) Cloned Envelope Glycoproteins: The first cloned gp 160 vaccine was produced in 1987 by MicroGeneSis, Inc. Until now , several groups have used genetic engineering techniques to produce gp 120 or gp 160 for immunisation by cloning the gp 120 gene or the entire gp 160 genes. On administering the cloned gp 160 vaccine, humoral immunity is induced. The antibodies produced i n the volunteers inhibit viral replication in vitro; this inhibition however, is always strain specific.

4) Recombinant Viruses Carrying HIV Genes: The development of this effective AIDS vaccine is based on the use of recombinant vectors. Vaccinia virus and the Sabin polio virus are live attenuated vaccines. These viruses can be engineered to carry genes from HIV -I, and the recombinant virus can then be used as a vaccine. As the recombinant virus is attenuated and not inactivated, it infects the host cells an d induces CTL activity. Vaccinia virus is large and can be engineered to carry a few dozen foreign genes without weakening its capacity of infecting the host cells and replicating within them. Engineered vaccinia virus can be easily administered by dermal _ scratching. The virus causes a limited localised infection in the host cells. The foreign genes are expressed by the vaccinia. If the foreign gene product is a viral envelope protein, it is inserted into the membrane of the infected host cell where it induces T-cell mediated immunity.

5.1.4.10. Diphtheria Antitoxin

Diphtheria vaccine is prepared by adsorbing formol toxoid on a mineral carrier. The Absorbed Diphtheria Vaccine (ADV) is a preparation obtained from the diphtheria formal toxoid containing 1500 Lines floculatainis [Lf] per mg of protein nitrogen and a mineral carrier (hydrated aluminium hydroxide or aluminium phosphate or calcium phosphate in a saline solution or another suitable solution isotonic with blood).

Preparation

1) The formal toxoid is prepared using the toxins produced by Corynebacterium diphtheriae in its growing phase. The toxoid has atleast 1500 Lf per mg of protein nitrogen.

2) Hydrated $Al(OH)_3$, $AlPO_4$, or $Ca_3(PO_4)_2$. are used as mineral carrier. The resultant solution obtained shows isotonicity with blood.

3) The antimicrobial preservatives (mostly phenolic type) which adversely affect the antigenic properties of antigens should not be added to the vaccine.

4) ADV-BP-1993 with atleast 30 units per dose should be used. It should be stored at 2 -8°C temperature, should not be frozen, and should be protected from light.

Variants of Diphtheria Vaccines

1) Adsorbed Diphtheria Vaccine (ADV) for Adults and Adolescents (BP - 1993) [Dip/Vac/Ads (Adults)]: This preparation should contain not less than 2 units per dose (i.e., 2.0 Lf per dose).

2) Diphtheria Toxoid (DT) (USP XXII): Corynebacterium diphtheriae in its growing phase is treated with formaldehyde and the product obtained is stored as a sterile solution. The preservative used should not be of the phenolic type. The preparation should be stored at 2 -8°C temperature, and should not be frozen.

3) Diphtheria Toxoid Adsorbed (DTA) (USP XXIII): This preparation is a plain diphtheria toxoid used either in precipitated form or that has adsorbed phosphate in addition, prepared as a sterile solution. The preparation should be stored at 2-8°C temperature, and should not be frozen.

Storage Conditions and Stability: Diphtheria vaccine should be stored and maintained at 2 -8°C temperature. The vaccine should not be frozen.

Properties of Diphtheria Toxin and Toxoid

Table 5.1: Properties of Diphtheria Toxin and Toxoid

Properties	Diphtheria Toxin	Diphtheria Toxoid
Toxicity	Highly toxic.	No specific toxicity.
Reactions	Causes an inflamed area around the site of injection.	Causes local reactions , especially in elderly subjects.
Stability	Not quite stable.	Quite stable at room temperature (2±2°C) for atleast 2 years.
Antibody Formation	Stimulate the formation of antibodies when injected.	Stimulate the formation of antibodies when injected.
Combination with Antitoxin	Positive	Positive

HYBRIDOMA TECHNOLOGY

5.2.1. Introduction

G. Kohler and C. Milstein in 1975 first discovered the hybridoma technology. It is used for producing hybrid cells by fusing B-lymphocyte with tumour or myeloma cells. The hybrid cells produced using hybridoma technology, are either cultured in laboratory or sub-cultured using mouse peritoneal cavity. Thus, due to the presence of B-lymphocyte genetic ma terial the hybrid cells can produce antibodies. The tumour cells used to produce hybrid cells make them undergo indefinite division in the culture.

The B-lymphocytes involved are pre -programmed to respond to a single type of antigen or antigenic determin ant, thus they produce a single type of antibody that shows specificity for a specific antigen. The reaction of an antigen with B-lymphocyte receptors triggers the rapid division of lymphocytes. As a result, a clone of B cells is produced that generate antibodies against that specific antigen.

This entire process is called clonal selection in which B-lymphocytes produce a single type of antibodies specific to a single type of antigen or antigenic determinant. However, a fully differentiated antibody produ cing B-lymphocytes (known as plasma cells) does not undergo division when cultured in a laboratory.

5.2.2. Principle

The myeloma cells used in hybridoma technology should not synthesise their own antibodies. The hybridoma cells are selected based on inhibiting the nucleotide (subsequently, the DNA) synthesising machinery. The mammalian cells can synthesise nucleotides either by De novo synthesis or salvage pathway (figure 5.1).

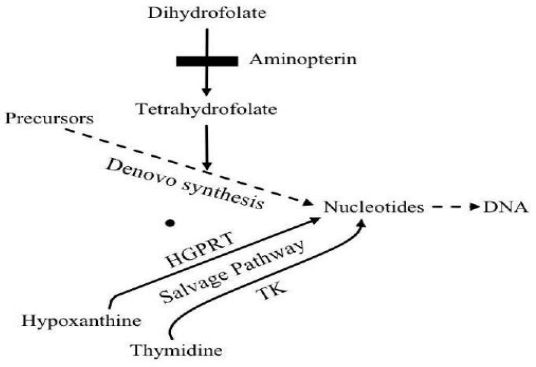


Figure 5.1: Pathways for the Nucleotide Synthesis (HGPRT-Hypoxanthine Guanine Phosphoribosyl Transferase; TK-Thymidine Kinase)

In the Denovosynthesis of nucleotides , tetrahydrofolate formed from dihydrofolate is required. Aminopterin (an inhibitor) is used to block the formation of tetrahydrofolate (and therefore nucleotides). In the salvage pathway, the purines and pyrimidines are converted into the corresponding nucleotides. The key enzyme involved in the salvage pathway of purines is the Hypoxanthine Guanine Phosphoribosyl Transferase (HGPRT), which converts hypoxanthine and guanine to inosine monophosphate and guanosine monophosphate, respectively. Thymidine Kinase (TK) is involved in the salvage pathway of pyrimidines and converts thymidine to Thymidine Monophosphate (TMP). If any of these enzymes (HGPRT or TK) undergo mutation, the salvage pathway gets blocked. When the mutated cells (i.e., the cells deficient in HGPRT) are grown in a medium containing Hypoxanthine Aminopterin and Thymidine (HAT medium), they fail to survive as the de novo synthesis of purine nucleotides is inhibited.

Thus, the cells deficient in HGPRT and grown in HAT medium die. The hybridoma cells possess the ability of myeloma cells to grow in vitro with a functional HGPRT gene obtained from the lymphocytes fused with myeloma cells. Thus, only the hybridoma cells can proliferate in HAT medium, and this procedure is used for their selection.

5.2.3. Production of MAbs

The establishment of hybridomas and production of MAbs involves the following steps (figure 5.2):

1) Immunisation,

2) Cell fusion,

3) Selection of hybridomas,

4) Screening the products,

5) Cloning and propagation, and

6) Characterisation and storage.

5.2.3.1. Immunisation

The first step in hybridoma technology is to immunise a mouse with a suitable antigen. The antigen and an adjuvant (like Freund's complete or incomplete adjuvant) are injected via subcutaneous route (adjuvants are non-specific potentiators of specific immune responses). The injections are repeated multiple times at many sites.

This increases the stimulation of B-lymphocytes which are responding to the antigen. Three days before the animal is slayed, a final dose of antigen is given via intravenous route. This approach gives rise to large number of immune-stimulated cells for synthesis of antibodies. The concentration of desired antibodies is assayed in the animal serum at frequent intervals during immunisation.

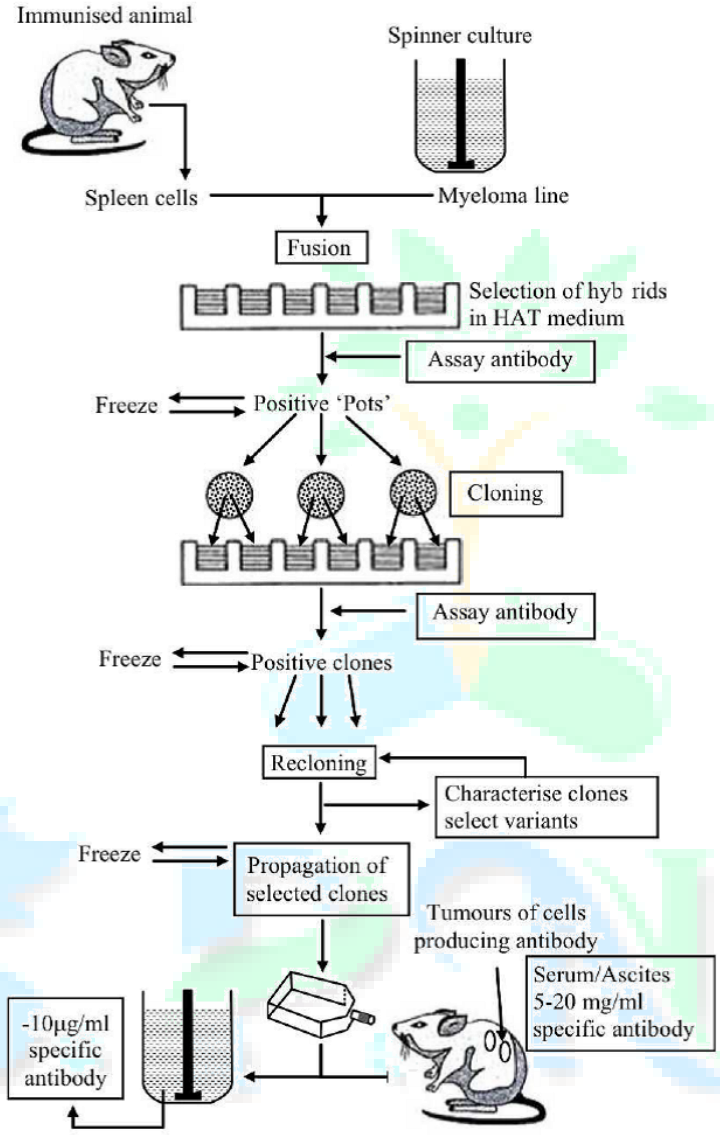
The animal is sacrificed when the concentration of antibodies in serum becomes optimal. The spleen is removed aseptically and disrupted mechanically or enzymatically to release the cells. The spleen lymphocytes are separated from the remaining cells by density gradient centrifugation.

5.2.3.2. Cell Fusion

The lymphocytes are thoroughly washed and mixed with HGPRT defective myeloma cells. The mixture of cells is treated with Polyethylene Glycol (PEG) but for a few minutes due to its toxicity. The cells are then washed to remove PEG and kept in a fresh medium. These cells contain a mixture of hybridomas (fused cells), free myeloma cells, and free lymphocytes.

5.2.3.3. Selection of Hybridomas

On culturing the cell s in HAT medium, the hybridoma cells grow and the remaining cells disappear slowly within 7 - 10 days. Selecting a single antibody producing hybrid cells is very essential, and is possible if the hybridomas are isolated and grown individually. The suspension of hybridoma cells is diluted to such intensity that the individual aliquots contain one cell each. These cells are grown in a regular culture medium to produce the desired antibody.



5.2: Protocol for the Derivation of Monoclonal Antibodies from Hybrid Myelomas

5.2.3.4. Screening the Products

The hybridomas should be screened for the secret 1on of the antibody of desired specificity, and the culture medium from each hybridoma culture should be tested occasionally (using ELISA and RIA techniques) for the desired antibody specificity. In both the techniques, i.e., ELISA and RIA, the antibody binds to the specific antigen (coated to plastic plates) and the unbound antibody and other components of the medium are washed off. Thus, the hybridoma cells producing the desired antibody are identified by screening. The antibody secreted by the hybrid cells is the monoclonal antibody.

5.2.3.5. Cloning and Propagation

The single hybrid cells producing the desired antibody are isolated and cloned by using the following two techniques:

1) Limiting Dilution Method: In this method, the suspension of hybridoma cells is serially diluted and aliquots of each diltion are transferred into microculture wells. The dilutions are made such that each aliquot in a well contains a single hybrid cell, thus ensuring that the antibody produced is monoclonal.

2) Soft Agar Method: In this method, the hybridoma cells are cultured in soft agar. Many cells can be grown simultaneously in a semisolid medium to form monoclonal colonies.

Practically, both these methods are used in combination to produce maximal MAbs.

5.2.3.6. Characterisation and Storage

The obtained monoclonal antibodies are subjected to biochemical and biophysical characterisation for the desired specificity. The MAbs should also be elucidated for the immunoglobulin class or sub -class, its specific epitope, and its number of binding sites.

The stability of the cell lines and the MAbs is also important. Both are characterised to check their ability to withstand freezing and thawing by freezing the desired cell lines in liquid nitrogen at several stages of cloning and culture.

5.2.4. Large Scale Production of MAbs

Production of MAbs in culture bottles is low (5-10µg/ml), thus to increase the yield the Hybrid cells are grown as ascites in the peritoneal cavity of mice. The ascitic fluid contains 5 -20mg of MAb/ml, which is much superior to the in vitro cultivation techniques.

Collection of MAbs from ascitic fluid has a heavy risk of contamination by pathogenic organisms of the animal. Also many animals have to be sacrificed to produce MAbs. Therefore, workers prefer in vitro techniques over using animals.

Encapsulated Hybridoma _ Cells for Commercial Production of MAbs

To increase the hybridoma cell density in suspension culture, the hybridomas are encapsulated in alginate gels using a Coating solution containing (poly-lysine) solution containing poly -lysine (figure 5.3); these gels a llow the nutrients to enter in and antibodies to come out of the hybridomas. In this way, the yield of MAb pr oduction can be Antibody production and separation increased (10-100 µg /ml).

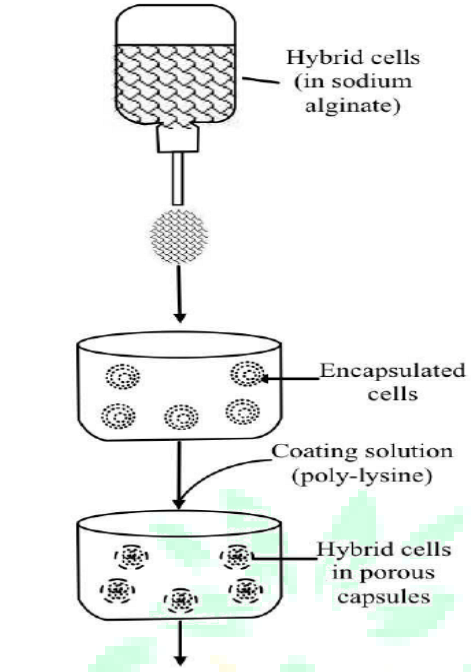


Figure 5.3: Production of Monoclonal Antibodies by Microencapsulation

4.2.5. Purification of MAbs

The desired antibodies should be extracted froma media sample of cultured hybridomas or a sample of ascites fluid. The contaminants in the cell culture sample consists of growth factors, hormones, and transferrins. The in vivo sample however, contains host antibodies, proteases, nuclea ses, nucleic acids, and viruses. Other secretions by the hybridomas (like cytokines) may be present in both the cases. Endotoxins may also be present in case of bacterial contamination as they are secreted by the bacteria.

For purification, the sample is conditioned by removing cells, cell debris, lipids, and clotted material s through centrifugation andthen filtration through a 0.45µm filter. Membrane fouling can be caused by the large particles in the further steps of purification. Moreover, the product concentration in the sample might not be sufficient, particularly in cases where the desired antibody is produced by a low -secreting cell line. Therefore, the sample is subjected to ultrafiltration or dialysis for condensation.

The charged impurities are mostly anions like nucleic acids and endotoxins, and are separated by ion exchange chromatography. At sufficiently low pH, cation exchange chromatography is conducted so that the desired antibody binds to the column and the anions flow through. While at sufficiently high pH, anion exchange chromatography is conducted so that the desired antibody flows through column and the anions bind to it.

Based on their isoelectric point (pl), many proteins and anions can be separated. For example, pl of albumin (4.8) is lower than the pl of most monoclonal antibodies (6.1). So, the average charge of albumin molecules at a certain pH is more negative. Size exclusion chromatography is used for removing transferrin.

Affinity purification is conducted to obtain maximum purity in a single step as the antigen used delivers antibody specificity. In this technique, the antibody - generating antigen is covalently bonded to agarose support. If the antigen is a peptide, it is synthesised with a terminal cysteine that allows selective attachment to a carrier protein (e.g., Keyhole Limpet Hemocyanin, KLH) during development and to promote purification. The media, containing antibody is incubated with the immobilised antigen in batch. It can also be incubated as the antibody is passed

through a column, at which it binds selectively and the impurities are rinsed. The purified antibody is recovered from the support by an elution using a low pH buffer or a gentle high salt elution buffer.

Sodium or ammonium sulphate is used to precipitate out the antibodies for their further selection. Antibodies precipitate at low salt concentrations, while other proteins precipitate at higher concentrations. To obtain best separation, salt should be added in sufficient amount, and the excess salt can be removed by desalting method like dialysis.

Chromatogram is used for the analysis of final purity. Presence of any impurities can be detected by the formation of peaks and the amount of impurity is indicated by the volume under the peak s. Gel electrophoresis and capillary electrophoresis can also be performed for the analysis of final purity. Presence of any impurities can be detected by the formation of bands of varying intensity.

5.2.6. Applications of MAbs

The application of monoclonal antibodies are:

1) Diagnostic applications,

2) Therapeutic applications,

3) Protein purification, and

4) Miscellaneous applications.

5.2.6.1. Diagnostic Applications

MAbs are used in biochemical analysis as diagnostic reagents and in imaging of diseases as diagnostic tools:

1) MAbs in Biochemical Analysis: In RIA and ELISA techniques, different diagnostic tests employing MAbs as diagnostic reagents are used. With these techniques, the c irculating concentrations of hormones (e.g., insulin, human chorionic gonadotropin, growth hormone, progesterone, thyroxine, triiodothyronine, gastrin, and renin) and many tissue and cell products (e.g., blood group antigens, blood clotting factors, interferons, interleukins, and tumor markers) are determined.

i) Pregnancy: MAbs are used in the detection of pregnancy by measuring the urinary levels of human chorionic gonadotropin.

ii) Cancers: MAbs are used in the estimation of plasma Carcinoembryonic Antigen (CEA) in colorectal cancer, Prostate Specific Antigen (PSA) in prostate cancer, and tumour markers for prognosis of cancers.

iii) Hormonal Disorders: MAbs are used in the analysis of thyroxine, triiodothyronine, and thyroid stimulating hormone for detecting thyroid disorders.

iv) Infectious Diseases: MAbs are used in the detection of infectious diseases by determining the circulatory levels of antigens specific to the infectious agent, e.g., antigens of Neisseria gonorrhoeae and herpes simplex virus for the diagnosis of STDs.

2) MAbs in Diagnostic Imaging: Immunoscintigraphy is the technique in which radiolabeled-MAbs are used in diagnostic imaging of diseases. When MAbs labelled with radioisotopes (e.g., 1odine-131 and technetium -99) are

injected through intr avenous route into the patients, they restrict at target sites (like a tumour) and then detected by radioactivity imaging.

i) Myocardial Infarction: In myocardial necrosis (i.e., cardiac cell death), the myosin (cardiac protein) gets exposed. Antimyosin MAb labelled with indium chloride-111 radioisotope is used in the detection of myosin and the myocardial infar ction site. Radiolabelled antimyosin MAb is used for detecting the location and the degree of damage to the heart. Hence, this technique can be used in diagnosing heart attacks.

ii) Deep Vein Thrombosis (DVT): It is the formati on of blood clots (thrombus) in the blood veins, especially in the lower extremities. Radioisotope labelled MAbs directed against fibrin or platelets are used for detecting DVT. The clots in thighs, pelvis, calves, and knees can be detected using fibrin specific MAbs.

iii) Atherosclerosis: It is the thickening and loss of elasticity of arterial walls. The atherosclerotic lesions can be detected by using radiolabelled MAb directed against activated platelets.

iv) Cancers: By using radioisotope labelled MAbs specific to the proteins(s), tumours of membrane origin can be detected. In table 5.2, some tumour markers used in MAb imaging of cancers are enlisted:

Table 5.2: Selected Tumour Markers along with the Associated Cancers used in MAb Imaging

Tumour Markers	Associated Cancer(s)
Carcinoembryonic Antigen (CEA)	Cancers of colon, stomach, and pancreas
α-fetoprotein	Cancers of liver and germ cells of testes
Human chorionic gonadotropin	Choriocarcinoma
Prostatic acid phosphatase	Prostate cancer
Epidermal growth factor receptor	Melanoma
Tumour-associated cell surface antigens	Various cancers

v) Bacterial Infections: Many attempts in recent years have been made to identify the infection sites by directing MAbs against bacterial antigens.

5.2.6.2. Therapeutic Applications

Monoclonal antibodies have the following therapeutic applications:

1) MAbs as Direct Therapeutic Agents: MAbs are used for direct enhancement of the immune function of the host. This minimises the toxicity to the target tissues or the host.

i) In Destroying Disease-Causing Organisms: MAbs aid effective opsonisation of pathogens (by coating with antibody) and enhance phagocytosis.

ii) In Cancer Treatment: MAbs are used in cancer treatment as they are effective against the antigens on the surface of cancer cells. The antibodies destroy the cancer cells by binding to them. With the help of MAbs, leukaemia, c olorectal cancer, lymphoma , and melanoma have been treated in many patients.

iii) In Immunosuppression of Organ Transplantation: Immunosuppressive drugs (e.g., cyclosporine and prednisone) overcome organ transplantation rejections. MAbs specific to Flymphocyte surface antigens are being used in the recent years forthe same purpose.

iv) In AIDS Treatment: AIDS most significantly causes immunosuppression by reducing CD4 cells of T-lymphocytes. The HIV with the help of surface membrane glycoprotein (gp120) binds to the specific receptors on CD4 cells.

v) In the Treatment of Autoimmune Diseases: By using MAbs directed against T -lymphocytes and B -lymphocytes, a few achievements have been made in the clinical trials of rheumatoid arthritis patients.

2) MAbs as Targeting Agents in Therapy: Tissue-specific MAbs can be coupled to many toxins, drugs, radioisotopes, etc. The resultant complex is then transferred to target tissues, thus delivering high concentration of drugs to the target site with minimum toxicity. MAbs are hence used for proper delivery of drugs or isotopes.

i) As Immunotoxins: Toxins and MAbs are conjugated to produce immunotoxins, e.g., Diphtheria toxin, Pseudomonas exotoxin, toxins in cancer treatment, etc. that can be used in therapy,

ii) In Drug Delivery: Generally, drugs are more effective in vitro (in laboratory when tested with cultured cells) in comparison to in vivo (inside the living body) because insufficient quantity of drug reaches the target tissue. This can be overcome by coupling the drugs with tissue - specific MAbs (directed against a cell surface antigen of the cells, like tumour) and targeting them to specific site of action.

iii) In the Dissolution of Blood Clots: The blood clots can be removed by using tissue Plasminogen Activator (tPA) asatherapeutic agent. The blood clots can be degraded by coupling tPA to MAbs directed against fibrin. The resultant MAb-tPA complex gets attached to the fibrin because of high affinity. As a result of high concentration of tPA at the target sites, plasminogen gets conve rted to plasmin that dissolves the blood clots (fibrin).

iv) In Radio Immunotherapy (RAIT): The radioisotopes coupled with MAbs are directed against tumour cells. This promotes the concentration of radioactivity at the specific sites of action and effective destruction of target tumour cells.

5.2.6.3. Protein Purification

MAbs can be prepared for any protein, a ndthe obtained MAb can be used for purifying the protein against which it was raised. MAbs columns can be developed by combining MAbs to cyanogen bromide activated Sepharose (chromatographic matrix). In this way , the immobilis ed MAbs can be used for the purification of proteins by immunoaffinity method.

Advantages

Protein purification using MAbs have many advantages, like specificity of the binding of MAbs to the desired protein, high degree of purification, and effective elution from the chromatographic column. For the purification of recombinant interferons, immunoaffinity chromatography is used which attains more than 5,000 fold purification of interferon-ap.

Disadvantages

However, achieving 100% purity of the target protein by immunoaffinity method is impossible because a small fraction of MAbs leaks into the elution. MAbs cannot differentiate between the intact target protein and a fragment of it with the antigenic site.

5.2.6.4. Miscellaneous Applications

Catalytic MAbs (Abzymes)

The antibody enzymes called abzymes are catalytic antibodies. Antibody recognition of an antigen and enzyme recognition of a substrate is different. The antibodies recognise in ground state, whereas the enzymes recognise in transition state (related with a conformational change of protein).

Researchers have developed a hapten-carrier complex similar to the transition state of an ester undergoing hydrolysis. This complex is used for producing anti-hapten monoclonal antibodies. These MAbs bring out hydrolysis of ester with a considerable degree of specificity (to the transition state to which MAbs were raised).

There are many other reactions apart from ester hydrolysis,in which antibodies can be used. Examples of such reactions are hydrolysis of amides and carbonates, cyclisation reactions, elimination reactiondio-molecular chemical reactionsetc.

Autoantibody Fingerprinting

In the recent years, researchers discovered a new class of Individual Specific (IS) autoantibodies that are produced after birth, reach maximum by 2 years, and become constant for the remaining life. For the detection and identification of IS autoantibodies in individuals, MAbs are produced, and this technique is termed autoantibody fingerprinting. The samples of autoantibodies are collected from blood, saliva, semen, and tears. This is helpfulin the detection of criminals, culprits etc.

IMMUNO-BLOTTING TECHNIQUES

Introduction

The technique of immobilisation of nucleic acids or proteins on a solid support like **nylon or nitrocellulose membranes** is referred to as blotting. It is an essential technique for hybridisation studies. Nucleic acid labelling and hybridisation on membranes have laid the base for various experimental techniques involving the understanding of gene expression, organisation, etc.

Identifying and measuring certain proteins in complex biological mixtures (like blood) are prevalent in scientific and diagnostic practices since ages. In recent years, identification of abnormal genes in genomic DNA has turned out to be progressively significant in clinical research and genetic counselling. Unique proteins and nucleic acid sequences are identified using blotting technique s that are extremely accurate, sensitive, and essential tools in molecular biology and clinical research.

General Principle

The blotting method involves the following four steps:

1) Electrophoretic extraction of protein or nucleic acid fragments in the sample,

2) Transfer and immobilisation on paper,

3) Binding of analytical probe to target molecule on paper, and

4) Bound probe visualisation.

By using electrophoresis the molecules in the sample are separated and moved on a support medium or membrane. As a result, the protein or DNA fragments get immobilised, providing a close copy of the original separation, and assisting subsequent biochemical analysis. Probes like antibodies or DNA that binds to the desired molecule are used to localise the immobilised protein or nucleic acid fragment s after they are transferred to the support medium. Autoradiography is used in the final step for visualis ing the position of | the probe bound tothe immobilised target molecule.

The three main blotting techniques that are discussed briefly are:

1) ELISA,

2) Western blotting, and

3) Southern blotting.

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISA technique uses an enzyme which is linked to the antigen/antibody that reacts with a colorless substrate and develops a colored reaction product. There is a large number of enzymes such as alkaline phosphatase, horse radish peroxidase, and p-nitrophenyl phosphatase which are employed in ELISA.

This is a cheaper and safer technique when compared to other immunoblotting techniques such as Radio-Immuno Assay (RIA).

On the basis of known concentration of antigen or antibody, a standard calibration curve is prepared from which the unknown concentration of sample is measured. A microtiter plate with numerous shallow wells is used in this method. It is very useful in testing for AIDS antibodies. However, now-a-days a number of ELISA kits have been developed and are in current use.

Table 6.1: Commonly Used Enzymes and their Substrates in ELISA

Enzymes	Substrates
Horseradish peroxidase	Hydrogen peroxide and *o*-phenylene diamine
Alkaline phosphatase	*p*-nitrophenyl phosphate
β-galactosidase	*o*-nitrophenyl-β-d-galactopyranoside

Table 6.2 enlists a few auto-antibodies detected by ELISA:

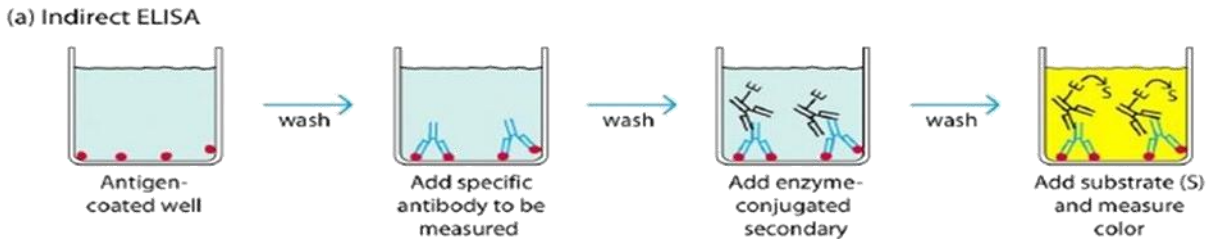
Table 6.2: Detection of Auto-Antibodies by ELISA

Antibodies	Target Auto-Antigens	Clinical Relevance
Thyroid microsomal antibody	Thyroid peroxidase	Auto-immune thyroid disease
Mitochondrial antibody (M2)	E2 pyruvate dehydrogenase complex	Primary biliary cirrhosis
Glomerular basement membrane antibody	C terminal end of type IV collagen	Good pasture's syndrome and anti-glomerular basement membrane nephritis
Double-stranded DNA antibody	dsDNA	Systemic lupus erythematosus
Phospholipid antibody	Cardiolipin	Primary phospholipid syndrome and systemic lupus erythematosus.

Types of ELISA:

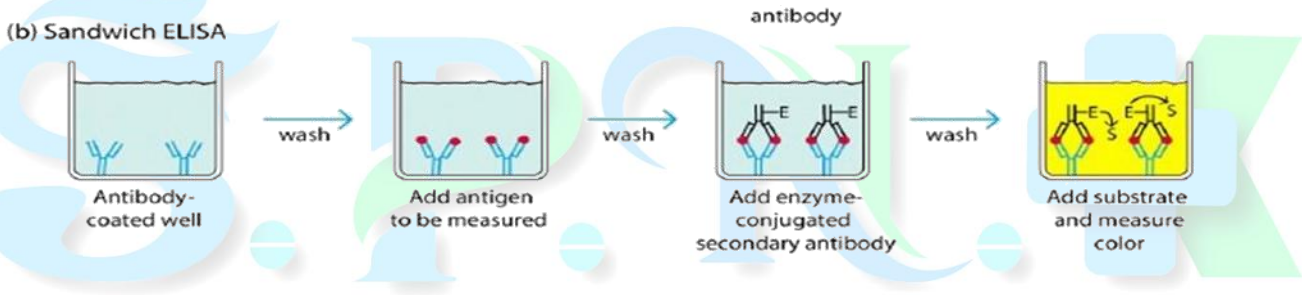
1. INDIRECT ELISA:

- It is used to measure **antibody**. Known antigen is coated on the plastic lining of the wells of microtiter plate which is made up of polystyrene latex.
- To test for the presence of antibodies against this antigen in the patient, his blood serum is added to the wells.
- If the patient's serum contains antibody specific to the antigen, the antibody will bind to the absorbed antigen otherwise not.
- After incubation, the wells are washed and the enzyme, labeled with anti-human gamma globulin (anti-Hgg), is added to the wells. Anti-Hgg can react with antigen-antibody complex. The mixture of wells is washed to remove the excess of unbound labeled anti-Hgg.
- Finally, the correct substrate for the enzyme is added which is hydrolyzed by the enzyme and develops a color. Varying concentrations of antibody in serum shows changes in the intensity of the color. This method is very useful in detection of antibodies to HIV, *Salmonella*, *Yersinia*, *Brucella*, *Treponema*, and *Streptococci*.



DOUBLE ANTIBODY SANDWICH ELISA:

- This method detects **antigen**. In this case antibody (antiserum) is immobilized on the surface of wells of microtiter plate.
- A test antigen is added to each well and allowed to react with the bound antibody. It is incubated during this period.
- If antigen combines specifically with antibody absorbed to wells, the antigen will be retained even after washing and unbound antigen would be made free.
- Thereafter, a second enzyme-linked antibody (eg. Alkaline phosphatase tagged to antibody) is added to react with bound antigen. Iti is again incubated for a few seconds, the enzyme labeled antibody reacts with the antigen-antibody complex already formed in the wells and results in the development of a sandwich.
- The mixture in wells is washed again to remove the excess of labeled enzyme. A chromogenic substrate ex. Nitrophenyl phosphate is added which reacts with the enzyme and develops yellow color.
- The reaction can be stopped by simply changing the pH or denaturing the enzyme. The change in color is measured visually or spectrophotometrically.
- Change in color shows the presence of desired antigen in the sample. This technique is useful in the detection of toxins of Vibrio cholera, E.coli, Staphylococcus enterotoxin-A and antigens of rotavirus.



COMPETITIVE ELISA:

This method is useful in estimation of antigen quantity. In this technique, antibody is incubated in an antigen containing sample solution. The antigen -antibody mixture is added to an antigen-coated micro titre well.

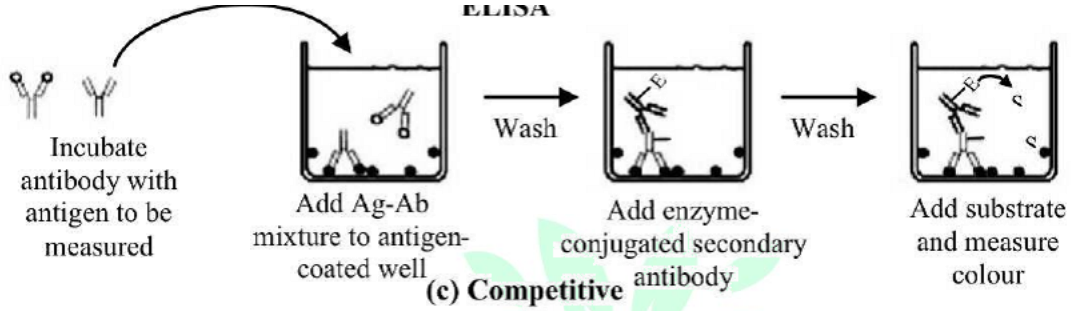
More the number of antigens present in the sample, less the number of free antibody available for binding to the antigen -coated well. Addition of an enzyme-conjugated secondary antibody (Ab2) specific for the isotype of primary antibody, determines the amount of primary anti body bound to the well. However, in competitive ELISA, higher the concentration of antigen in the original sample, lower is the absorbance.

Advantages

i) This method is highly specific as two antibodies are used.

ii) T his method is highly sensitive as both direct and indirect detection methods are used.

iii) This method is suitable for complex samples as the antigen is not purified before measurement.



(c) Competitive ELISA

Applications

The technique of ELISA has the following applications:

1) It can be used to detect the presence of antigen or antibody in a sample.

2) It can be used to determine serum antibody concentrations in a virus test.

3) It can be used in food industry to detect potential food allergens.

4) It can be used in disease outbreaks to track the spreading of diseases, e.g., HIV, bird flu, common, colds, cholera, STDs, etc.

WESTERN BLOTTING

The method of western blot was discovered by **George Stark** at Stanford, and the name western blot was laid down by **W. Neal Burnette**. This method, also known as immunoblot or protein blot, detects specific proteins in a sample of tissue homogenate or extract.

Western blotting is a qualitative and semi-quantitative technique for protein analysis. This technique is useful in cell and molecular biology. It aids in the identification of desired protein from a mixture of proteins extracted from cells. It also facilitates evaluation of the size and amount of protein. The technique of western blotting requires SDS-PAGE (**S**odium **D**ioctyl **S**ulfate- **P**olyacrylamide **G**el **E**lectrophoresis).

Principle

Western blotting is a fast and sensitive method of assay that detects and characterises proteins. It works on immunochromatography principle, wherein separation of proteins as per their molecular weight occurs in polyacrylamide gel.

The separated proteins are transferred or electro -transferred on nitrocellulose membrane, and_ then detected by specific primary antibody and secondary enzyme labelled antibody and substrate.

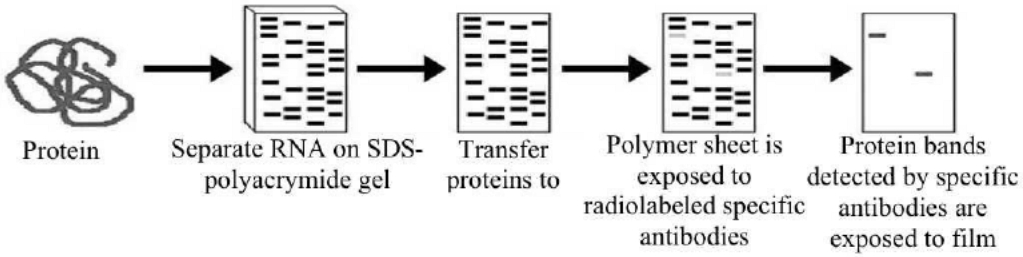


Figure 6.3: Principle of Western Blotting

Procedure

The steps involved in the procedure of western blotting are:

1) Tissue Preparation (Preparation of Sample Lysate): In the sample, ice cold PBS and lysis buffer (e.g., RIPA buffer that provides maximum protein yield) are added. The lysis buffer is selected based on the localisation of the desired protein. Unlike isolated cytoplasmic proteins, the solubilisation of membrane bound proteins is achieved by stronger extraction detergents.

Lysis buffer should possess protease inhibitors that avoid the degradation of the desired protein. Lysis of cells is done by incubating on ice and then applying shear pressure through pipette. Later the cell mixture is centrifuged and the pellet is discarded. The supernatant obtained is the lysate of use.

2) Gel Electrophoresis: The individual proteins in the obtained sample lysate are separate d based on their molecular weight. The separation is achieved by using a positive electrode that attracts a negatively charged protein. This is done by introducing the prepared protein sample into a polyacrylamide gel (commercially available). Gels of fixed percentages or gradients of acrylamide are commercially available.

Higher the acrylamide percentage, smaller the pore size of the gel matrix. Hence for low molecular weight proteins, higher percentage of gels is preferred; whereas for large proteins, low percentage of gel is considered better. Gradient gels have varied range of pore size, thus are useful for proteins of all sizes.

The steps involved in gel electrophoresis are:

i) The gel is prepared by inserting itin the electrophoresis apparatus and filling with running buffer.

ii) The wells of the gel are washed with running buffer and the chambers are added with buffer.

iii) The samples are loaded into the wells. A pre-stained molecular weight ladder is loaded in one well toaid in monitoring protein separation during electrophoresis and verifying protein weight int he sample later

during analysis.

iv) The electrophoresis unit is closed and a power supply is connected. The majority of units run for 45-60 minutes at 200 volts or until the loading buffer seeps to the bottom of gel. In this time period,

the negatively charged sample proteins migrate toward s the positive electrode through the polyacrylamide gel matrix.

3) Transfer: The separated proteins from gel are transferred to a solid membrane or blot. This works on the principle of applying electric field whereby negative proteins shift towards a positive electrode. Wet or semi-dry conditions are used for this transfer.

The wet transfer method involves the following steps:

i) The gel is removed from its cassettby cutting the top portion of the wells.

ii) The top left corner is marked to indicate gel direction.

iii) The gel is allowed to float in transfer buffer while preparing the transfer sandwich using a cassette, sponges, filter paper, the gel, and nitrocellulose membrane.

iv) The top left corner is marked on the blotting paper to indicate blot direction.

v) The membranes are incubated for 10 minutes in transfer buffer.

vi)A stack is prepared by placing sponge, filter paper, gel, membrane, filter paper, and sponge from the black negative cathode towards red positive anode.

vii) The gel or membrane should not be touched with bare hands and clean tweezers or spatula should be used, since touching the membrane can contaminate the blot. As a result, excessive background signals will appear

viii) A clean roller should be employed at every layer to remove bubbles or else they will interfere with efficient protein transfer.

ix) The cassette is locked and placed in the transfer apparatus containing cold transfer buffer positioned from negative to positive.

x) The heat development can be avoided by transferring with a cold pack in the apparatus or operating in a cold room with the spinner bar at the bottom of the chamber.

xi) The chamber is closed and power supply is connected.

xii) Transfer is done as per the manufacturer’s instructions (usually, 100 volts for third to 120 minutes is recommended).

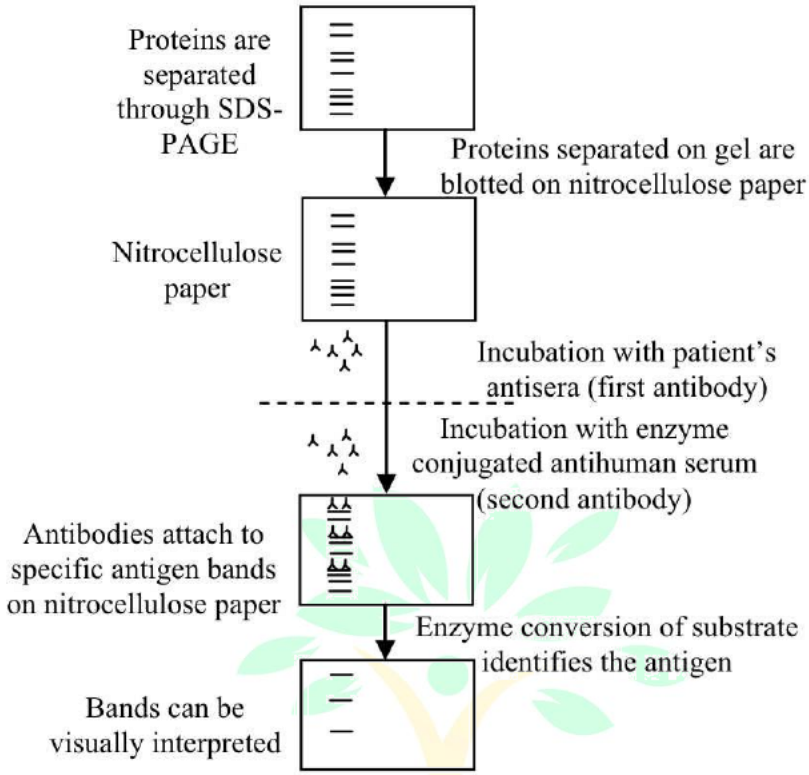


Figure 6.4: Western Blot Test

Immuno-Blotting: When protein is electrically transferred to a membrane, the blot is blocked using a protein specific primary antibody. Later, a secondary antibody is added that identifies the primary antibody.

The immuno-blotting method involves the following steps:

i) The membrane is detached from the cassette and rinsed thrice with water.

ii) The blot areas which do not contain protein are blocked to avoid non-specific antibody binding and to minimise overall background signal.

iii) The commonly used blocking buffers are 5% non-fat dry milk or BSA in a TBS -Tween solution. Milk solution is avoided when probing with phosphor-specific antibodies to prevent high background from its endogenous phosphoprotein, casein.

iv) The membrane along with blocking solution is incubated for an hour at room temperature with slight agitation.

v) The blocking solution is decanted and washed for 5 minutes with TBS tween.

vi) Before adding the primary antibody, it is diluted with a blocking buffer (in specified concentration) and incubated overnight with gentle shaking at 4°C temperature.

vii) After incubation the primary antibody is decanted. The membrane is washed five times forfive minutes each using large volumes of TBS tween with vigorous agitation to eradicate nonspecific background signals.

viii) Thereafter, the secondary antibody is diluted in blocking solution. The membrane is incubated for an hour at room temperature at the specified concentration.

ix) The membrane is decanted and washed five times for five minutes each with large volumes of TBS tween followed by vigorous agitation.

5) Detection: In this final step, signal development is achieved by electro-chemiluminescence (or ECL reaction). It is a common, sensitive and inexpensive method of detection invhich HRP enzyme conjugated to the secondary is used to catalyse the ECL reaction and give out light. This emitted light is projected onto X-ray film and developed or digitised using specialised and sensitive camera.

The detection method involves the following steps:

i) Equal parts of ECL reagents are mixed in 1:1 ratio as per the manufacturer's instructions.

ii) The membrane is incubated for 3-5 minutes with no agitation.

iii) The ECL mixture is decanted after incubation, and the excess solution is wiped off from the membrane corner.

iv) In order to avoid drying, the membrane is wrapped in a clear plastic –like sheet protector.

v) A roller is used to remove bubbles (if formed) or any excess solution.

vi) Without further delay, the membrane is developed.

vii) Manual adjustment of the exposure time is done by using film and camera systems to obtain a picture perfect western blot.

viii) Quantification of relative band densities is done using the commercially available software.

ix) Molecular weight verification can also be achieved through comparis on between the band sizes and the molecular weight ladder.

Applications

The technique of western blotting has the following applications:

1) It can be used to determine the size and amount of protein in the sample.

2) It can be used in disease diagnosis by detecting the antibody (produced against virus or bacteria) present in serum.

3) It can be used to identify defective proteins, e.g., in Prions disease.

4) It is the confirmatory test for HIV as it determines the presence of anti –HIV antibody in the serum.

5) It is a perfect test to determine the prevalence of Creutzfeldt-Jacob disease, Lyme disease, Hepatitis B, and Herpes.

SOUTHERN BLOTTING

E.M. Southern invented the technique of southern blotting. This method is useful in molecular biology. In this method, hybridisation analysis is performed by transferring the DNA from a gel to a membrane. The DNA is cleaved using suitable **restriction enzymes** and made to run on a gel. Then it is treated with sodium hydroxide that denatures the DNA to give a single strand.

Principle

Southern blotting is a hybridisation technique used for identifying particular size of DNA from a mixture of other similar molecules. This technique relies on the principle of separating DNA fragments by gel electrophoresis and identifying them by labelled probe hybridisation.

The DNA fragments are separated basedontheir size and charged using electrophoresis. Thes eparated DNA fragments are transferred on nylon membrane, and the desired DNA is detected using specific DNA probe complementary to the desired DNA. A hybridisation probe is a short (100-500bp), single stranded DNA labelled with a marker so that it can be detected after hybridisation.

Procedure

In the method of southern blotting, the DNA is transferred from a gel to a membrane for hybridis ation analysis. Then the DNA is cut with suitable restriction enzymes and run on a gel. On treating w ith NaOH , the DNA undergoes denaturation to yield a single strand.

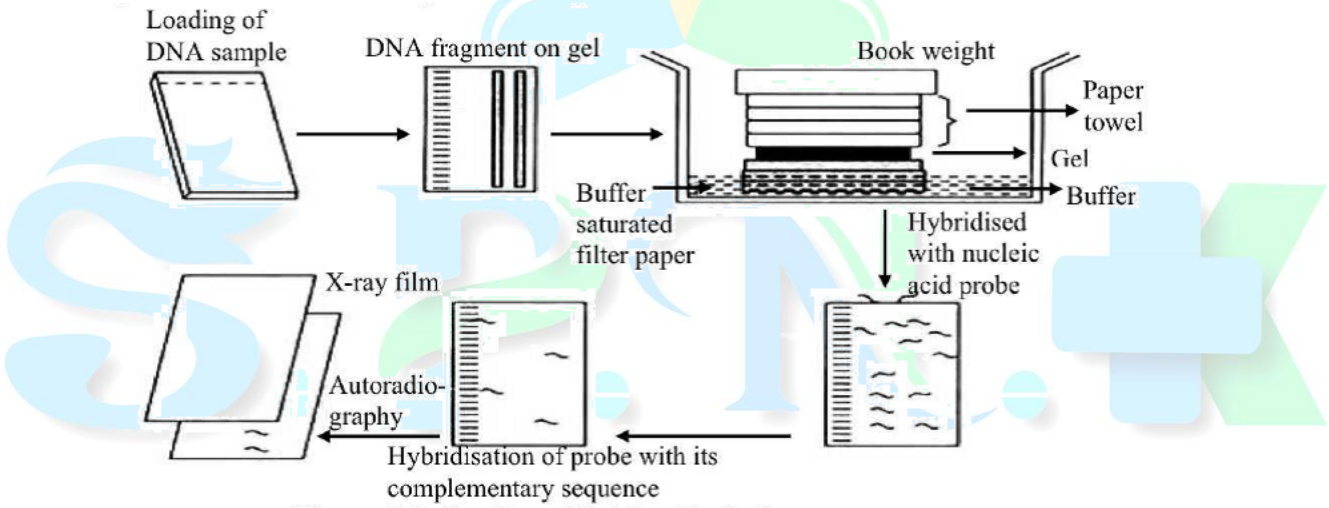


Figure 6.5: Southern Blotting Technique

The DNA is transferred from agarose gel to the membrane by capillary action. The gel is placed ona filter paper saturated with buffer, and the nitrocellulose membrane is placed above the gel and covered with 2-3 layers of dry filter paper towel (figure 6.5). The buffer reaches the top papers by flowing through the gel and membrane. While flowing it carries the DNA fragment with it, but DNA is retained on the paper as it cannot pass through the membrane. This membrane with trapped DNA is_ exposed overnight to a solution containing the radio labelled cDNA probe. The binding of probe to its complementary sequence is detected by autoradiography.

Applications

The technique of southern blotting has the following applications:

1) It is used to detect DNA in the sample.

2) DNA fingerprinting (an example of southern blotting) is used for paternity testing, criminal identification, and victim identification.

3) It is used to isolate and identify the desired genes.

4) It is used in restriction fragment length polymorphism.

5) It is used to identify mutation or gene rearrangement in the DNA sequence.

6) It is used in diagnosing diseases caused by genetic defects.

7) It is used to identify infectious agents.

GENETIC ORGANISATION OF EUKARYOTES AND PROKARYOTES

Introduction

Living organisms are categorised into prokaryotes and eukaryotes based on their cellular features, especially the nature of membrane bound organelles and genetic material organisation. The genetic materials in prokaryotes as well as in eukaryotes are organised into chromosomes. Although the term chromosome is applicable in eukaryotes only, yet the prokaryotic genetic materials are also described in the same way. The prokaryotic and eukaryotic chromosome s are different and their differences are enlisted in table 6.3:

Table 6.3: Difference between Prokaryotic and Eukaryotic Chromosomes

Prokaryotic Chromosome	Eukaryotic Chromosome
The chromosome formation is not present.	The genetic material is organised into chromosomes.
A single chromosome is present in each cell.	Two or more chromosomes are present in each cell.
It is shorter.	They are larger.
It contains a covalently closed circular DNA (cccDNA).	They contain a linear DNA with two ends.
It codes for a few proteins.	They code for numerous proteins.
It occupies the centre region of the cell and is not enclosed by the nucleus.	They are enclosed in the nucleus.
It is in direct contact with the cytoplasm as the nucleus is absent.	They are separated from the cytoplasm by the nuclear membrane.
It is associated with the mesosomes of the plasma membrane.	They are not associated with the plasma membrane and stay away from it.
DNA is not associated with histone proteins.	DNA is associated with histone proteins.
Nucleosomes are not formed.	Nucleosomes are formed by the association of DNA with t he histone proteins.
It contains a single origin of replication (Ori).	They contain many origin or replications.
The DNA replication can occur at any stage of the life cycle.	The genetic material replicates at the S phase of cell cycle.
It has a negative charge that is nullified by Mg^{2+} ions.	They have a negative charge that is nullified by the positively charged histone proteins.
It is circular, thus telomere is absent.	Telomere is present in their tip.
Centromere, kinetochore, secondary constriction and chromosomal arms are not formed.	They contain centromere, kinetochore , and chromosomal arms.

Eukaryotes

In eukaryotes, the major part of DNA is found in the chromosomes , which themselves are present within the nucleus (a distinct, double –membrane bound structure that makes up about 10% of the cell volume). The eukaryotic cell membrane has pores and is continuous with the endoplasmic reticulum.

The eukaryotes have varied number of chromosomes which is fixed for a biological species. During cell division the physical characteristics of chromosomes vary.

A eukaryotic chromosome has a single very large and linear double stranded DNA (ds-DNA) molecule. For example , a diploid human cell that contains 46 chromosomes (22 pairs of autosomes andi pair of sex chromosomeshas a total of 6 x 10^9 base-pairs. In individual human chromosomes the length of DNA molecules ranges from 1.5-8.7cm. These large DNA molecules are packed in chromosomes ranging from a few microns m length and breadth This is achieved by the formation of protein DNA complex known as chromatin(in eukaryotic cell.

The proteins that bind to DNA are histones and non-histone proteins. Histones are basic in nature due to the presence of basic amino a cids (such as lysine and arginine). Since they carry positive charges, they bind to the negatively charged DNA molecules forming characteristic structural units, i.e., the nucleosomes.

The long double stranded DNA molecule in each chromosome systematically surrounds the histones to form nucleosomes. The structure of nucleosome resembles beads connected to each other through linker DNA. A nucleosome bears a histone core having 8 sub-units (octamer) of 4 different histones, i.e., H»A,H»B, H3, and Hy with two molecules of each.

This histone protein core is encircled by two turns of ds-DNA molecule, thus forming a nucleosome. The DNA molecule runs as a continuous thread from one nucleosome to another. The dominant part of DNA between the two nucleosomes is the linker. Nucleosome width is 1lmm, and it repeats itself at intervals of 200 nucleotide pairs of DNA. The linker between two nucleosomes may have varied length. These features of eukaryotic DNA are shown in the figure 6.6:

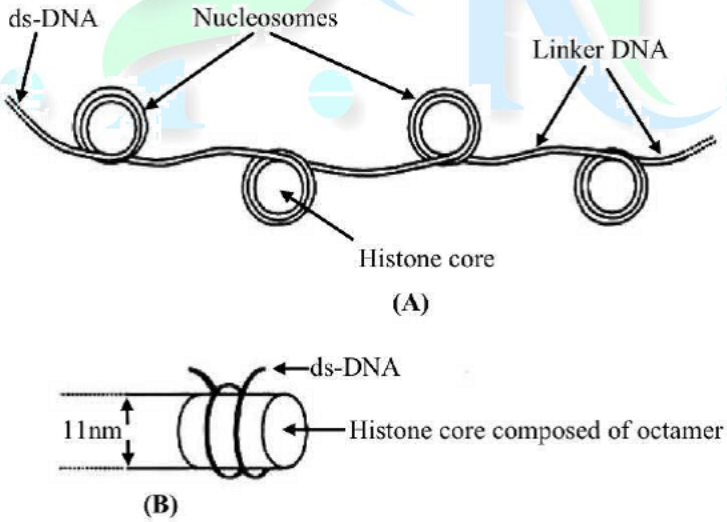


Figure 6.6: (A) Nucleosomes Connected by Linked DNA; (B) Side View of a Nucleosome with its Histone Octamer Core Wrapped by Turns of a Running ds-DNA

The nucleosomes are basic structures that build chromatin. They are organised as closely packed 30 nm chro matin fibres, which can be observed under high resolution electron microscope.

Prokaryotes

The internal structure and genomic organisation of prokaryotic cells are simple than that of the eukaryotic cells. The cells of prokaryotes do not have complex compartmentalisation as in the cells of eukaryotes. Some prokaryotic c ells show specialised membranes that are plasma membrane in foldings participating in metabolic functions.

The prokaryotic genome is structurally different from eukaryotic genome. On an average it has only about one -thousandth of DNA as in eukaryotic genome. In prokaryotes, mostly the genome has a DNA ring with few proteins linked to it.

This genetic material ring is the prokaryotic chromosome. In eukaryotes, the chromosome lies within the nucleus, whereas in prokaryotes, the chromosome is in a nucleoid region (a portion of cytoplasm which in electron micrographs appears lighter than the surrounding cytoplasm).

In prokaryotes and eukaryotes, fundamental similarity is observed in DNA replication, transcription, and translation; however, some differences also exist. For example, ribosomes in prokaryotic cell are smaller than those in eukaryotic cells. Also the protein and RNA content of these ribosomes is different. These differences are important for some antibiotics (like erythromycin and tetracycline) as they bind to ribosomes and block protein synthesis. This blocking action takes place only in prokaryotes and notin eukaryotes. Hence these antibiotics kill bacteria without harming the human cells.

Prokaryotes (like E. coli) lack a well -defined nucleus. Hence, the negatively charged DNA binds to each other through some positively charged proteins in the nucleoid region. The DNA in this region is arranged within the nucleus as large loops connected through proteins.

MICROBIAL GENETICS

Introduction

Microbial genetics is the study of genes and gene function in bacteria, archaea, and other microorganisms. Microorganisms acquire genes and undergo recombination, in which the genetic material from two organisms combines to form a new chromosome having a genotype different from that of the parent. This new arrangement of genes exhibié new physical or chemical properties. The microorganisms are known to undergo several recombination types, of which general recombination is the most common one. It involves a reciprocal exchange of DNA (between a pair of DNA sequences) on the microbial chromosome and is characterised by the exchanges occurring in bacterial transformation, bacterial recombination, and bacterial transduction. Another type of recombination is site-specific recombination , in which a viral genome is integrated into the bacterial chromosome.

Replicative recombination is another type which involves movement of genetic elements by switching position from one place to another on the chromosome. The principles of recombination apply only to prokaryotic (and not to eukaryotic) microorganisms. Eukaryotes exhibit a complete sexual life cycle, during which new combinations of a particular gene are formed by crossing over between homologous chromosomes. This process is not seen in bacteria, where only a single chromosome exists.

Transformation

Transformation is a kind of genetic recombination which involves the passage of only gene carriers (i.e., the DNA molecules of donor cell) into the recipient cell through the liquid medium. It was described by **Frederick Griffith** (an English bacteriologist) in 1928. He performed an experiment on laboratory mice and two types of Diplococcus pneumoniae (organism causing pneumonia). He established that one type has rough(R) non-capsulated cells and another one had smooth (S) capsulated cells. The R -type is non-pathogenic, while the S-type is pathogenic.

The experiment showed the following results (figure 7.1):

1) On injecting mice with live non-pathogenic (R-type) cells, they remain alive.

2) On injecting mice with dead pathogenic (S-type) cells, they remain alive.

3) On injecting mice with pathogenic (S-type) cells, they suffered from pneumonia and died.

4) On injecting mice with live non -pathogenic (R-type) cells mixed with dead pathogenic (S-type) cells, they suffered from pneumonia and died.

5) On isolating the dead tissue of mice, the smooth (S) capsulated cells are found on agar.

Thus in the above experiment, the conversion of R -type toS -type is called transformation.

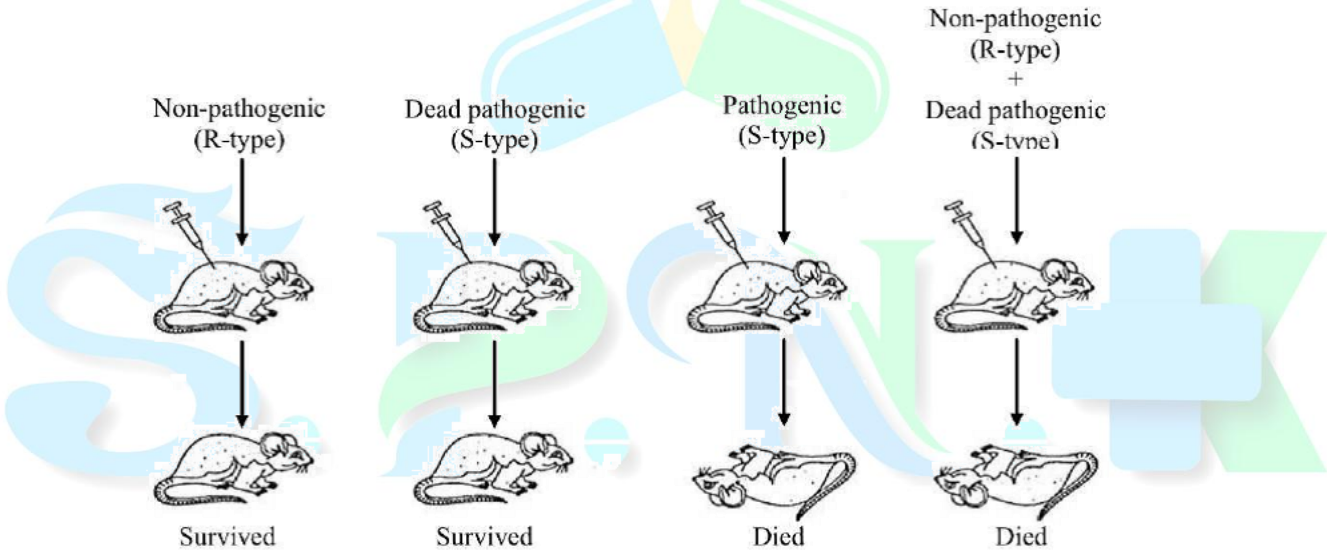


Figure 7.1: Griffith's Experiment with Mice and *Diplococcus Pneumoniae*

Later in 1932, **James L. Alloway** used fragments from dead smooth -type cells and transformed the R -type cells to S-type, thus confirming the work of **Griffith**. Then in 1944, **Oswald T. Avery, Colin M. MacLeod,** and **Maclyn N. McCarty** also found that DNA isolated from the fragments can induce transformation. The result of their experiment was the first proof that DNA is the genetic material in living organism.

The mechanism involved in transformation can be described as:

1) Transformation takes place in a few cells of the mixed population.

2) A few donor cells break apart, resulting in an explosive release and fragmentation of DNA.

3) A fragment of double stranded DNA (10 -20 genes) gets attached to the recipient cell for entry into the recipient cell.

4) During entry one strand of the fragment is dissolved by the enzyme, thus only the second strand is left, which passes into the recipient cell through cell wall and cell membrane.

5) Within the recipient cell, a portion of single strand of double stranded DNA of recipient cell is displaced by the enzyme and then replaced by the DNA of donor cell.

6) The displaced DNA is dissolved by another enzyme.

7) Thus, the recipient cell becomes transformed displaying the characters of its own as well as of the newly incorporated DNA.

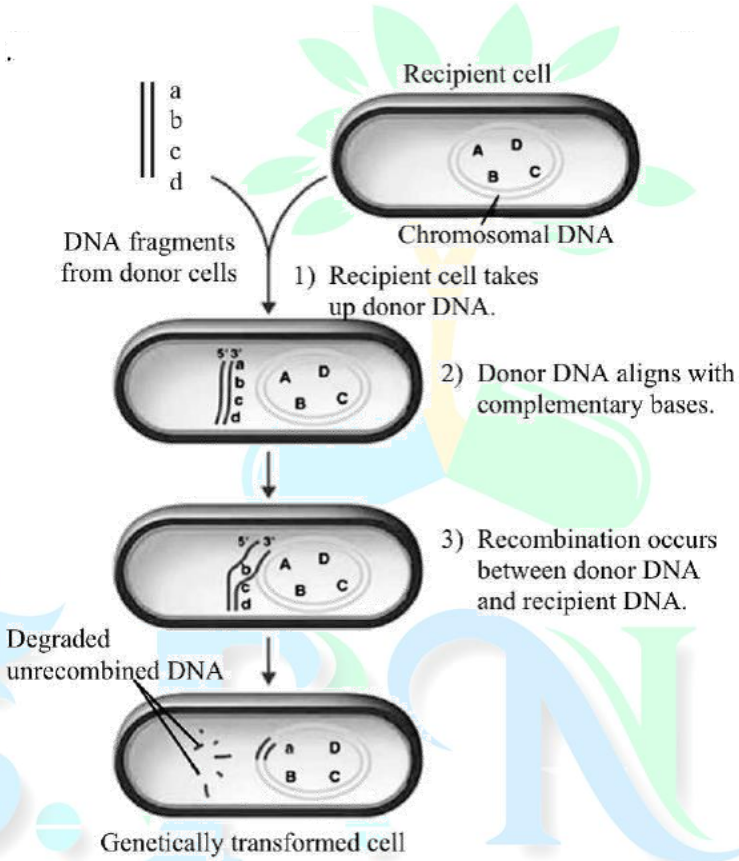


Figure 7.2: Bacterial Cell showing Transformation

Transduction

Transduction is a genetic recombination method which involves transfer of genetic material f rom the donor to the recipient cell through a non-replicating bacteriophage. This process was discovered by **Joshua Lederberg and Norton Zinder** in 1952 during their research with Salmonella typhimurium.

In transduction, a small fragment of bacterial DNA is incorporated into an attacking bacteriophage (i.e., virus which infects bacteria). When this bacteriophage infects a new bacterial cell, it transfers the genetic material into it, and thus genetic recombination takes place.

Specialised Transduction

The specialised transduction process occurs as follows:

1) The bacteriophage gets attached to a bacterial cell wall at the receptor site and its nucleic acid is transferred into the cytoplasm of the host cell (figure 7.3A).

2) The phage does not cause lysis of the host bacterium.

3) The phage nucleic acid codes for the synthesis of repressor proteins in the bacterial cell. These proteins prevent the virus from producing the material required for its replication.

4) In the bacterial cell, the viral DNA exists either as a fragment in the cytoplasm or attaches itself to the chromosome, known as prophage (figure 7.3B).

5) The bacterial cell which carries the prophage is lysogenic and the phenomenon in which the phage DNA and bacterium exist together is termed lysogeny.

6) The bacterial cell may remain lysogenic for many generations and during this period the viral DNA replicates a number of times with the bacterial chromosome.

7) However, later the phage stops the synthesis of repressor proteins in the bacterial cell, followed by the synthesis of phage components.

8) Thereafter, the phage DNA separates from the bacterial chromo some and induces the synthesis of phage proteins (figure 7.3C).

9) During this separation, a number of genes of the bacterium get attached to it.

10) These attached genes replicate along with the phage DNA (figure 7.3D) and later develop into phage particles, which come out from the bacterial cell by bursting (figure 7.3E).

11) When the new phage particle (figure 7.3F) infects a new bacterial cell (figure 7.3G and H), the attached bacte rial genes along with the phage particle enter the chromosome of the new bacterium and cause recombination (figure 7.31).

12) Thus, the new bacterial cell contains genes of its own as well as from the parent bacterial cell.

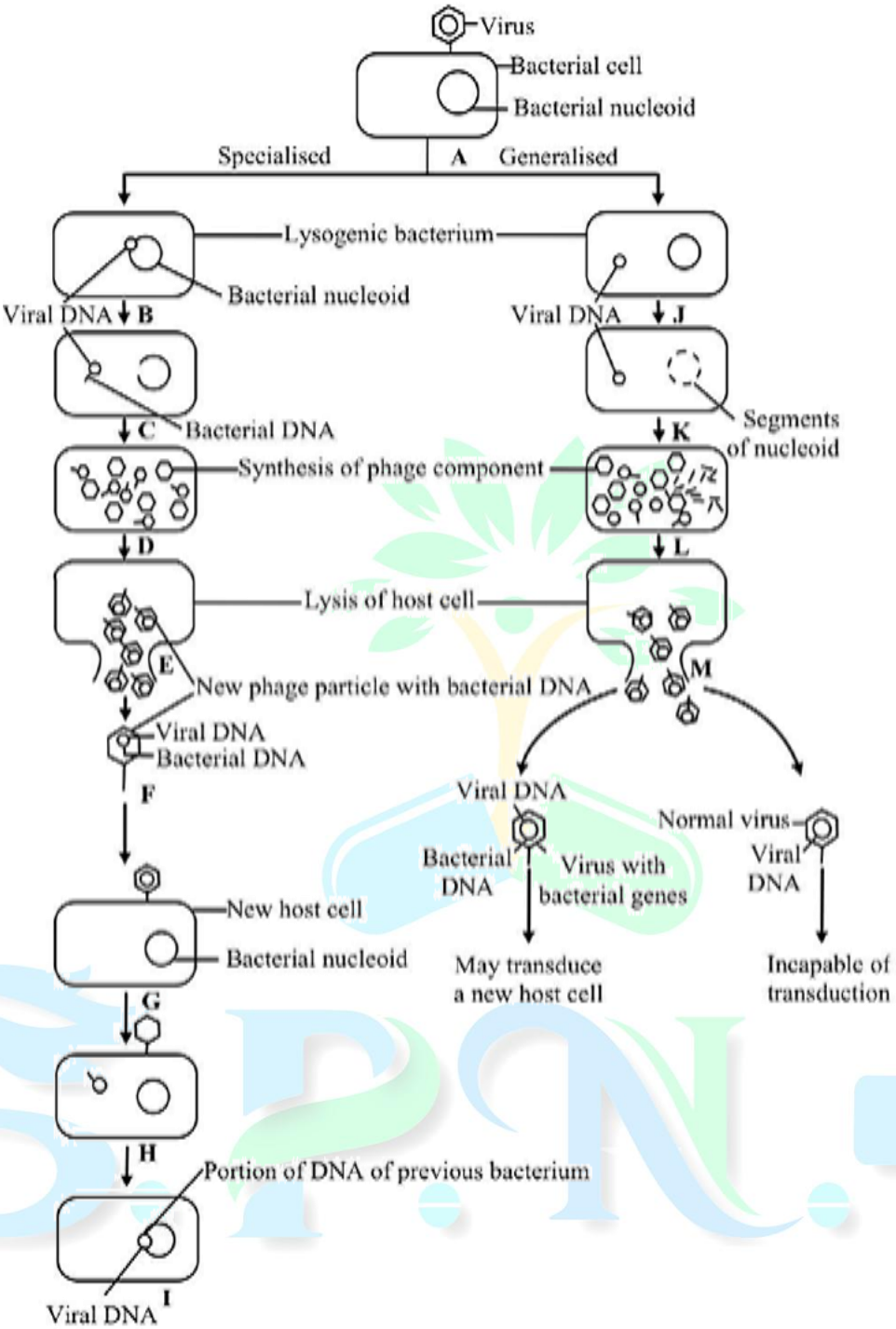


Figure 7.3: Diagrammatic Representation of Transduction

Generalised Transduction

Generalised transduction occurs more commonly than the specialized transduction, and involves the following steps:

1) The prophage particle is present in the cytoplasm of the infected bacterial cell (figure 7.3J).

2) The phage DNA starts synthesising new phages.

3) The chromosome of bacterial cell gets fragmented (figure 7.3K).

4) Some fragments get attached to the DNA of some new phage particles, w hile the others remain with phage DNA (figure 7.3L).

5) The newly formed phage with frag ment of bacterial chromosome in its DNA (figure 7.3M) attacks a new bacterium.

6) As aresult, the gene of the parent bacterium is transferred to the new bacterium and causes recombination.

Conjugation

Conjugation involves exchange of genetic material between two bacterial cells through a conjugation tube. This process was first postulated in 1946 by **Joshua Lederberg and Edward Tatum** in Escherichia coli. Later conjugation was also demonstrated in Salmonella, Vibrio, and Pseudomonas.

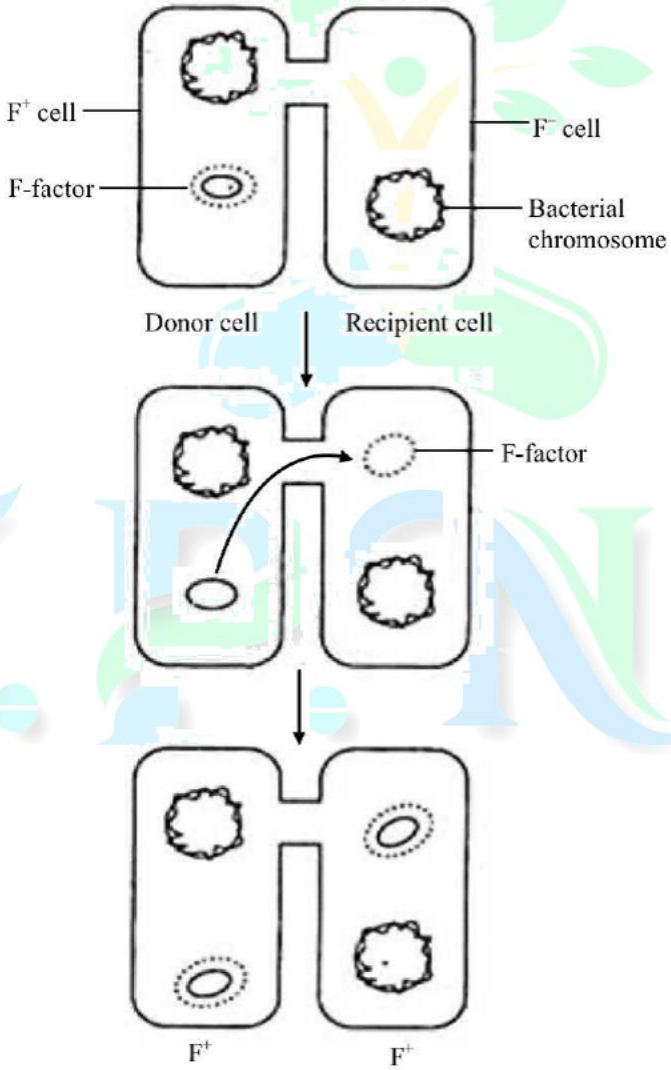


Figure 7.4: Recombination by F-factor in Conjugation

There are two mating types of bacteria; one is the male type or F^+or donor cell that donates DNA, while the other is female type or F^- or recipient cell that receives the DNA. The recipient cell on receiving the DNA behaves as a donor cell. The F -factor is the fertility factor, sex -factor, or F -plasmid that is found in the cellof F^+ (the donor cell or male type). The plasmid taking part in conjugation is the episome.

The steps involved in conjugation process are:

1) In conjugation, two cells of opposite mating type (i.e., F^+ and F^-) temporarily attach with each other via sex pilus (figure 7.4).

2) There is a hole of 2.5um diameter in sex pilus, through which DNA can pass from the donor to the recipient cell.

3) The F -factor or F -plasmid is a double stranded DNA loop found in the cytoplasm along with the nucleoid.

4) After the conjugation tube is established, the F-factor prepares for replication by the rolling circular mechanism.

5) The two strands of F -factor separate from each other and one of them passes to the F^- cell (recipient cell).

6) On reaching the F cell, the enzymes synthesize a complementary strand to form a double helix, which bends into a loop. Thus, the conversion process is completed.

7) In F^+ cell (donor cell) also, anew DNA strand is formed to complement the left over DNA strand of the F-factor.

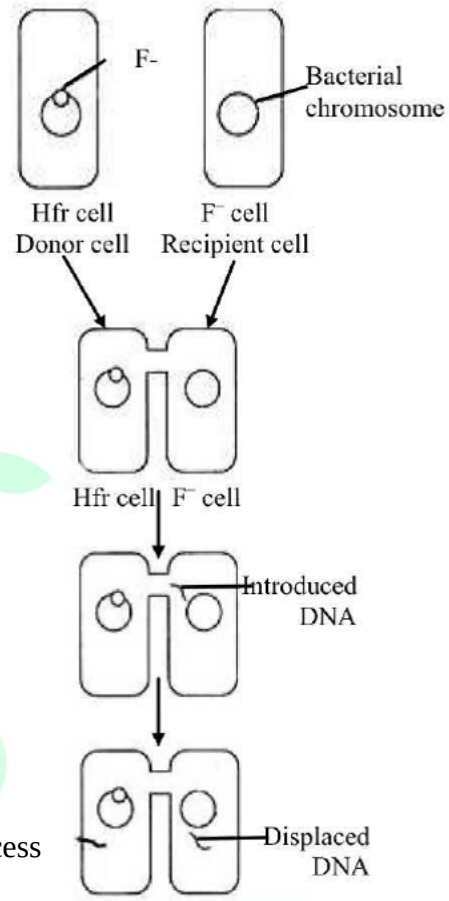


Figure 7.5: Recombination by Fragment of DNA in Conjugation

In another type of conjugation , nucleoid DNA is passed from one cell to another via conjugation tube. Bacterial strains are known as Hfr (High frequency of recombination) strain. In the 1950s, **William Hayes** discovered such strains of E. coli. This Hft factor is also called episome. In Hfr strain, the F-factor is attached to the nucleoid DNA, i.e., the bacterial chromosome.

The steps involved are:

1) The Hfr and F - cells attach with each other through sex pilus (figure 7.5).

2) The bacterial chromosome opens at the point of attachment of F-factor, and a copy of one strand is formed by the rolling circular mechanism.

3) A portion of single stranded DNA passes into the recipient cell through the pilus.

4) Due to agitation in medium, the pilus may DNA break and the conjugation tube may not survive for long. Thus, the entire DNA fails Fragment of DNA in Conjugation to enter the recipient cell.

Plasmids

Apart from bacterial chromosome (nucleoid), genetic elements are also found to be present in the cytoplasm of bacterial cells. These genetic elements, called plasmids, replicate separately from the chromosome.

In 1952, **Lederberg** demonstrated the existence of plasmids in bacterial cytoplasm while working on conjugation process in bacteria. He invented the term plasmid to indicate the genetic elements that were transmissible from one bacterial cell to another and determine the maleness in bacteria.

Properties

Given below are the properties of plasmids:

1) They are specific to one or a few particular bacteria.

2) They replicate separately from the bacterial chromosome.

3) They code for their own transfer.

4) They act as episomes and reversibly integrate into bacterial chromosome.

5) They may pick-up and transfer certain genes of bacterial chromosome.

6) They may affect some of the characteristics of bacterial cell.

7) They differ from viruses as:

i. They do not cause damage to cells and are beneficial.

ii. They do not have extracellular forms and exist inside the cells as free and circular DNA.

Physical Nature

The physical nature of plasmids is relatively simple. They are small double -stranded DNA molecules and mostly circular; however, many linear plasmids are also known. Naturally occurring plasmids have a varying size ranging from 1 kilobase to more than 1 megabase, and the size of a plasmid DNA is 5% less than the size of bacterial chromosome.

Most of the plasmid DNA isolated from bacterial cells exists in supercoil configuration (the most compact form for DNA to exist within the cell). The copy number indicates that different plasmids occur within the cells in different numbers. Some plasmids are present in the cell in 1 -3 copies, whereas others are present in over 100 copies. This copy number is controlled by the genes on plasmid and by interactions between the host and the plasmid.

Types

Given below are the main types of recognized plasmids:

1) F-Plasmid (or F-Factor): The 'F' stands for fertility. This plasmid was the first to be described, is very well characterised, and plays a major role in conjugation in E.coli. It confers 'maleness' on the bacterial cells and due to this property the term sex-factor is also used to refer to F-plasmid. F-plasmid is a circular ds-DNA molecule containing 99,159 base pairs. Its genetic map is shown in figure 7.6. One region of the plasmid contains rep genes involved in the regulation of DNA replication; the other region contains transposable elements (Iis3, Tn 1000, IS3 and IS2 genes) having the ability to function as an episome; and the third large region termed the tra region contains **tra genes** involved in the transfer of plasmids during conjugation.

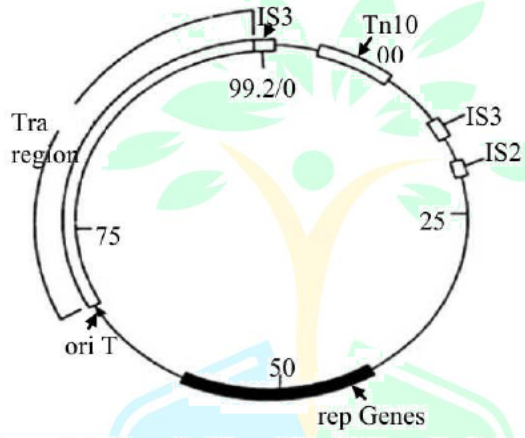


Figure 7.6: Genetic Map of the F-Plasmid of *E.*

2) R-Plasmids: These are the most widespread and well-studied group of plasmids. They are termed resistant plasmids as they confer resistance to antibiotics and various growth inhibitors. R -plasmids contain genes coding for enzymes that can destroy and modify antibiotics. They are not integrated into the host chromosome. Some R -plasmids possess a single resistant gene while others may even possess eight resistant genes.

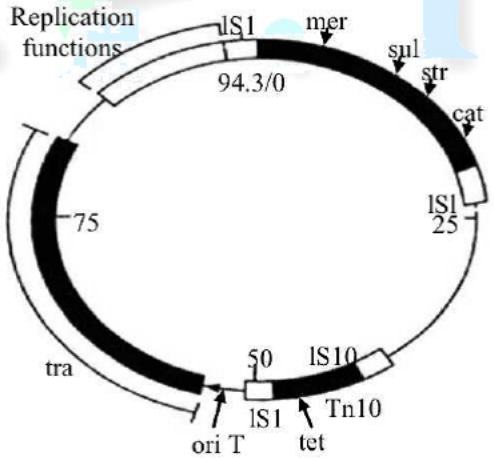


Figure 7.7: Genetic Map of the R-Plasmid. R100, cat = Chloramphenicol Resistance Gene; Str = Streptomycin Resistance Gene; sul = Sulfonamide Resistance Gene; mer = Mercury Ion Resistance Gene; IS = Insertion Sequences.

For example, plasmid R 100 is a 94.3 kilobase -pair plasmid (figure 7.7) carrying resistant genes for sulfonamides, streptomycin, spectinomycin, chloramphenicol, tetracycline, etc. It also carries genes conferring resistance to mercury.

3) Virulence-Plasmids: These plasmids confer pathogenicity on the host bacterium. They make the bacterium more pathogenic as it has the ability to resist host defence or produce toxins. For example , Ti - plasmids of Agrobacterium tumefaciens induce crown gall disease of angiospermic plants; enterotoxigenic strains of F. coli cause traveller's diarrhoea because of a plasmid that codes for an enterotoxin inducing extensive secretion of water and salts into the bowel.

4) Col-Plasmids: These plasmids carry genes that confer the host bacterium the ability to kill other bacteria by secreting bacteriocins (a type of protein that kill cells by creating channels in the plasma membrane, thus increasing its permeability). These plasmids can also degrade DNA or RNA or attack peptidoglycan and weaken the cell wall.

Bacteriocins act against closely related strains for example Col El plasmid of E. coli code for the synthesis of bacteriocin (called colicins) for killing other susceptible strains of E. coli. Col plasmids of some E. coli also code for the synthesis of bacteriocin (calleccloacins) for killing Enterobacter species. Lactic acid bacteria produce bacteriocin NisinA which inhibits the growth of various gram-positive bacteria and is also used as a preservative in food industries.

5) Metabolic Plasmids: These plasmids (also called degradative plasmids) contain genes to code enzymes that degrade unusual substances such as toluene (aromatic compounds), pesticides (2,4-dichloro-phenoxyacetic acid), and sugars (lactose). An example is the TOL (=pWWO) plasmid of Pseudomonas putida. Some metabolic plasmids that occur in certain strains of Rhizobium induce nodule formation in legumes and carry out fixation of atmospheric nitrogen.

Transposons

A transposon is a DNA sequence that can move or insert itself at a new location in the genome. This phenomenon of transposon movement to a new site in the genome is termed transposition. Transposons can encode transposase (a special protein) that catalyses the transposition process. Transposons are specific for different groups of organisms. They constitute a fairly accountable fraction of genome of organisms like fungi, bacteria, plants, animals, and humans. They can even alter the genetic composition of organisms.

Transposons or transposable genetic elements are also referred to as mobile genetic elements. They can be categorised based on their mode of transposition or on the organisms in which they are present.

Types

Transposons are categorised into the following types based on their transposition mechanism:

1) Cut and paste transposons,

2) Replicative transposons, and

3) Retro elements.

Cut and Paste Transposons

These transposons transpose by cutting the transposable sequence from one position in the genome and inserting it into another position within the genome. (figure 7.9).

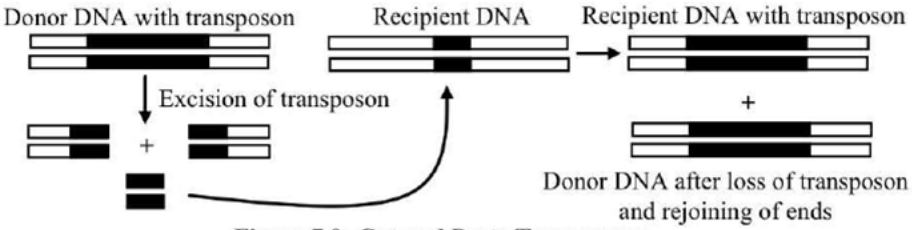


Figure 7.9: Cut and Paste Transposons

In the cut and paste transposition, two transposase sub -units are involved and each binds to the specific sequences at the two ends of transposon. Then these sub-units of transposase protein come together and lead to the excision (cutting) of transposon. The so formed excised transposon-transposase complex gets integrated to the target recipient site. Hence, in this way the transposon is cut from one site and pasted on the other site by a mechanism mediated by transposase protein (figure 7.10).

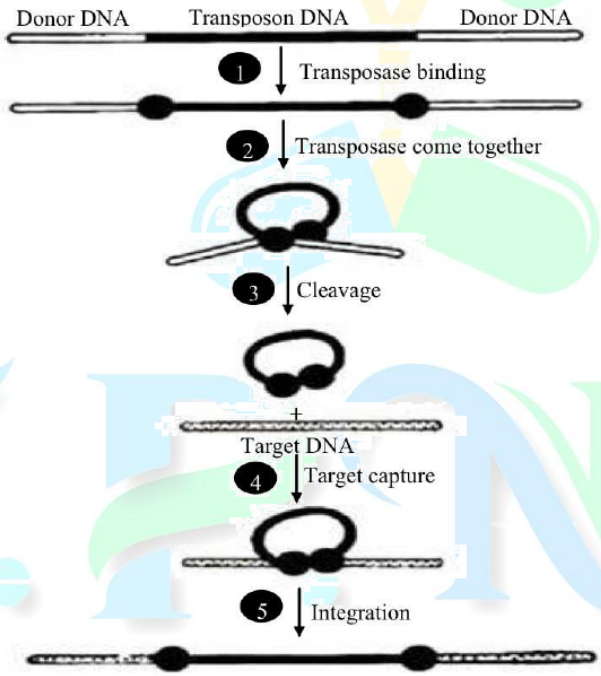


Figure 7.10: Role of Transposase Protein in Cut-and-Paste Transposition

IS-elements, P -elements in maize, hobo -elements in Drosophila, etc. are the examples of cut and paste type of transposons.

Replicative Transposons

These transposons transpose by replicating the transposable sequence, and inserting the so formed copy of DNA into the target site without altering the donor site (figure 7.11). In this type of transposition, one copy of transposon is gained, and after transposition the donor as well as the recipient DNA molecule exhibit one transposable sequence each.

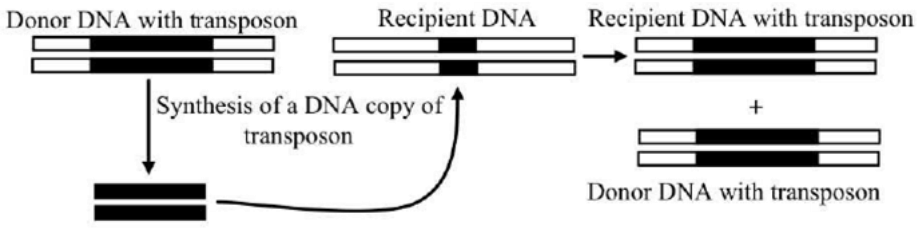


Figure 7.11: Replicative Transposons

Tn3-elements found in bacteria are the examples of replicative transposons.

Retro Elements

These elements transpose by DNA synthesis via reverse transcription (i.e., RNA to DNA) by using elements RNA as the template (figure 7.12). The transposable elements requiring reverse transcriptase for their movement are the retro transposons.

In this type of transposition, an RNA intermediate is involved, and the transposable DNA is transcribed to produce an RNA molecule. Then this RNA is used as a template for producing a complementary DNA by the activity of reverse transcriptase enzyme. The so formed single stranded DNA copy is made double stranded and inserted into the target DNA site.

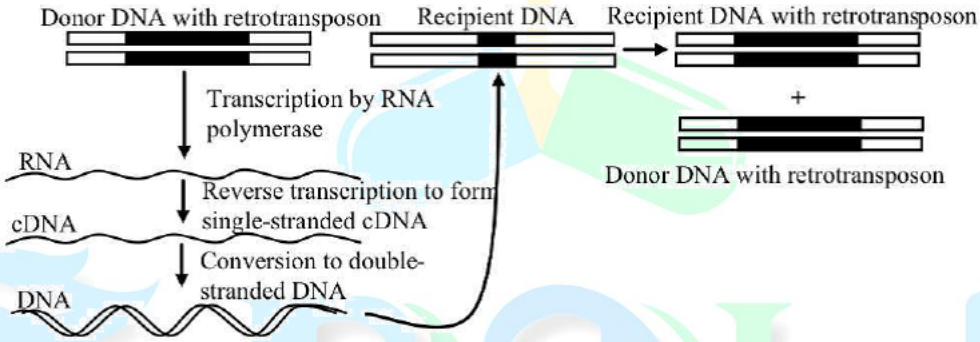


Figure 7.12: Transposition Involving Reverse Transcription

Tn3-elements found in bacteria are the examples of replicative transposons.

Retro Elements

These elements transpose by DNA synthesis via reverse transcription (i.e., RNA to DNA) by using elements RNA as the template (figure 7.12). The transposable elements requiring reverse transcriptase for their movement are the retro transposons.

In this type of transposition, an RNA intermediate is involved, and the transposable DNA is transcribed to produce an RNA molecule. Then this RNA is used as a template for producing a complementary DNA by the activity of reverse transcriptase enzyme. The so formed single stranded DNA copy is made double stranded and inserted into the target DNA site.

Significance

Transposons have the following significant values:

1) They have the ability to alter the structural and functional characteristics of genome by changing their position in the genome.

2) They can cause mutation by insertion, deletion, etc.

3) They can contribute in evolution as they can tremendously alter the genetic organisation of organisms.

4) They can be used as cloning vectors in gene cloning. For example, P -elements are often used as vector for introducing transgenes into Drosophila.

5) They can also be used as genetic markers while mapping the genomes.

6) Transposon-mediated gene tagging can help in searching and isolation of a particular gene.

MICROBIAL BIOTRANSFORMATION

Introduction

Microorganisms are capable of modifying a wide range of organic compounds enzymatically. Bio-transformations (bi-conversions or microbial transformations) are the processes in which microorganisms convert the organic compounds into structurally related products. It can also be said that biotransformation involves microbial conversion of a substrate into a product with a few number of enzymatic reactions.

Biotransformation is vital to survival as it converts the absorbed nutrients (food, oxygen, etc.) into substances required for normal body functions. In some pharmaceuticals, the metabolite (and not the absorbed drug) is therapeutically effective; for example, the biotransformed metabolite of phenoxybenzamine (a drug for hypertension) is the active agent. Biotransformation also serves as an important defence mechanism in which the toxic xenobiotics and body wastes are converted into less harmful substances that can be removed from the body.

Types of Reactions

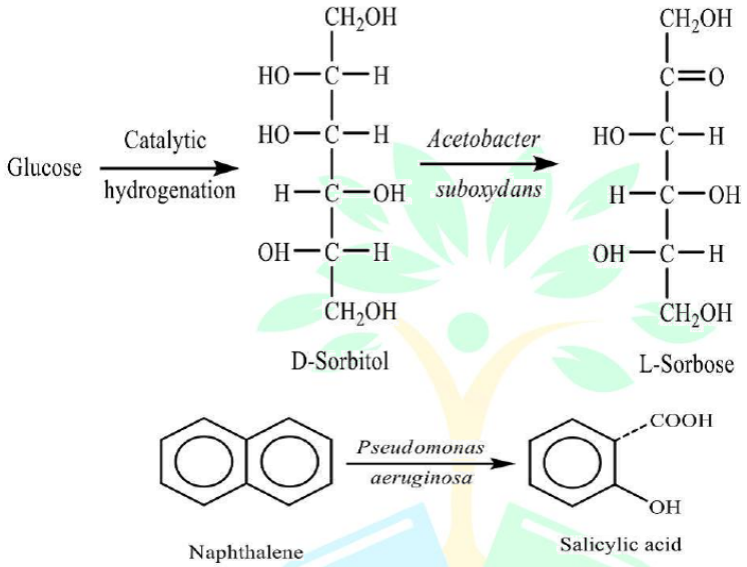
Microorganisms perform different types of simple or mixed reactions. Some important biotransformation reactions and the involved microorganisms are listed in the table 7.1:

Table 7.1: Selected List of Important Biotransformation Reactions

Types of Reaction	Examples	Commonly used Microorganisms
Oxidation	Tryptophan→ 5-Hydroxytryptophan	*Bacillus subtilis*
	Naphthalene → Salicylic acid	*Corynebacterium* sp.
Reduction	Benzaldehyde → Benzyl alcohol	*Saccharomyces cerevisiae*
	Nitropentachlorobenzol → Pentachloroaniline	*Streptomyces aureofaciens*
Hydrolysis	Anhydrotetracycline → Tetracycline	*Streptomyces aureofaciens*
	Methyl laureate → Menthol	*Mycobacterium phlei*
Condensation	Streptomycin → Streptomycin-phosphate	*Streptomyces griseus*

1) Oxidation: A rule framed based on the results of earlier studies stated that if a secondary hydroxyl group of a polyol is located between a primary and a secondary group, which is in the cis-position with respect to the oxidisable group, then the hydroxyl group is oxidised to give a ketone.

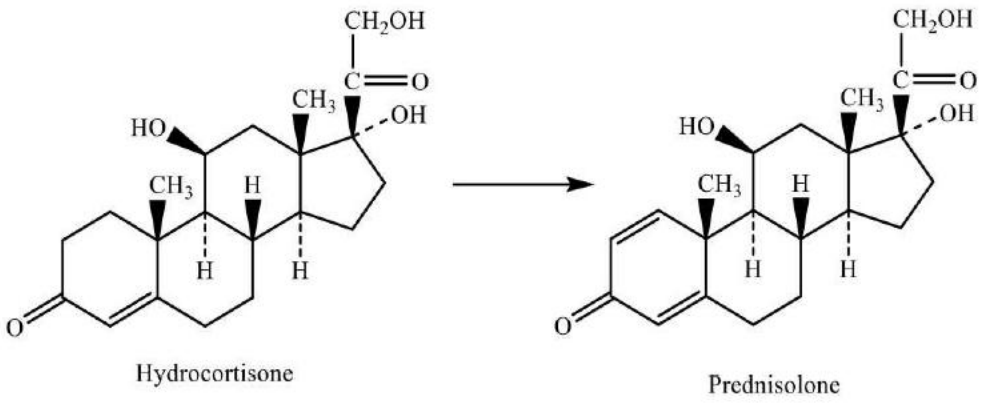
For example, conversion of D -sorbitol to L -sorbose by microbial oxidation is an important step in the synthesis of L -ascorbic acid; microbial conversion of p-cymene to cumic acid and p-xyleneto p-toluic acid; conversion of naphthalene into salicylic acid by oxidation by Pseudomonas aeruginosa.



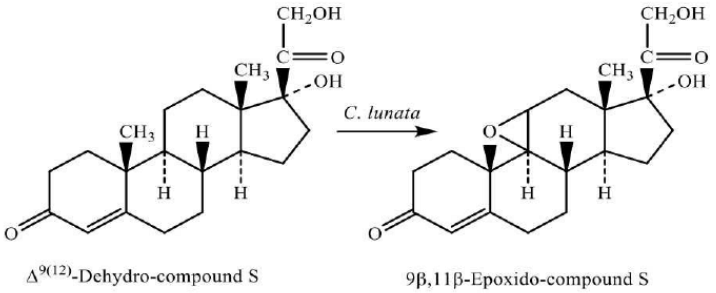
2) Hydroxylation: Hydroxylation of steroid is a monooxygenase reaction. It has been established that the carbon atoms can be hydroxylated by different organisms. The stereospecific microbial hydroxylations are practically available in all the carbon atoms of steroid molecule. 11alpha-hydroxylation, beta-hydroxylation, 16 alpha-hydroxylation, and 21- hydroxylation are the industrial ly important hydroxylation reactions. The

increased demand for cortisone and hydrocortisone can be met by microbial hydroxylation of steroid intermediates at C-11.

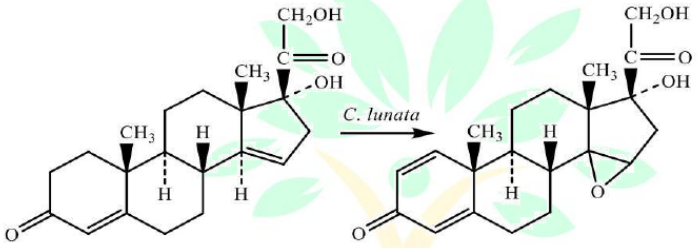
3) C-1 Dehydrogenation: The therapeutic properties of cortisone and cortisol were modified by introducing a double bond be tween C -1 and C -2, and producing prednisone and prednisolone, respectively.



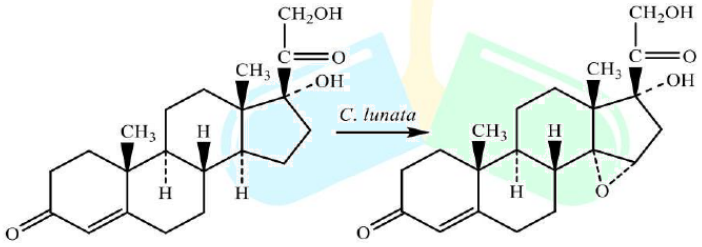
4) Epoxidation: When an unsaturated steroid is incubated with a microorganism capable of introducing an axial hydroxy group into the nucleus at unsaturation point, epoxidation of the isolated double bond takes place. Some examples of epoxidation through the action of C.lunata are:



$\Delta^{9(12)}$-Dehydro-compound S — 9β,11β-Epoxido-compound S



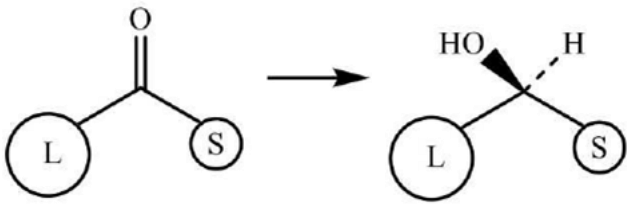
Δ^{14}-Dehydro-compound S — 14α,15β-Epoxido-compound S



Δ^{14}-Dehydro-compound S — 14α,15β-Epoxido-hydrocortisone

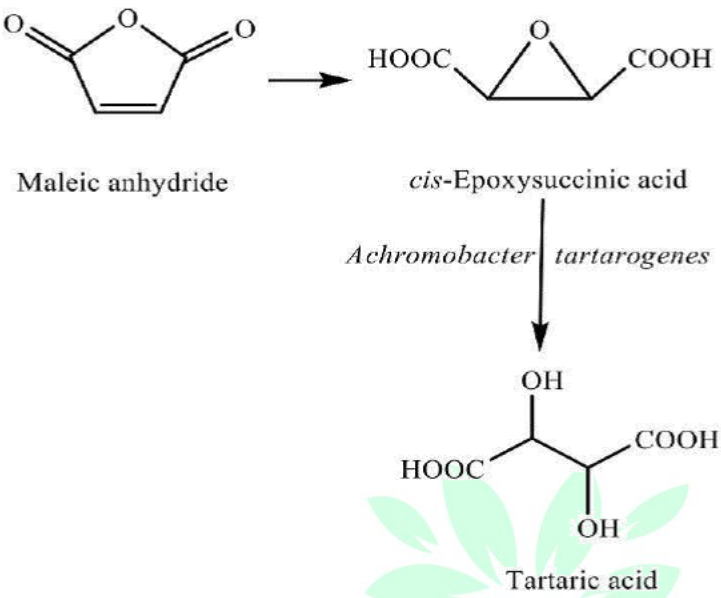
5) Reduction: The size and nature of the substituents in the substrates influence the stereospecific microbial reduction of ketonic substrates. For example, a highly stereospecific reduction of racemic decalone and hexahydroindanone derivatives and of related di - and tri-cyclic ketones by Curvularia falcata.

Stereospecific reduction occurs when the ketone is flanked by large (L) and small (s) groups and gives alcohol of S-configuration.

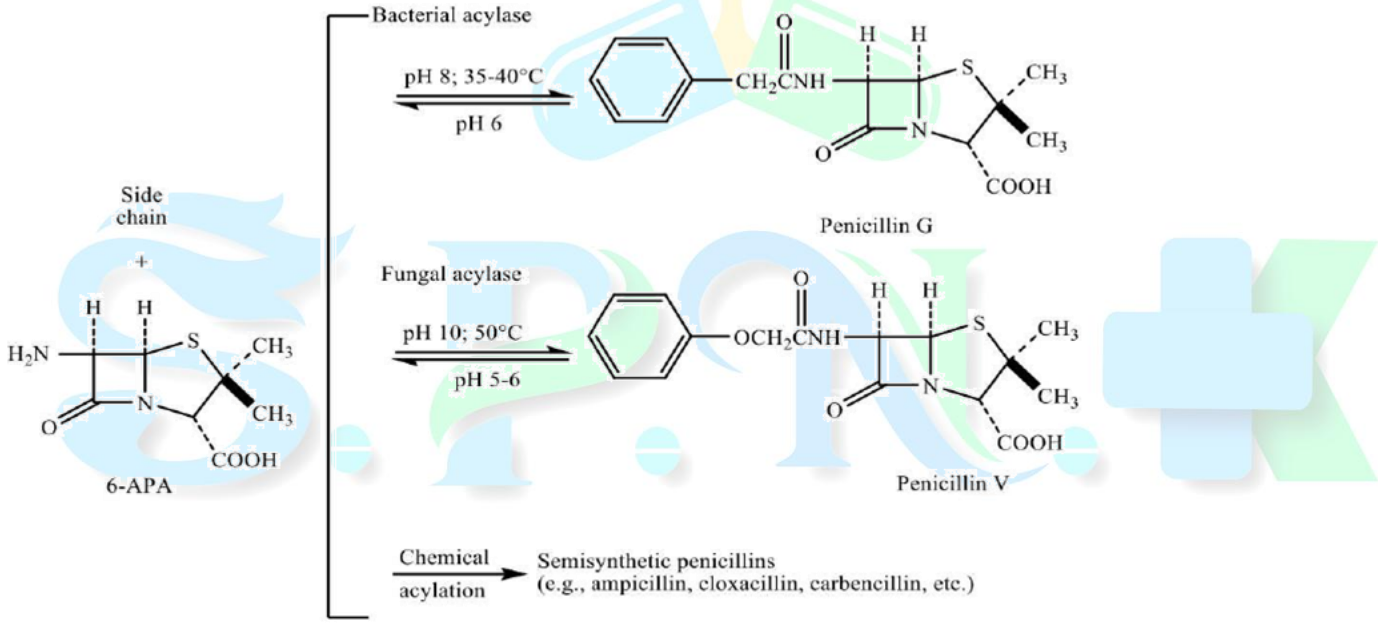


Microorganisms can also establish selective reductions of 8-diketones important in steroid synthesis.

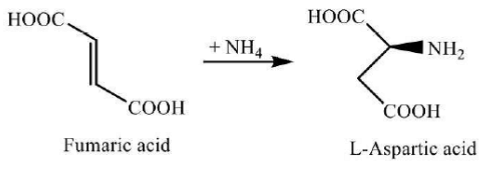
6) Hydrolysis: A large number of esters, glycosides , epoxides, lactones, Beta- lactams, and amides can be hydrolysed by microorganisms. For example, tartaric acid is produced from maleic anhydride by Achromobacter tartarogenes.



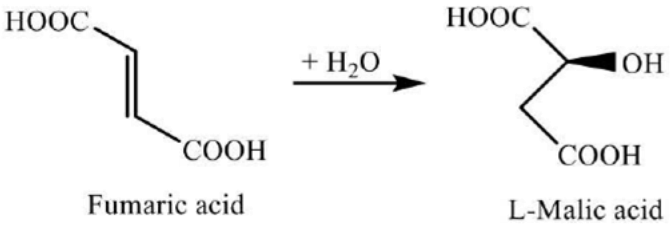
Large scale manufacture of 6 -APA (the principal intermediate in the manufacturer of semi -synthetic penicillins) by selective hydrolysis of penicillin G or V is catalysed by penicillin acylase enzyme. This enzyme may be either bacterial acylase (which hydrolyses penicillin G) or fungal acylase (which hydrolyses penicillin V).



7) Hydration and Amination: Asymmetric addition of water or ammonia to fumaric acid yields optically active products. Therefore, aspartase –producing bacteria have been used for manufacturing L-aspartic acid.



Pre-treatment with det ergents is recommended to suppress the formation of succinic acid, and this result into hydration to L-malic acid (with 70% yield).



Fumaric acid L-Malic acid

Amination is involved in the manufacture of many -amino acids and of guanosine-5'-monophosphate (5 '-GMP) with the hel p of two mutants of Brevibacterium ammoniagenes.

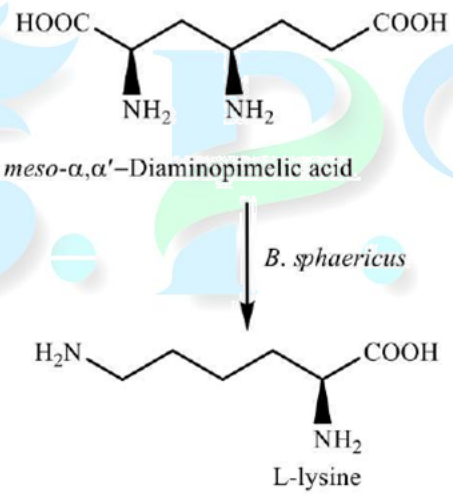
8) Dehydration: Microbial dehydration of fatty acids, histidine, cis-terpene hydrate, L-phenylalanine, etc. are some commonly occurring reactions.

9) Deamination: Microbial deamination is either of the two types:

i) Hydrolytic: Forexample, conversion of L -tyrosine into 4 - hydroxyphenylacetic acid, or

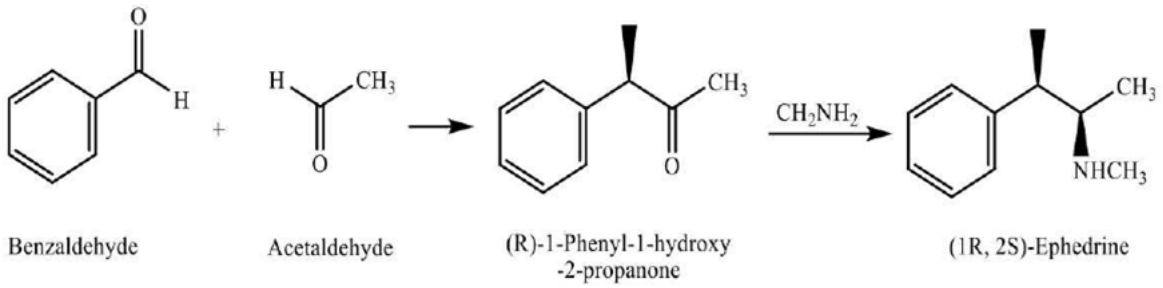
ii) Oxidative: For example, conversion of isoguanine into xanthine and formycin A, which further converts into formycin B.

10) Decarboxylation: An important decarboxylation reac tion is of aromatic and linear carboxylic acids. Another example is the synthesis of L-lysine by stereospecific decarboxylation (catalysed by Bacillus sphaericus) of meso- (but not DL)-α, α' -diaminopimelic acid (DAP) to L-lysine.



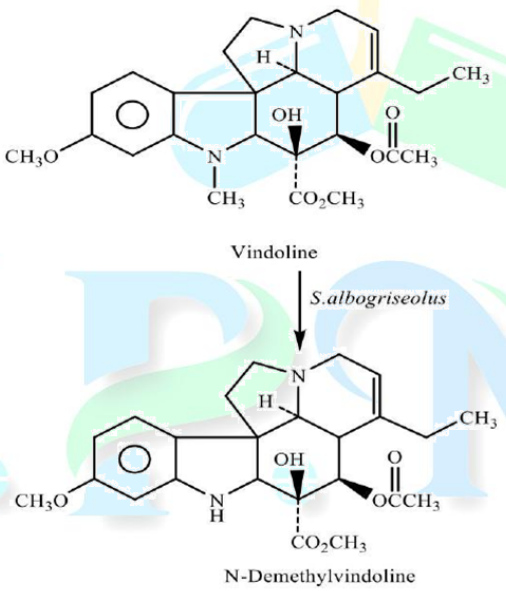
L-lysine

11) Condensation: Condensation is the reaction of substituted benzaldehyde utilised in 1934 for synthesising natural (1R,2S) -ephedrine. Acetaldehyde (produced from glucose by microorganisms) is reacted with benzaldehyde added to the fermenting yeast, to yield (R) -l-phenyl-l-hydroxy-2propanone. Propanone undergoes reductive chemical condensation with methylamine to yield (1R, 2S)-ephedrine.

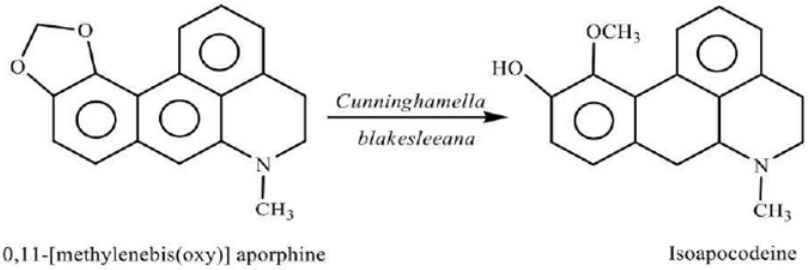


12) Transglycosylation: This reaction was used in 1944 to prepare sucrose from glucose-1-phosphate and fructose by sucrose phosphorylase of Pseudomonas saccharophila. Tri- and tetra-saccharides from sucrose, lactose, maltose, and cellobiose can also be obtained by this reaction utilising various bacterial, yeast, and fungal enzymes.

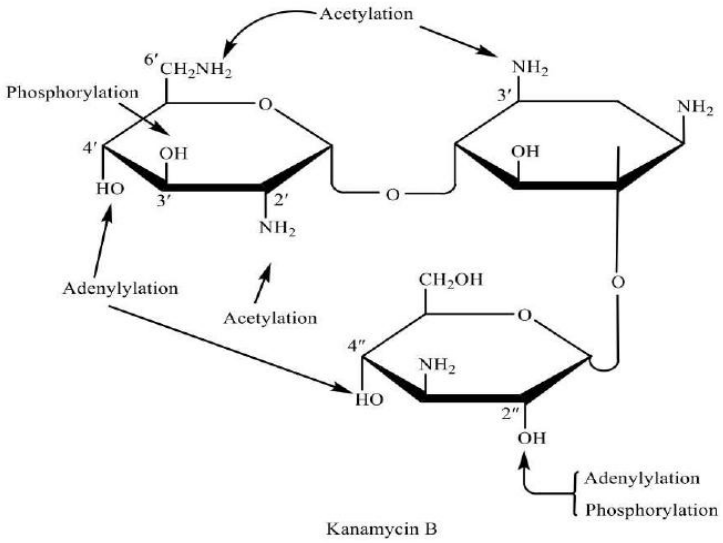
13) N- and O-Demethylation: This conversion of natu ral products is preferred over chemical treatments due to its high selectivity. Vindoline (a monomeric vinca alkaloid) is used for synthesising an anti -tumour compound, and is converted to N-demethyl vindoline by Streptomyces albogriseolus.



O-demethylation of 10,11 -[methylenebis(oxy)] aporphine by Cunninghamella blakesleeana proceeds with high regioselectivity and yields isoapocodeine.

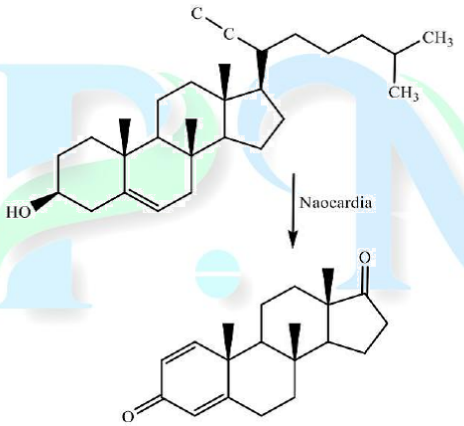


14) **N-Acetylation, O -Phosphorylation, and O -Adenylyation:** Through these conversions, therapeutically valuable and partially or completely inactive aminocyclitol antibiotics, e.g., streptomycin, kanamycin, gentamicin, neomycin, etc. can be obtained. For example, kanamycin B is inactivated at several places by any of the three mechanisms.



Kanamycin B

15) Cleavage of Side Chains: In 1913, the first cholesterol degradation by microorganism was reported. Later it was observed that various microorganisms cause total degradation of cholesterol. It was noticed that since enzymatic reactions involved in side chain degradation and ring cleavage occur independently and simultaneously, the ring cleavage activity can be inhibited selectively by adding specific inhibitors and modifying substrate molecule.



16) Isomerisation: Isomerisation of steroids is useful for manufacturing high fructose syrup (used as a food sweetener). Using a similar process, inexpensive carbohydrates are hydrolysed to glucose, which is isomerised in 50% solution by a Streptomyces strain. Elucidation of these mechanisms has allowed chemical modification of the sites where inactivation occurs. A number of such bioactive analogues (e.g., dibekacin and amikacin) have been prepared and are not subjected to inactivation; thereby inhibiting the microorganisms against which the parent antibiotics are ineffective. This approach can fight the problem of drug resistance in case of antibiotics and anti-malarials. It can be thus concluded that microbial transformations (bioconversions) are employed in the non -chemical synthesis of various compounds at most

competitive prices. Sometimes chemical synthesis can be supplemented with microbial transformation. This method is useful even when a compound cannot be synthesised chemically.

Sources of Biocatalysts

Biotransformation reactions employa large range of biological catalysts, including growing cells, resting cells, killed cells, immobilised cells, cell-free extracts, enzymes, and immobilised enzymes.

Given below are the important sources of biocatalysts and the biotransformation procedures:

1) Growing Cells: The desired cells are cultivated in a medium. As the cells start growing (6-24 hours), the culture is added with a concentrated substrate. Emulsifiers (e.g., Tween, organic solvents) can also be added to solubilise the substrates and/or products, e.g., steroid biotransformation. With the help of spectroscopic or chromatographic techniques, the conversion of substrate into products can be monitored. On achieving optimum product, biotransformation is stopped.

2) Non-Growing Cells: The following reasons make the use of non –growing cells for biotransformation reactions desirable:

i) Substrate can be used in high concentration, and this high substrate concentration stops the cell growth.

ii) Cells can be washed before use, thus removing any contaminating substances.

iii) The conversion efficiency of substrate into product is high.

iv) Biotransformation can be optimised by maintaining desirable environmental conditions (pH, temperature, etc.).

v) Isolation and recovery of the product is easy.

3) Immobilised Cells: These cell scan be used to carry out biotransformation reactions continuously. Also, the same cells can be used repeatedly. Some bioconversions with single or multistage reactions are carried out using immobilised cells, e.g., commercial production of L - alanine and malic acid.

4) Immobilised Enzymes: The cell -free enzyme systems are used in bioconversions in the form of immobilised enzymes, which have the following advantages:

i) Undesirable side reactions do not occur.

ii) Desired products do not get degraded.

iii) No transport barrier is present across the cell membrane for the substrate or product.

iv) Isolation and recovery of the product is simple and easy.

v) Several immobilised enzyme systems have been developed for bio - transformations, e.g., glucose isomerase and penicillin acylase.

Applications

Microbial biotransformation is used in the following processes:

1) While studying the fate of organic chemicals in the environment, it has been found out that a large reservoir of enzymatic reactions with a large potential in preparative organic synthesis, has been exploited for several oxygenases on pilot and even on industrial scale.

2) Novel catalysts can be obtained from metagenomic libraries and DNA sequence based approaches.

3) There are increasing capabilities in adapting the catalysts to specific reactions and process requirements by rational and random mutagenesis, thus broadening the scope for application in fine chemical industry and in the field of biodegradation.

4) These catalysts are exploited in whole cell bioconversions or in fermentations, calling for system -wide approaches to understand strain physiology and metabolism and rational approaches to the engineering of whole cells as they are increasingly put forward in the area of systems biotechnology and synthetic biology.

5) Biological processes are employed in the removal of contaminants and pollutants from the environment.
6) Some microorganisms possess a catabolic adaptability to degrade or transform such compounds. New methodological breakthroughs in sequencing, genomics, proteomics, bioinformatics and imaging are producing information in large amounts.

7) In the field of environmental microbiology, genome-based global studies provide exceptional views of metabolic and regulatory networks and evidences to the evolution of biochemical pathways. These evidences are relevant to biotransformation and to the molecular adaptation strategies to changing environmental conditions.

8) Bio-transformation is also used for producing steroids with the help of Streptomyces lavendulae and Aspergillus and Penicillium species.

MUTATION

Introduction: An abrupt alteration in the phenotype of an individual is termed mutation. Molecularly, mutation denotes any permanent and rare change in genome i.e., the number or sequence of nucleotides. Wright in 1791 revealed mutation in male lamb with short legs. **Hugo de Vries** in 1901 coined the term mutation defining heritable phenotypic changes. In each species, the chromosomes present a characteristic structure and number. A mutagenic agent (like ionising radiation), a chemical compound, irregularities during cell division, crossing over, and fertilis ationmay sometimes lead to spontaneous breakage of chromosomes. A minute alteration in the structure or number of chromosome may lead to phenotypical changes. The change s in the genetic material associating a chromosome or its part are referred to as chromosomal aberrations or chromosomal mutations.

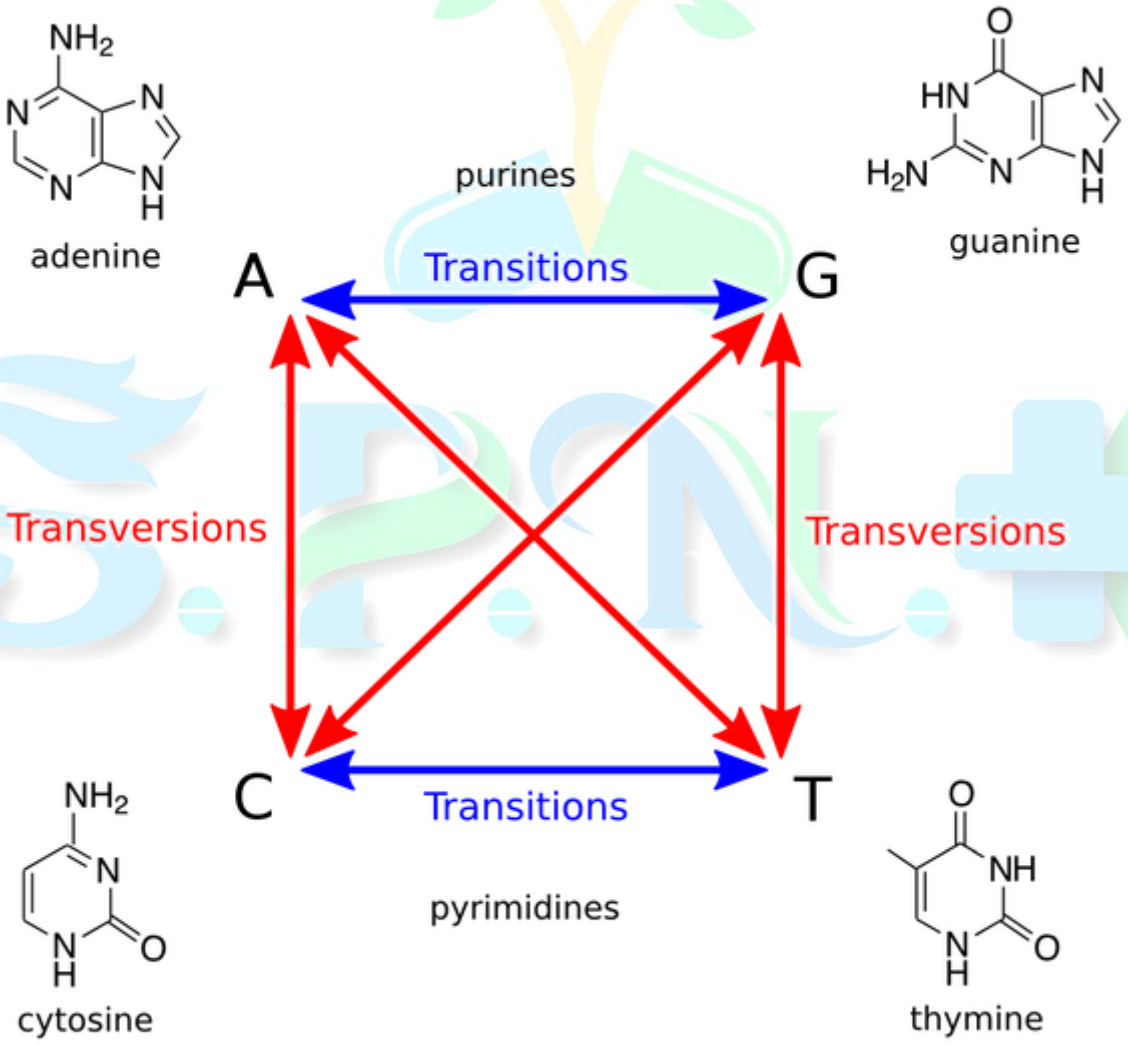
Types of Mutation

Mutation is of the following types:

1) Point Mutation: In this type, a single nucleotide is changed in a DNA sequence. Point mutation is determined by comparing the DNA sequences in wild type and the mutant. If one base has been replaced with another, it is called base substitution When studying — these changes at the level of nucleotide, they are categorised into transition and transversion (figure 8.1):

i) Transition Mutation: In this type, a purine or a pyrimidine is replaced with another purine or pyrimidine, respectively. For example, guanine replaces adenine or adenine replaces guanine ; cytosine replaces thymine and thymine replaces cytosine.

ii) Transversion Mutation: In this type, a pyrimidine is replaced with a purine on the same strand or vice versa. For example, cytosine or thymine replaces guanine; cytosine or thymine replaces adenine; thymine is changed by adenine or guanine; adenine or guanine replaces cytosine.



Base-pair substitution leads to any of the three mutation types that influence the process of translation:

i) Nonsense Mutation: In this type, one DNA base pair undergoes change. Here one amino acid is not replaced with another, but the altered DNA sequence prematurely signals the cell to stop the protein synthesis (figure 8.2). This type of mutation forms a shortened protein that is either slightly functional or non-functional.

ii) Missense Mutation: In this type, one DNA base pair undergoes change causing replacement of one amino acid with another in the protein made by a gene (figure 8.2).

iii) Silent Mutation: In this type, sequence of bases ina DNA molecule undergoes change; however, the amino acid sequence of a protein is not altered (figure 8.2).

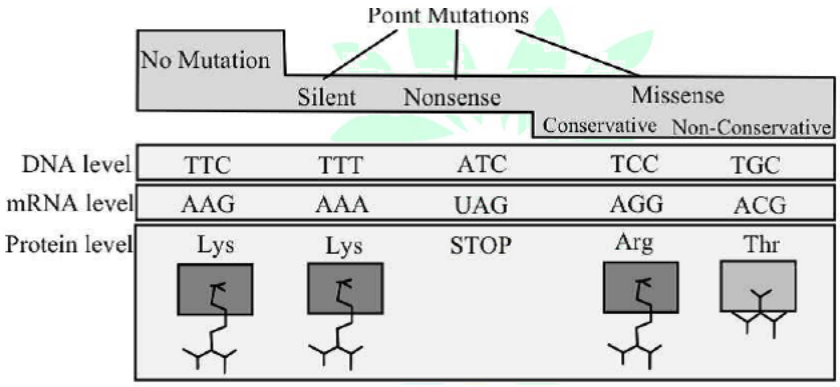
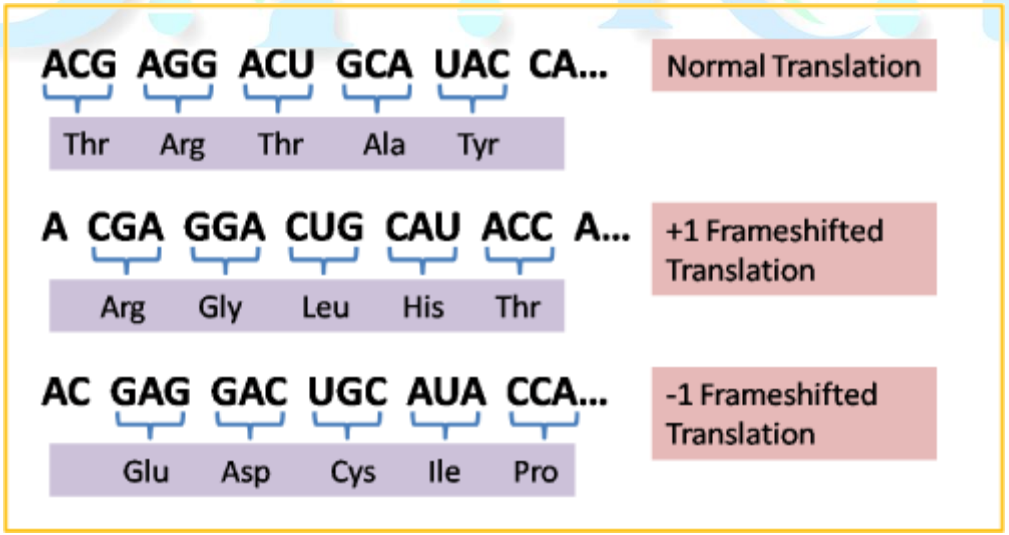


Figure 8.2: Types of Point Mutations

2) Neutral Mutation: In this type, the replacement of amino acid due to base substitution does not cause any functional change in the protein. For example, the amino acid serine is converted to threonine; valine is converted to alanine, etc.

3) Frameshift Mutation: In this type, addition or removal of DNA bases alters a reading frame of genes that comprises of groups of 3 bases each coding for a single amino acid. Frameshift mutation shifts the grouping of these bases and alters the amino acids coding toforma non-functional protein. Frameshift mutation includes insertions, deletions, and duplications.



4) Deletion Mutation: In this type, the deletion can be short (point where one base pair is involved) or long (removal of a large fragment). Bacteria are haploid containing a copy of each gene and cannot remove the essential genes. Bacterial genome generally bears long sequence s that can be deleted with no loss of bacterial viability. Recombination of directly repeated sequences in the bacterial genome causes deletion. Reversion (replacement of the lost or deleted original sequence) of deletion mutants is rarely possible. The deletion of three bases (codon) forming mutated polypeptide is shown in figure 8.4:

Template DNA	→	TAC	AAA	TGG	CTG	.....	ATA	CCT	TTT	.....
mRNA	→	AUG	UUU	ACC	GAC	.....	UAU	GGA	AAA	.....
Polypeptide	→	Met	Phe	Thr	Asp	.....	Tyr	Gly	Lys	.....
					↓ Deletion mutation					
Template DNA	→	TAC	AAA	TGG	.....		ATA	CCT	TTT	.....
mRNA	→	AUG	UUU	ACC	.....		UAU	GGA	AAA	.....
Polypeptide	→	Met	Phe	Thr	.....		Tyr	Gly	Lys	.....

Figure 8.4: Deletion Mutation due to the Deletion of Three Bases or a Codon

5) Duplication: In this type, a piece of DNA is abnormally copied multiple times. This unusual copying result in functional changer the protein formed.

6) Inversion: Occasionally, a region of DNA reversibly (but still inherited) aligns into the opposit e orientation. If the signals needed for expressing an adjacent gene are located in the inverted region, inversion will shift the associated gene on or off. Repeated sequences in a genome may present inversion as well. For example, variation in the flagellar antigens is seen in Salmonella typhimurium.

7) Insertion Mutation: In this type, integration ofa large sequence fragment occurs through insertion of sequence elements or transposons. Insertion sequences give rise to insertion mutations by inserting into other DNA sequences. A large amount of spontaneous mutations occurs through genes inactivation by inserting a copy of an insertion sequence element rather (and not by replication errors). Transposons are DNA elements greater than insertion sequences. They switch from one region to another ina DNA sequence. Insertion of a transposon into a gene disorders the gene function. Transposons have one or more recgnisable genetic markers. The exclusively studied transposons bear antibiotic resist ance genes, facilitating the isolation of insertion mutants.

Template DNA	→	TAC	AAA	TGG	CTG	.....	ATA	CCT	TTT	.....
mRNA	→	AUG	UUU	ACC	GAC	.....	UAU	GGA	AAA	.....
Polypeptide	→	Met	Phe	Thr	Asp	.....	Tyr	Gly	Lys	.....
					↓ Insertion mutation					
Template DNA	→	TAC	AAA	TGG	GCT	CTG	.....	ATA	CCT	TTT	.....
mRNA	→	AUG	UUU	ACC	CGA	GAC	.....	UAU	GGA	AAA	.....
Polypeptide	→	Met	Phe	Thr	Arg	Asp	.....	Tyr	Gly	Lys	.....

Figure 8.5: Insertion Mutation due to Insertion of Three Bases or a Codon

8) Substitution Mutation: In this type, an exchange between two bases (i.e. switching of achemical letter, like T to C) occurs. This alters a codon encoding a different amino acid, thus producing a different protein. At times the protein structure is not affected by substitutions; such mutations are named as silent mutations. Whereas if substitution s alter an amino acid coding codon to a single stop codon, an incomplete protein is produced. This disturbs the protein structure that may totally change the organism. For example, in sickle cell anaemia substitution occurs in codon (GAG mutates to GTG and changes Glu to Val).

9) Repeat Expansion: In this type, the number of repetition of short DNA sequence is increased. Thus, the formed protein functions differently than the original. Nucleotide repeats are t he short DNA sequences repeated many times in a row. For example, a tri-nucleotide repeat comprises of 3 base-pair sequences, and a tetra-nucleotide repeat comprises of 4 base-pair sequences.

Types of Mutants

Mutant is a mutation product. It could be a genotype, a cell, a polypeptide, or an individual. The four categories of identifiable mutants are:

1) Morphological Mutants: These mutants show alteration in their form (shape, size, and colour). Some examples of morphological mutants are albino spores in Neurospora, curly wings in Drosophila, dwarf - sized peas, and sheep with short legs.

2) Lethal Mutants: In these mutants, the new allele is recognized by its mortal or lethal effect on the organism. Lethal mutant alleles lead to the death of individuals carrying them; however, semi-lethal or sub - vital alleles let the individuals survive.

3) Conditional Lethal Mutants: Under specific environmental conditions, some alleles generate a mutant phenotype called restrictive mutants. When the conditions are different, they produce a normal phenotype called permissive that can be grown under permissive conditions and later transferred to restrictive conditions for further evaluation.

4) Biochemical Mutants: In these mutants, their biochemical function of the cell is lost. The normal function of the cell is regained upon enriching the medium with suitable nutrients. For example, adenine auxotrophs can be grown in adenine -rich med ium, whereas wild type can grow in adenine - deficient medium.

Agents of Mutation - Mutagens

Physical or chemical agents that increase the frequency of mutations are termed mutagens. Many radiations (physical mutagens) and chemicals (chemical mutagens) act as mutagens. Various physical and chemical mutagens are presented below:

1) Physical Mutagens: These include radiations like X -rays, Gamma-rays, Alpha-rays, Beta-rays, fast and thermal (slow) neutrons, and UV rays (table 8.1).

Table 8.1: Physical Mutagens (Radiation), their Properties and Mode of Action

Types of Radiation	Properties	Mode of Action
X-rays	Penetrating and non-particulate.	Induce mutations by forming free radicals and ions; c ause addition, deletion, transition and transversion.
γ-rays	Highly penetrating and non - particulate.	Induce mutations by ejecting atoms from the tissues; c ause addition, deletion, transition and transversion.
α-rays	Less penetrating, particulate, and positively charged.	Induce mutations by ionisation and excitation; c ause chromosomal and gene mutations.
β-rays	More penetrating than α-rays, particulate, and negatively charged.	Induce mutations by ionisation and excitation; c ause chromosomal and gene mutations.
Fast and thermal neutrons	Highly penetrating, particu late, and neutral particles.	Cause chromosomal breakage and gene mutations.
UV rays	Low penetrating and non - ionising.	Cause chromosomal breakage and gene mutations.

2) Chemical Mutagens: These are categorized into four groups (table 8.2):

Table 8.2: Some Commonly used Chemical Mutagens and their Mode of Action

Groups of Mutagen	Chemicals	Mode of Action
Alkylating agents	Ethyl methane sulphonate Ethyl ethane sulphonate	AT ↔ GC transitions GC ↔ AT transitions
Base analogues	5-Bromo uracil 2-Amino purine	AT ↔ GC transitions AT ↔ GC transitions
Acridine dyes	Acriflavin Proflavin	Deletion, addition , and frame shifts.
Others	Nitrous acid Hydroxylamine Sodium azide	AT ↔ GC transitions GC ↔ AT transitions Transitions

Physical Mutagens

Physical mutagens include various types of radiations, i.e., X-rays, gamma rays, alpha particles, beta particles, fast and thermal (slow) neutrons , and UV rays (table 8.3).

Table 8.3: Physical Mutagens (Radiation), their Properties and Mo de of Action

Types of Radiation	Properties	Mode of Action
X-rays	Penetrating and non - particulate	Induce mutations by forming free radicals and ions; cause addition, deletion, transition and transversion.
γ-rays	Highly penetrating and non-particulate.	Induce mutations by ejecting atoms from the tissues; cause addition, deletion, transition and transversion.

α-rays	Less penetrating, particulate, and positively charged.	Induce mutations by ionisation and excitation; cause chromosomal and gene mutations.
β-rays	More penetrating than α-rays, particulate, and negatively charged.	Induce mutations by ionisation and excitation; cause chromosomal and gene mutations.
Fast and thermal neutrons	Highly penetrating, particulate, and neutral particles.	Cause chromo somal breakage and gene mutations.
UV rays	Low penetrating and non - ionising.	Cause chromosomal breakage and gene mutations.

Radiations are the most important physical mutagen. They cause damage in the DNA molecules falling in the wavelength range below 340nm and photon energy above 1eV (electron volt). The destructive radiation consists of ultraviolet UV-rays, X -rays, Gamma-rays, Alpha-rays, Beta-rays, cosmic rays, neutrons, etc.

Radiations cause three types of damage, i.e., lethal damage (killing the organisms), potentially lethal damage (can be lethal under certain ordinary conditions), and sub-lethal damage (cells do not die till the radiations reach a certain threshold value).

On exposing the bacteria to radiation, their ability to develop colonies is lost gradually.

This gradual loss of viability is graphically expressed on a plot of the surviving colonies against the increasing exposure time. This dose-response graph is termed as **survival curve** (figure 8.6).

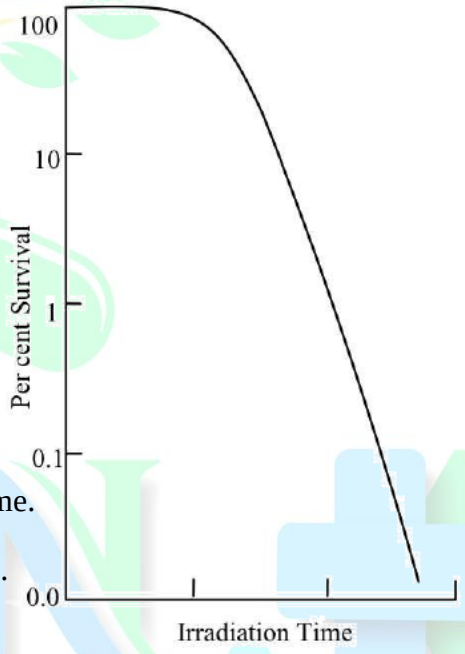


Figure 8.6: Typical UV Light Survival Curve for a Bacterium

This curve is analysed by a simple mathematical theory called **hit theory**. Every organism possesses a sensitive target site, which is damaged or hit by radiation photons (light particles), thus inactivating the organisms. The related equation can be derived based on this theory. These equations are used for calculating the survival curve for the populations containing 'N' identical organisms exposed to 'D' dose of damage causing radiation.

$$N=No\ e^{-KD} \qquad \text{----------> (1)}$$

The surviving fraction (S):

$$S=N/No=e^{-KD} \qquad \text{----------> (2)}$$

On plotting S against D, a straight line with a slope of K is obtained (figure 8.6). This curve is termed an exponential or single hit curve, and is obtained when the phages are irradiated with X-rays.

If in a population of different organisms, each organism consists of 'n' sites, each site should be hit to inactivate the organism. Therefore, each organism is hit by 'n' times. The probability of one unit being hit by a dose 'D" is:

$$P=1-e^{-KD}$$

So the probability of Pn will be:

$$Pn = (l-e^{-KD})^{n}$$

The surviving fraction (S) of the population is:

$$1—pn \text{ or } S= 1-(1-e^{-KD})n \text{ ----------> } (3)$$

On expanding equation (3):

$$S=1-(1-ne^{-KD} +e^{-nKD})$$

At the large value of D, the higher order terms become negligible. Therefore, at high dose (D):

$$S=ne^{-KD} \text{ (or)}$$

$$\text{In } S = \text{In } n—KD \text{ ----------> } (4)$$

On plotting equation (3) for K -1, the different values of n reveals that for small a values of D, In S gradually changes. (figure 8.7). At large value of D, equation (4) dominates and the curve becomes linear.

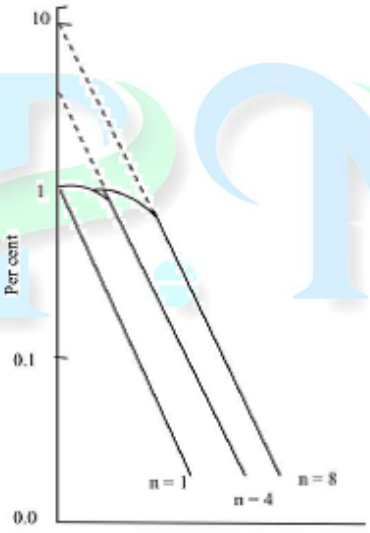


Figure 8.7: Survival Curve for Different Values of n (Hits' Time)

Ultraviolet (UV) Radiation

UV radiation damages the DNA duplex of bacteria and phages. They get absorbed and excite the macromolecules. The absorption maxima of nucleic acid (280nm) and protein (260nm) are almost the same.

UV rays generally target the DNA molecule and not the proteins. However, the absorption spectrum of RNA and that of DNA are quite similar.

The DNA on excitation causes cross-linking, breaks the single strands and damages the base as minor lesions, while it generates nucleotide dimer as a major lesion. Purines are more radio-resistant than pyrimidine, while thymine is more reactive than cytosine.

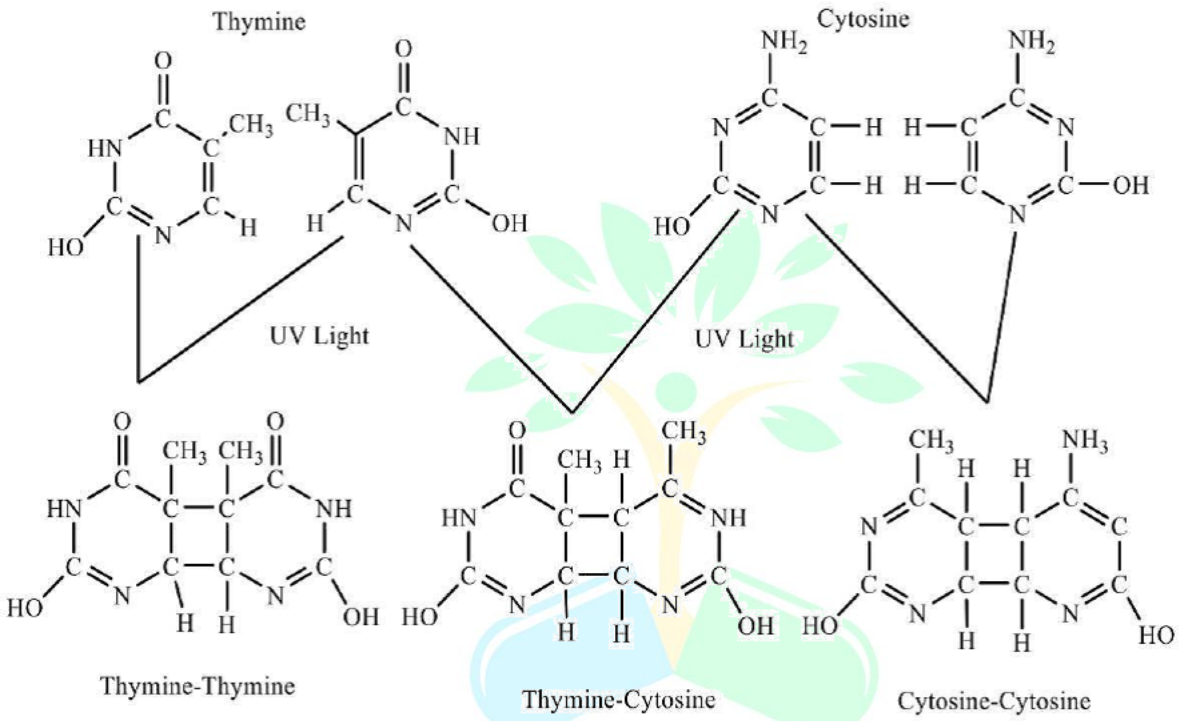


Figure 8.8: Formation of Pyrimidine Dimer Induced by UV Radiation

The ratio of thymine -thymine (TT), thymine -cytosine (TC), and cytosine - cytosine (CC) dimer (figure 8.8) is 10:3:3, respectively. A few dimers of TU and UU also appear. The initial step in pyrimidine dimerization is the hydration of their 4:5 bonds.

The DNA helix is distorted by the formation of thymine -thymine (TT) dimer as they are pulled towards each other. This distortion weakens the hydrogen bonding to adenines in the opposing strand. This structural distortion inhibits the advance of replication fork.

X-Rays

The X-rays break the phosphate ester linkages in the DNA at multiple points; thus, resulting in the deletion or rearrangement of a large number of bases in the DNA molecule.

The X-rays break either one or both the strands of DNA. If both the DNA strands break, the condition becomes fatal. The DNA segment between the two breaks is removed, thereby causing deletion. Since both the X -rays and UV rays damage the DNA molecule, they are used in sterilization of bacteria and viruses.

Chemical Mutagens

The chemical mutagens are divided into the following four groups (table 8.4):

Table 8.4: Some Commonly used Chemical Mutagens and their Mode of Action

Groups of Mutagen	Chemicals	Mode of Action
Alkylating agents	Ethyl methane sulphonate Methyl methane sulphonate Ethyl ethane sulphonate Ethylene imines	AT ↔ GC transitions Transitions GC ↔ AT transitions Transitions
Base analogues	5-Bromouracil 2-Aminopurine	AT ↔ GC transitions AT ↔ GC transitions
Acridine dyes	Acriflavine, proflavine	Deletion, addition, and frame shifts.
Others	Nitrous acid Hydroxylamine Sodium azide	AT ↔ GC transitions GC ↔ AT transitions Transitions

Alkylating Agents

An alkyl group' is added to the hydrogen bonding oxygen of guanine (N, position) and adenine (at N 3 position) residues of DNA with the help of alkylating agents. This result in alkylation, which increases the probability of ionisation with the introduction of pairing errors. Hydrolysis of linkage of base - sugar occurs that causes a gap in one chain.

Depurination is the name given to this phenomenon of loss of alkylated base from the DNA molecule (by breakage of bond joining the nitrogen of purine and deoxyribose). Depurination is not always mutagenic. The gap caused by the loss of purine can be repaired.

Given below are the commonly used alkylating agents:

1) Dimethyl Sulphate (DMS),

2) Ethyl Methane Sulphonate (EMS, -$CH_3CH_2SO_3CH_3$)

3) Ethyl Ethane Sulphonate (EES, -$CH_3CH_2SO_3CH_2CH_3$)

EMS can specifically remove guanine and cytosine from the chain and create a gap in which any base (A, T, G, and C) can be inserted. During replication, chain without gap will form a normal DNA. In the second round of replication, the second gap is filled by a suitable base.

On inserting a correct base, normal DNA sequence is produced. On inserting incorrect bases, transversion or transition mutation occurs. For example, methyl nitrosoguanidine adds methyl group to guanine , thus causing it to mispair with thymine. After subsequent replication, GC converts into AT.

Base Analogues

A chemical compound that is similar to one of the four DNA bases is termed a base analogue. It can be introduced in a growing polynucleotide chain when normal replication occurs. Base analogues have base pairing properties that differ from the bases. They replace the bases and cause stable mutation.

5-Bromouracil (5-BU), an analogue of thymine, is a very commonly used base analogue that functions like thymine and pairs with adenine (figure 8.9A).

5-BU undergoes tautomeric shift from keto form to enol form caused by bromine atom. The enol form exist for a long t ime for5 -BU than for thymine (figure 8.9B). If 5-BU replaces a thymine, it forms a guanine during replication, which specifies cytosine causing G:C pair (figure 8.9A).

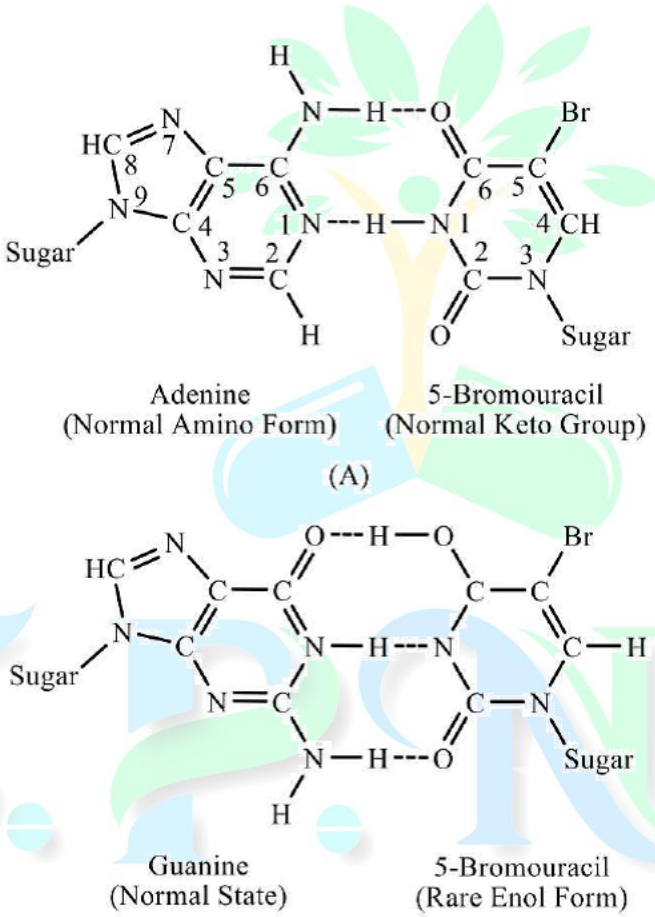


Figure 8.9: Mutagenesis by Base Analogue 5-bromouracil. (A) The Keto Form of 5-BU Pairs with Adenine; (B) 5-BU is Tautomarised to Enol Form and Pairs with Guanine Rather than Adenine

During replication, the keto form of 5-BU substitutes for T and the replication of an initial AT pair becomes an A: BU pair (figure 8.10A). The enol form of 5-BU pairs with G (the first mutagenic step of replication). In the next round of replication, G pairs with C. Thus, transition is completed from AT—GC pair. 5-BU can also convert GC into AT. The enol form acts as a cytosine analogue (and not thymine analogue). Due to error, GC pair converts into a G:BU pair, which becomes an AT pair (figure 8.10B). Due to the presence of

such pairing properties, 5-BU is used in chemotherapy of cancer and viral infections.5 –BU pairs with guanine and disturbs the replication in microorganisms.

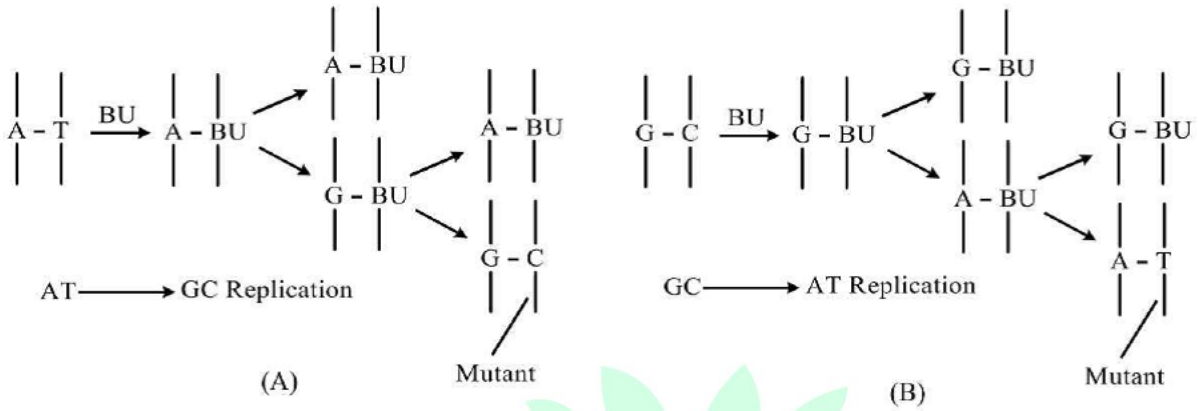


Figure 8.10: Mechanism of 5-Bromouracil (BU)-Induced Mutagenesis. (A) AT → GC Replication; (B) GC → AT Replication

5-Bromodeoxyuridine (5-BDU) can replace thymidine in DNA molecule. The 2- Amino Purine (2. -AP) and 2,6 -Di-Amino Purine (2, 6-DAP) are the purine analogues. 2-AP pairs with thymine but can form a sin gle hydrogen bond with cytosine, thus resulting in AT to GC transition. The effectiveness of 2 -AP and 2,6-DAP is not as much as 5-BU and 5-BDU.

Chemicals Changing the Specificity of Hydrogen Bonding

Many chemicals are incorporated into DNA to change the specificity of hydrogen bonding. The chemicals used as mutagens are nitrous oxide (HNO_2); hydroxylamine (HA), and ethyl-methane-sulphonate (EMS).

1) Nitrous Oxide (HNO_2): It converts the amino group of bases into keto group by oxidative deamination. The frequency order of deamination (removal of amino group) is adenine > cytosine > guanine.

2) Deamination of Adenine: It forms hypoxanthine, whose pairing behavior is similar to that of guanine. Thus, it pairs with cytosine (and not thymine) and replaces AT pairing with GC pairing (figure 8.11A).

3) Deamination of Cytosine: This forms uracil by replacing the —NH_2 group with — OH group. Since the affinity for hydrogen bonding of uracil is similar to that of thymine, C-G pairing is replaced with U-A pairing (figure 8.11B).

4) Deamination of Guanine: This forms non-mutagenic xanthine, which behaves like guanine as there is no change in pairing behaviour. Xanthine pairs with cytosine, therefore, G-C pairing is replaced with X-C pairing.

5) Hydroxylamine (NH_2OH): It hydroxylates the C4 nitrogen of cytosine and converts into a modified base via deamination which causes to base pairs like thyamine. Therefore, GC pairs are converted to AT pairs.

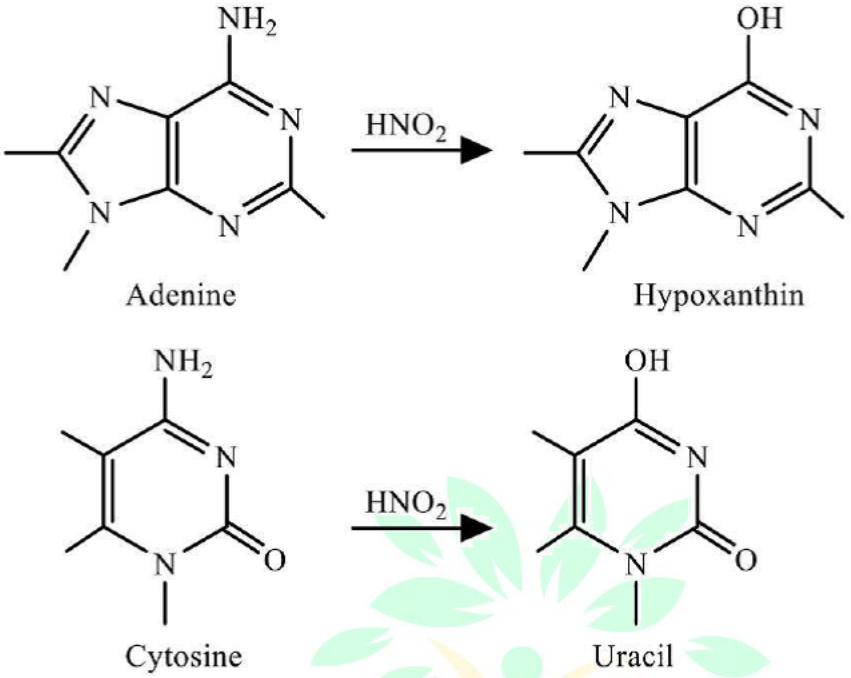


Figure 8.11: Deamination by nitrous oxide of adenine into hypoxanthin (A), and cytosine into uracil (B)

Intercalating Agents

Some dyes, e.g., acridine orange, proflavine , and acriflavine, are three ringed molecules having dimensions similarto those of purine pyrimidine pairs (figure 8.12). These dyes in aqueous solution can insert themselves in between the DNA bases (i.e., intercalate the DNA) in adjacent pairs; and this process is termed intercalation, and the dyes are called intercalating agents.

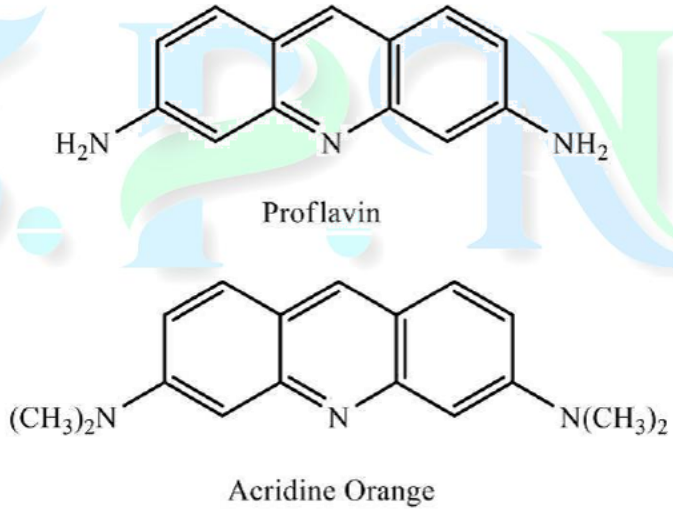


Figure 8.12: Chemical Structure of Two Mutagenic Acridine Derivatives

Acridines are planer (flat) molecules that can be intercalated between the DNA base pairs. They distort the DNA and causes deletion or insertion after replication of DNA molecule. Due to deletion or insertion of intercalating agents, frameshift mutations occur (figure 8.13).

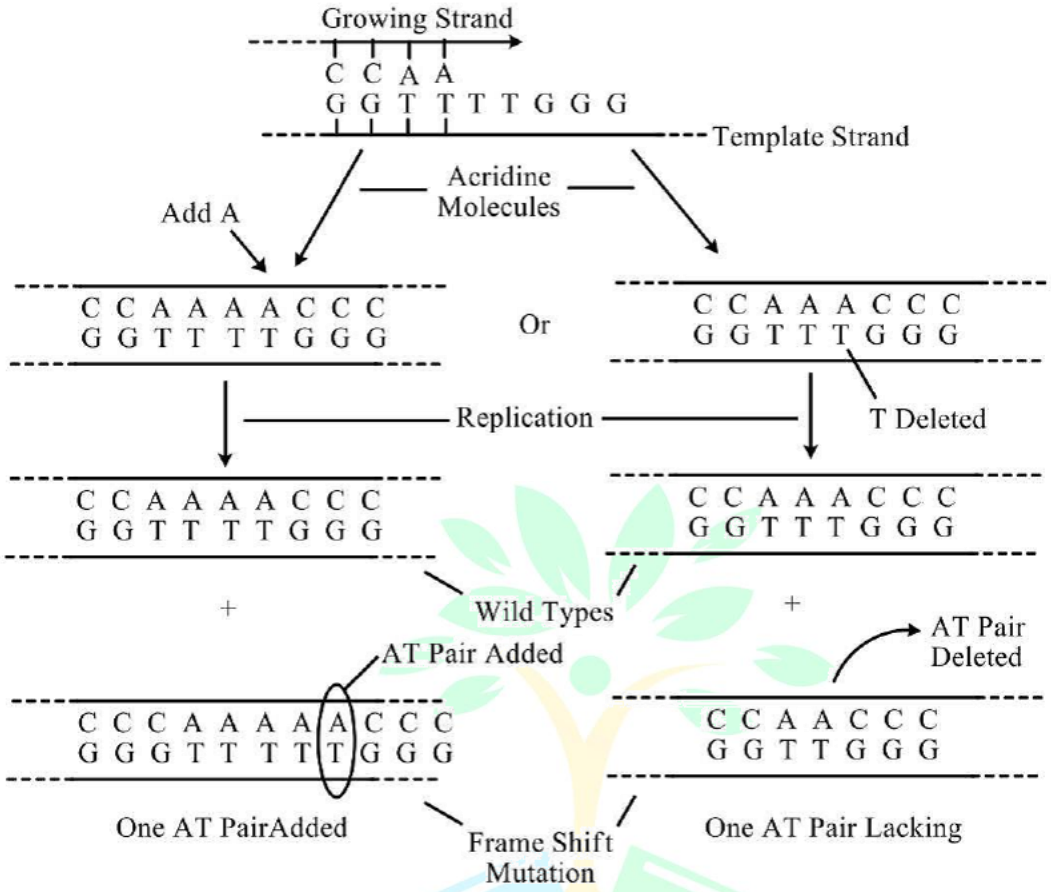


Figure 8.13: Mechanism of Intercalation of an Acridine Molecule in the Replication Fork

FERMENTATION

Introduction

The process of fermentation involves biochemical activity of organisms during their growth, development, reproduction, senescence, and death. Fermentation technology employs organisms to produce food, pharmaceuticals, and alcoholic beverages in industries on a large scale.

The principle involved in industrial fermentation technology is that organisms are grown under optimum conditions and are provided with raw materials and other necessary requirements like carbon, nitrogen, salts, trace elements, and vitamins. The end products formed due to their metabolism during their life span are released into the media. These end products are extracted by human beings as they are commercially valuable.

Some major end products of fermentation produced on a large scale industrial basis are wine, beer, cider, vinegar, ethanol, cheese, hormones, antibiotics, complete proteins, enzymes, and other beneficial products.

Fermentation Methods

The three different processes of fermentation are:

1) Batch fermentation,

2) Fed-batch fermentation, and

3) Continuous fermentation.

1. Batch Fermentation

In batch fermentation process, the microorganisms are inoculated in a fixed volume of batch culture medium. The organisms during their growth consume the nutrients, and the growth products (i.e., biomass and metabolites) start accumulating. Since the nutrient environment within the bioreactor is continuously changed, the rate of cell metabolism also changes, and ultimately, cell multiplication stops due to limitation of nutrients and accumulation of toxic excreted waste products.

The complex nature of batch growth of microorganisms is shown in figure 9.1. In the initial lag phase, no apparent growth is observed; however, biochemical analyses show metabolic turnover signifying that the cells are acclimatizing to the environmental conditions and will start growing. Then comes the transient acceleration phase when the inoculum begins to grow. This phase is quickly followed by the exponential phase where the organisms are growing at the fastest rate as the nutrients are in excess, environmental conditions are optimum, and growth inhibitors are absent.

In batch fermentation process, the exponential growth occurs for a limited period. With the change in nutrient conditions, the growth rate decreases and begins the deceleration phase followed by the stationary phase at which the growth stops completely because of nutrient exhaustion. The death phase when the growth rate has come to an end is the final phase of the cycle. Mostly the biotechnological batch processes are stopped before this stage because of decreasing metabolism and cell lysis.

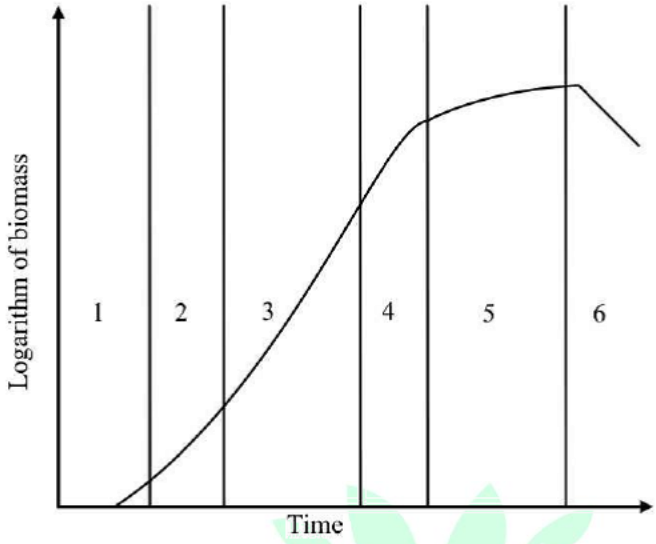


Figure 9.1: Growth Characteristics in a Batch Culture of a Microorganism. 1) Lag Phase, 2) Transient Acceleration, 3) Exponential Phase, 4) Deceleration Phase, 5) Stationary Phase, 6) Death Phase

Advantages

1) Requires less space,

2) Can be easily handled, and

3) Less chances of contamination.

Disadvantages

1) Time consuming,

2) Requires more time for cleaning, sterilisation, and cooling, and

3) Product yield is low.

2. Fed-Batch Fermentation

In fed -batch fermentation process, freshly prepared culture media is added periodically without removing the culture fluid, thus increasing the volume of fermentation culture. This fermentation type is used for producing proteins from recombinant microorganisms.

3. Continuous Fermentation

In continuous fermentation process, a near -balanced growth is obtained with a little fluctuation in nutrients, metabolites, cell numbers, or biomass. In this process, fresh medium is added to the batch process at the exponential phase of growth and simultaneously withdrawing the medium along with cells. Continuous methods of cultivation allow the organisms to grow under steady state (unchanging) conditions, in which growth occurs at a constant rate in a constant environment. In a completely mixed continuous culture system, sterile medium is passed into the bioreactor (figure 9.2) at a steady flow rate, and culture broth (containing medium, waste products, and organisms) emerges from it at the same rate, thus the volume of the total culture in the bioreactor remains constant.

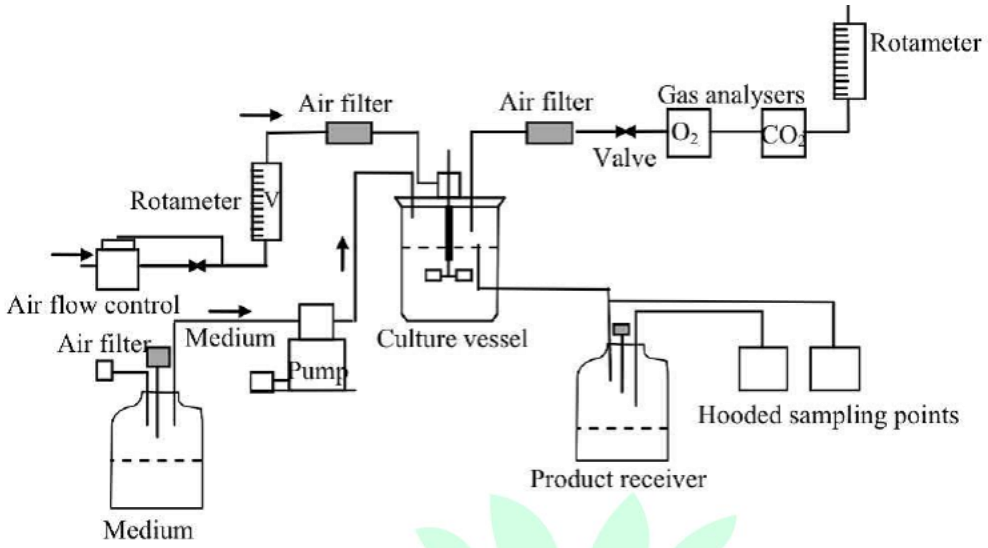


Figure 9.2: A Simple Laboratory Fermenter Operating on a Continuous Basis

The three types of continuous fermentation processes are:

1) Single Stage Continuous Fermentation: In this process, the fermented is continuously used. Media is added at a specific rate and the product formed is removed at the same rate. Thus, a balance is maintained between the input of media and output of fermented media.

2) Single Stage Recycle Continuous Fermentation: In this process, a single fermenter is continuously used. The inoculation rate of fermentation media and the withdrawal rate of fermented broth are same. In this process, a portion of fermented broth plus withdrawal culture is recycled to the fermenter.

3) Multi-Stage Continuous Fermentation: In this process, two or more fermenters are continuously operated in a sequence. The first fermenter is used for growth of cells because in some fermentation processes the product is formed after cell multiplication. The effluent of first fermenter serves as an in-fluent for the next fermenter.

Advantages

1) Product forms continuously,

2) Product yield is good,

3) Inoculation of culture is done only once, and

4) Saves time and labor.

Disadvantages

1) Complex and difficult to operate,

2) More chances of contamination, and

3) Operators with brief knowledge on fermentation and microbial behavior and growth are required.

General Requirements

Water forms the leading component of the media in which the microorganisms grow. On achieving optimum production in liquid fermentation processes, removal of water is a major factor in the cost of bioproduct recovery and downstream processing. The water quality is highly significant as it affects microbial growth and production of some bioproducts. In media production, there is quality control of raw materials. In respect of volume, water is an important raw material in many biotechnological processes, and its supply and use should be carefully monitored and controlled.

A source of energy or carbon, a nitrogen source, inorganic elements, and specific growth factor s (for some cell types) are the basic nutritional requirements of microorganisms. Carbon and nitrogen sources are mainly derived from complex mixtures of cheap natural products or by-products in most of the biotechnological processes (table 9.1):

Table 9.1: Carbohydrate and Nitrogen Sources for Industrial Media

Carbohydrate Sources	Form	Nitrogen Sources (% Nitrogen by Weight)
Glucose	Pure glucose monohydrate and hydrolysed starch	Barley (1.5-2.0) Beet molasses (1.5-2.0)
Lactose	Pure lactose and whey powder	Corn-steep liquor (4.5)
Starch	Barley, groundnut meal, oat flour, rye flour, and soybean meal	Groundnut meal (8.0) Oat flour (1.5-2.0) Pharmamedia (8.0)
Sucrose	Beet molasses, cane molasses, crude brown sugar, and pure white sugar	Rye flour (15-2.0) Soybean meal (8.0) Whey powder (4.0)

The nutrients availability and their type exert strong physiological control over fermentation reactors and product formation. Input of raw materials to fermentation depends on the material cost at the particular time because their market price alters with seasonal and other variables. Sterilisation practices for biotechnological media should destroy maximum contaminating microorganisms at minimum temperature and causing minimal damage to the medium components. Bat ch-wise sterilisation in the bioreactor is being widely used, although continuous methods are also coming in use.

Preparation of the media in an appropriate manner is the basis of the entire fermentation process. Poor media design leads to low efficiency of growth and poor product formation.

4. Study of Media

Media is a mixture of various nutrients prepared artificially to support the growth and multiplication of microorganisms. A large variety of media are available in the laboratory. The media mostly are available commercially, have pre –mixed components, and require addition of water followed by sterilization. Media are continuously being developed and/or revised to be used for isolating and identifying desired bacteria in various fields (like food, water, and clinical microbiology). Culture media are required for growing organisms from infected material so that the causative agent can be identified.

A culture media has the following constituents:

1) Water: It acts as a source of hydrogen and oxygen.

2) Electrolyte: Sodium chloride or other electrolytes.

3) Peptone: It is a complex mixture of partially digested proteins, and contains proteases, amino acids, polypeptides, phosphates, minerals (potasstum and magnesium), and accessory growth factors like nicotinic acid and riboflavin.

4) Meat Extract: It contains protein degradation pro ducts, inorganic salts, carbohydrates, and growth factors.

5) Blood or Serum: 5-10% defibrinated sheep blood is most commonly used for enriching the culture media. In some media, serum is also used.

6) Agar: It is obtained from sea weed (Algae gelidium species), and contains a long-chain polysaccharide, a small amount of protein-like material, and various inorganic salts.

Types of Media

The media used in fermentation are classified as follows:

1) Based on Physical State

i) Liquid media,

ii) Semisolid media, and

iii) Solid media.

2) Based on the Presence of Molecular Oxygen and Reducing Substances in the Media

i) Aerobic media and

ii) Anaerobic media.

3) Based on Nutritional Factors

i) Simple media,

ii) Complex media,

iii) Synthetic media, and

iv) Special media:

a) Enriched media, b) Enrichment media, c) Selective media, d) Differential media,

e) Indicator media, f) Transport media, and g) Sugar media.

Culture Media for Lactobacilli and E. coli

Autotrophs can be cultivate d using chemically defined media, which are also used for defining the nutritional requirements of heterotrophs. However, chemically defined media is not employed and instead some complex raw materials (e.g., peptones, meat extract, and yeast extract) are used for the routine cultivation of heterotrophs. The resulting media support the growth of a wide variety of heterotrophic bacteria. To obtain a solid medium, agar is added which serve as a non-nutritive solidifying agent. Table 9.2 contains a description of these raw materials:

Table 9.2: Composition of Media Supporting Growth of Lactobacilli and *E. coli* (Heterotrophic Bacteria)

1) **Medium for Cultivation of Lactobacilli**	
i) Casein hydrolysate	5gm
ii) Glucose	10gm
iii) Solution A	10ml
iv) Solution B	5ml
v) L-Asparagine	250ml
vi) L-Tryptophan	50mg
vii) L-Cystine	100mg
viii) DL-Methionine	100mg
ix) Cysteine	100mg
x) Ammonium citrate	2gm
xi) Sodium acetate (anhydrous)	6gm
xii) Adenine, guanine, xanthine, and uracil	10mg each
xiii) Riboflavin, thiamine, partothenate, and niacin, each	500µg
xiv) Pyridoxamine	200µg
xv) Pyridoxal	100µg
xvi) Pyridoxine	200 µg
xvii) Inositol and choline, each	10µg
xviii) *p*-Aminobenzoic acid	200µg
xix) Biotin	5µg
xx) Folic acid (synthetic)	3µg
xxi) Make up to 1lt with distilled water.	
Solution A: K_2HPO_4 and KH_2PO_4; each 25 gm; dissolved in distilled water to a volume of 250ml **Solution B:** $FeSO_4.7H_2O$, 0.5 gm; $MnSO_4.2H_2O$, 2.0 gm; NaCl, 0.5 gm; and $MgSO_4.7H_2O$, 10gm; Dissolved in distilled water to a volume of 250ml	
2) **Medium for Cultivation of *E. coli***	
i) $NH_4H_2PO_4$	1gm
ii) Glucose	5gm
iii) NaCl	5gm
iv) $MgSO_4.7H_2O$	0.2gm
v) K_2HPO_4	1gm
vi) H_2O	1,000ml

3. Culture Media for Viruses

For the growth of viruses, the following three basic types of cell culture are in use:

1) Primary Culture: This culture is prepared by dispersing cells, obtained from freshly removed host tissues , with trypsin. Generally, they cannot grow for more than a few passages in culture.

2) Secondary Culture: This culture includes diploid cell lines that have undergone a change allowing their limited culture (up to 50 passages) but retaining their normal chromosome pattern.

3) Continuous Cell Culture: This culture is capable of more prolonged indefinite growth that has been derived from diploid cell lines or from malignant tissues. They invariably have alteredan irregular number of chromosomes. The type of cell culture u sed for viral cultivation depends on the sensitivity of cells to a particular virus.

5. Study of Equipments

The apparatus that maintains optimal conditions for the growth of microorganisms is a fermenter. It is used in large-scale fermentation and in the commercial production of antibiotics and hormones. It is a bio-reactive equipment utilised for microbe cultivation. In fermenters, various microbes will grow, undergo metabolism, and generate fermentative products under optimum environmental conditions.

Fermenters are being widely used in industries where enzymes, antibiotics, airline monosodium glutamate, citric acid, solvent, pharmaceuticals, food, etc. are prepared.

A fermenter is made up of the following parts:

1) Agitator (Impeller): Its size and position in the vessel depends on the fermenter size. An ideal impeller should be $1/3^{rd}$ or 1/2 of the vessel diameter (D) above the base of the vessel. More than one impeller is required in tall vessels in order to obtain suff icient aeration agitation. However, the number of impeller may vary with the vessel size.

2) Stirrer Gland and Bearing: Four basic types of seals assembly are used:

i) The packed gland seal,

ii) The simple bush seal,

iii) The mechanical seal, and

iv) The magnetic drive.

3) Baffles: These are metal strips, $1/10^{th}$ of the vessel diameter, and attached radially to the walls in agitated vessel (of all sizes) to prevent a vortex and improve aeration efficiency.

4) Sparger: It is a device equipped to introduce air into the liquid in a fermenter. It should be determined before the operation that whether sparger is to be used manually or with mechanical agitation as it is capable of influencing the equipment design to determine initial bubble size. The three basic types of sparger that have been used are:

i) The porous sparger,

ii) The orifice sparger, and

iii) The nozzle sparger.

5) Sampling Point: It is a valve equipped for withdrawing samples for in process laboratory tests.

6) Drain Point: It is a drain present at the bottom to remove the completed fermentation broth for further processing.

Sterilization Methods

Sterilization is an important operation which differentiates biochemical and chemical processes. The process aims to provide a contamination –free environment. Fermentation is a biochemical process of producing metabolic products by the action of a microorganism or a group of microorganisms on a substrate, in the presence of nutrients in the medium. Fermentation proceeds only with the involvement of:

1) A microorganism,

2) A medium,

3) A fermenter,

4) Nutrients/other additives, and

5) Air in the case of aerobic processes.

Thus, a sterile environment is required; or else, the contaminants would dominate forming a contaminated product (having toxicants) or no product at all. A contaminant may affect the fermentation process in the following ways:

1) It may contaminate the final product.

2) The medium would be consumed to support the growth of contaminating organisms.

3) The contaminated product may be greater than the desired product.

4) The contaminated product may interfere with the recovery of the desired product.

5) Unsterile air in aerobic fermentations may spoil the fermentation product.

The above mentioned problems can be overcome only if a contamination -free environment is maintained by sterilization.

1. Sterilisation of Fermenter

A fermenter is sterilised separately if the medium is sterilised outside and introduced into the fermenter. Joints, crevices, pits or flange joints in a fermenter are the potential hazardous sites where the nutrients and microorganisms accumulate, thus causing contamination. A fermenter is sterilised by heating as steam at 15

psig is introduced in it for 20 minutes. All entry and exit points of air, medium, substrate, etc., are sterilised . All the condensate exit points should have slopes towards the discharging end so that formation and accumulation of steam condensate in the pipelines is avoided. The condensers, thermowells, pressure transducers, analytical probe points, etc., have pits and crevices where contaminant microorganisms accumulate, thus should be sterilised.

After sterilisation, the fermenter should be flushed with sterile air under positive pressure, providedthat on cooling it does not develop vacuum and suck in the non sterile atmospheric air. The medium sterilised in a different vessel should be introduced into the sterilised fermenter asepticallfhe transport lines, etc., should be maintained under aseptic conditions, and in case of failure contamination results.

2. Sterilisation of Media

The medium used in industrial fermentation processes should be sterilised prior to use, so as to avoid contamination by microorganisms which may otherwise:

1) Consume the nutrients in the medium,

2) Alter the chemical structure of the nutrients,

3) Alter the pH,

4) Create more foam in the fermentation which ultimately affects aeration,

5) Produce metabolic products which affect the growth of fermentation microorganisms,

6) Alter the oxidation-reduction potential of the medium, and

7) Convert, degrade or destroy the desired fermentation product.

Sterilisation of medium can be done by:

1) Boiling in water,

2) Passing steam, or

3) Autoclaving.

The media used in fermentation are sterilised thermally for which various techniques have been developed. Thermal sterilisation s hould be a batch or continuous process or a HTST (High Temperature Short Time) process. In cases when the sterilisation cost is high and it destabilises the process economics, industrial fermentations are carried out at low pH, and by using c ontamuination inhibitors (e.g., lactic acid).

Synthetic media in comparison to crude media require less sterilisation efforts. The former requires a small amount of heating for sterilisation, while the latter contains some heat-resistant bacterial spores, thus requiring prolonged heating.

3. Sterilisation of Air

For industrial aerobic fermentation processes, clean and sterile air is required in large quantity. Table 9.3 lists the representative species of air-borne bacteria:

Table 9.3: Representative Species of Air-Borne Bacteria

Species	Width (Microns)	Length (Microns)
Enterobacter aerogenes	1.0-1.5	1.0-2.5
Bacillus cereus	1.3-2.0	8.1-25.8
Bacillus licheniformis	0.5-0.7	1.8-3.3
Bacillus megaterium	0.9-2.1	2.0-10.0
Bacillus mycoides	0.6-1.6	1.6-13.6
Bacillus subtilis	0.5-1.1	1.6-4.8
Micrococcus aureus	0.5-1.0	0.5-1.0
Proteus vulgaris (spores)	0.5-1.0	1.0- 3.0

A cubic metre of air contains around $4x10^3$ to $20x10^3$ particles with a maximum possibility of $12x10^3$. However, air contains 10^3 to 10^4 particles/m^3 on an average, and the average size of the dust particle is approximately 0.6 microns.

Sterilisation of air can be done by:

1) Heating,

2) Using UV rays and other electromagnetic waves,

3) Using germicidal sprays, or

4) Filtration.

7. Large-Scale Production Fermenter Design

Industrial fermenters are either of anaerobic or aerobic classes. Anaerobic fermenters do not require much special equipment, except for the removal of heat generated during the fermentation process; on the contrary, aerobic fermenters require much elaborate equipment for adequate mixing and aeration. The industrial fermentation processes are mostly aerobic, thus a typical aerobic fermenter is shown in figure 9.4 and its parts have been discussed below.

Cooling Jacket

Large-scale industrial fermenters are made up of stainless steel. A fermenter is a large cylinder closed at the top and fitted with various pipes and valves at the bottom. The fermenter is fitted with a cooling jacket on the external surface, and through this jacket steam (for sterilisation) or cooling water (for cooling) is passed.

Cooling jacket is an essential part because sterilisation of the nutrient medium and removal of the heat generated are mandatory for completing the fermentation process successfully. In large fermenters, heat transfer occurs insufficiently through the jacket, and thus, internal coils are provided to pass through either steam or cooling water.

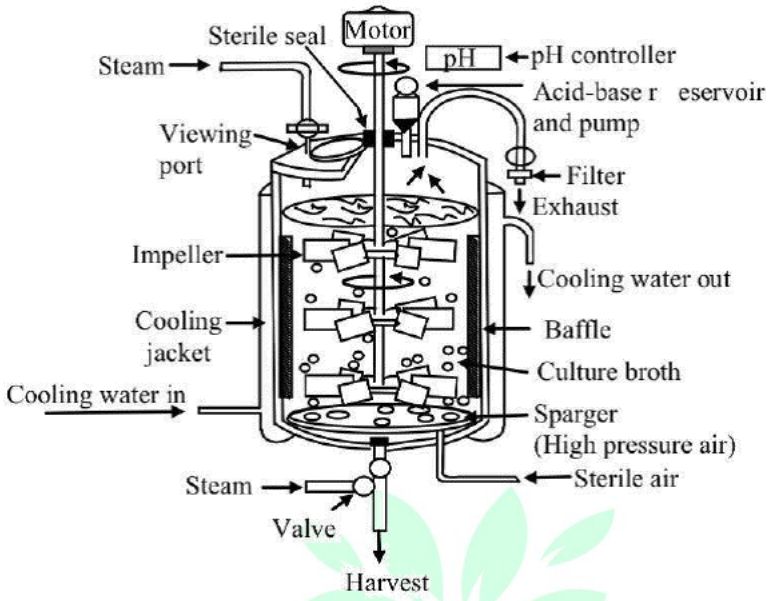


Figure 9.4: Internal View of an Industrial Aerobic Fermenter

Aeration System and Stirring

Aeration system forms an essential part of a fermenter. The oxygen demand by the culture, in a fermenter with a high microbial population density, is tremendous; but since oxygen is poorly water -soluble, it barely transfers rapidly through the growth medium. Therefore, precautions should be taken and two separate aeration devices (i.e., sparger and impeller) should be used to ensure proper aeration and oxygen availability in the fermenter throughout the culture.

1. Sparger is a series of holes in a metal ring or a nozzle through which filter-sterilised or oxygen -enriched air enters the fermenter under high pressure as a series of tiny bubbles from which the oxygen passes by diffusion into the liquid culture medium.

2. Impeller (or agitator) is an agitating device required for stirring of the fermenter. Stirring achieves mixing of the gas bubbles and of the microbial cells through the liquid culture medium. Thus, stirring ensures uniform access of microbial cells to the nutrients.

The impeller's size and position in the fermenter depends on the fermenter's size. Ideally, the impeller should be $1/3^{rd}$ of the fermenter's diameter and should be positioned above the fermenter's base. The number of impeller to be fitted also depends on the size of fermenter. More than one impeller is equi pped in tall fermenters in order to obtain satisfactory aeration and agitation.

3. Baffles

Baffles are equipped in the fermenters of all sizes. They prevent vortex and improve the efficiency of aeration. They are metal strips of $1/10^{th}$ of fermenter's diameter and are attached radially to the walls.

4. Controlling Devices for Environmental Factors

In microbial fermentation, growth and product formation should be measured and also the environmental parameters (such as temperature, oxygen concentration, pH, cells mass, levels of key nutrients, and product concentration) should be controlled throughout the process using various devices in a fermenter.

5. Use of Computer in Fermenter

Computer technology has put a remarkable influence in fermentation process and computers are used to model fermentation processes in industrial fermenters. Integration of computers into fermentation systems is based on their capacity for process monitoring, data acquisition, data storage, and error -detection. Some online data analysis functions include the acquisition measurements, data verification, filtering, unit conversions, calculation of indirect measurements, differential integration calculations of estimated variables, data reduction, tabulation of results, graphical presentation d results, process stimulation, and data storage.

Various Controls of a Fermenter

Process monitoring and control are the issues to be faced during fermenter development. With the recent advents in bio analytical techniques, a vast array of probes has been developed and being utilised. The controls applicable in the fermentation process are enlisted in table 9.4, and the process monitoring parameters are given in table 9.5:

Table 9.4 : Typical Fermenter Control Ranges

Control Parameters	Range
Temperature	8°C above coolant to 60°C ± 1°C
Agitator Speed	0-1000 rpm
Stability	>98%
pH range	2-12 ± 0.1
Pressure	2000mbar
DO_2 range	0-100%
Air flow	0-6 litres/minute

Table 9.5: Fermentation Process Control Monitoring Parameters and Methodology

Process Controls	Monitoring Devices
Air Flow	Flow meter
Coolant Water Flow	Flow meter
Power Input	V.O.H.; torque
Temperature	Resistance thermocouples thermistors diodes.
Rheology	Tube viscometer, cone and plate viscometer, concentric cylinder viscometer, infinite sea viscometer foam control.
Redox Potential	pH dissolved oxygen (DO_2), polarographic probes, galvanic probes, permeable tube/oxygen analyser, dissolved carbon dioxide (DCO_2).
State of Culture	Enzyme probes metabolic heat substrate analysis.
Cell Concentration	Gravimetric dry weight turbidity.
Immunocytometry	Cell number, impedance, carbon dioxide production, oxygen production, DNA content, cell particle size distribution.
Gas Analysis	Oxygen analysers, carbon dioxide analysers, mass spectrometry, gas chromatography.

Factors Affecting Fermenter Design

In designing fermentation processes, the major component is media (i.e., water) in which the microorganisms grow. A wide variety of fermenters are commercially available, and thus while selecting a definite type of a fermenter, the following factors should be considered:

1) The type of reaction to be carried out, and

2) The suitable operating conditions for that reaction.

Some other factors involved in the designing of a fermenter are:

1) Selection of the Microorganism: This is the most important step in which an appropriate strain of a particular microorganism is to be selected. This determines the growth phase in which the product is formed, the temperature and pH ranges, the aeration degree required, and the contamination effects.

2) Selection of Fermenter Configuration: After the micro organism, an appropriate fermenter configuration is to be selected. Thus, the merits and demerits of different fermenter configuration such as batch stirred –tank fermenter, continuous stirred -tank fermenter, tubular fermenter, etc. should be understood well.

3) Determination of the Fermenter Dimensions: Size of the fermentation batch manages the volume and diameter of the fermenter. Similarly, the type of fermentation determines the values of the operating variables such as concentration, temperature and pH, process time (for batch fermentation), and flow rate (for continuous fermentation).

4) Heat Transfer and Safety Factors: The extent of heat transfer surface and the requirements of mixing devices, power and aeration, facilities for monitoring and control, and the safety factors should be determined.

5) Materials of Construction: The materials of construction and mechanical design, and the devices required for maintaining aseptic conditions should be selected.

6) Media Design of Various Fermentation Processes: There are several essential factors that govern and also play an important role in the media design of various fermentation processes, such as:

i) Quality of Water: The predominant water quality is of great importance as it affects the resultant microbial growth and also the production of specific bioproducts.

ii) Quality Control of Raw Materials: Apart from water, other chemical constituents, e.g., pasteurised wort (malt extract solution), salts, acids, etc., should also be of better grade and quality to obtain faultless optimised fermentation and yield desired bioproducts.

iii) Nutritional Requirements: The vital nutritional requirements of microorganisms are an energy or carbon source, an available nitrogen source, inorganic elements, and specific growth factors (only for some particular cell-types).

iv) Sterilisation Practices: The various conventional, time -tested and widely adopted sterilisation practices meant for the biotechnological media should kill maximum contaminating microorganisms to cause

minimum damage to the medium components. Among th e two frequently employed steri lisation practices, the batch wise sterilization in the bioreactor is considered to be most widely used, while the continuous sterilisation methods are also gaining acceptability and adaptability.

Production of Penicillins

Penicillin is the first antibiotic which was clinically used in 1941. Firstly it was obtained from the fungus Penicillium notatum; but presently it is obtained from a high yielding mutant of P. chrysogenum. The nuc leus of penicillin consists of fused rings of thiazolidine and beta-lactam, and these rings have side chains attached through an amide linkage (figure 9.5).

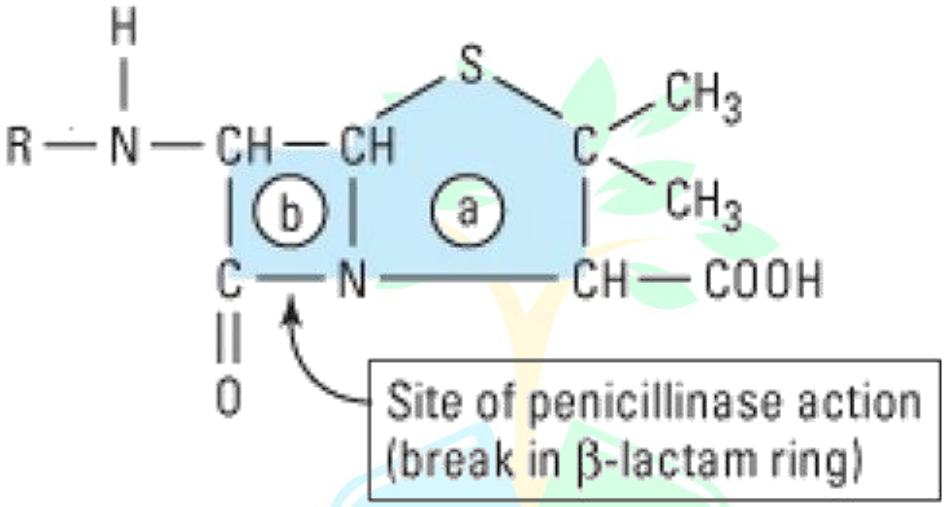


Fig 9.5: Chemical Structure of Penicillin. a. Thiazolidine Ring, b. Beta lactam ring.

Penicillin fermentation is an aerobic process with a volumetric oxygen absorption rate of 0.4-0.8mm/min. The aeration rate required varies with the stain, the type of fermenter used, and the impelle r system. However, it ranges between 0.5 to 1.0 vvm. It is produced by fed batch submerged fermentation in a stirred tank fermenter.

This process of penicillin production by fermentation has been described under the following headings:

1) Strain development,

2) Inoculum production,

3) Inoculation,

4) Medium, and

5) Extraction and purification.

1. Strain Development

The variety of moulds yielding a greater amount of penicillin is called the high yielding strain, developed from the wild P. chrysogenum by sequential genetic selection process. In this process, an improved mutant is developed step by step by treating the wild strain of P. chrysogenum with a series of mutagenic agents or

exposing to UV radiation either individually or in combination with X -rays and chemical mutagens. This process can also be named as strain improvement.

Strain development is a strenuous and lengthy process. The selected mutant in comparison to the wild type has a greater capacity for antibiotic production.

Table 9.6: Significant Stages in Strain Improvement Programme in *P. Chrysogenum*

Strains	Methods	Penicillin ($mgml^{-1}$)	Characteristics
P. notatum (Fleming)	*P. chrysogenum*	3	
NRRL 1951	Original isolate	60	Yellow pigment
NRRL 1951.B25	Selection	125	Yellow pigment
X-1612	X rays	300	Yellow pigment
WIS Q 176	UV-light	800-1000	Yellow pigment
B 13-D 10	UV-light	–	Pigment-free
WIS 47-638	Selection	–	Pigment-free
WIS 47-1564	Selection	800-1000	Pigment-free, 95-100% G*
WIS 48-701	Selection	–	Pigment-free, 95-100% G*
WIS 49-133	Nitrogen mustard gas	1500-3000	Pigment-free, 95-100% G*
WIS 49-2166	Nitrogen mustard gas	1500-3000	Pigment-free, 95-100% G*
WIS 50-535	Nitrogen mustard gas	1500-00	Pigment-free, 95-100% G*
WIS 51-20	Selection	2000-3000	Pigment-free, 95-100% G*
EIS.1	NM and Selection	7000	Pigment-free, Penicillin G

Different biosynthetic penicillins with enhanced features can be formed by adding side chain precursors to the fermentation medium or by chemically modifying the natural penicil lins. Penicillins now are mainly produced semi- synthetically by chemical modification of the natural penicillin yielded from the fermentation of P. chrysogenum strains.

Natural penicillins are modified by removing their natural acyl group and leaving behind the 6 -APA (6-Amuinopenicillanic Acid) to which other acyl groups are added to confer new properties. This is done by passing the natural penicillin (with removed natural acyl group) through a column of immobilised penicillin acylase (obtained from E. coli) at neutral pH.

For example, penicillin G is converted to 6 -APA and phenylacetic acid; the 6 -APA is then acylated by adding an appropriate side chain to obtain a semi - synthetic penicillin.

2. Inoculum Production

Inoculum is the microorganism used in fermentation. Generally, a high yielding strain of P. chrysogenum is used. A strain of the fungus is sub -cultured from stock culture for inoculums development. Spores from primary source are suspended in either water or a dilute solution of a non-toxic wetting agent (e.g., 1:10000

sodium lauryl sulphate). Then the spores are added to flasks or bottles containing wheat bran and nutrient solution. These flasks or bottles are incubated at 24°C for 5 -7 days to support heavy sporulation. This complete process is repeated a number of times to have more sporulation. The spores obtained are used directly to inoculate the inoculum tanks or stirred fermenters. The incubation temperature is maintained at 24 -27°C for 2 days with continuous agitation and aeration to support heavy mycelial growth, which may be added to a 2^{nd} or 3^{rd} stage of fermentation. The resulting inoculum employed in a production tank is tested by microscopic examination as well as by sub-culturing method.

Many sporulation media have been designed to obt ain a large number of spores.Moyer and Coghill developed a sporulation media in 1946, which is most widely used (table 9.7).

Table 9.7: Composition of Moyer and Coghill Sporulation Medium

Components	Concentration (gm/lt)
Glycerol	7.5
Cane molasses	7.5
Corn steep liquor	2.5
$MgSo_4.7H_2O$	2.5
KH_2PO_4	0.050
Peptone	0.060
NaCl	5.00
Fe-tartrate	0.005
$CuSO_4.5H_2O$	0.004
Agar	2.50
Distilled water	1.0

3. Inoculation

Inoculation is the process of introducing pure inoculum into the fermenters or production tanks by any of the following three methods:

1) Dry Spores may be used as Inoculum: The spores of P. chrysogenum are hydrophobic, thus either the spores are blown deep into the medium or a wetting agent (e.g., sodium lauryl sulphate) is used.

2) Suspension of Non-Germinated Spores: This suspension (prepared with 1:10000 sodium lauryl sulphate solution) is fed into the fermenter by using spray guns or pipettes. Then the fermentation medium is agitated and aerated so that the spores get uniformly distributed in the entire medium.

3) The medium in the fermentation tanks are fed with pre -germinated spores or mycelial pellets (prepared by the germination of spores) after2 -3 days of spore inoculation.

Fermenters with a capacity of 40,000 to 2 lacs litres are used for penicillin production. Tanks with larger capacities are not used due to difficulties with the oxygen supply. Some manufacturers use Waldh of fermenters or air lift fermenters, but this is only possible in mutants generating low viscosity.

Depending on the producti on strain, the operational temperature is maintained between 25-27°C. A flow chart for penicillin production is shown in figure 9.6:

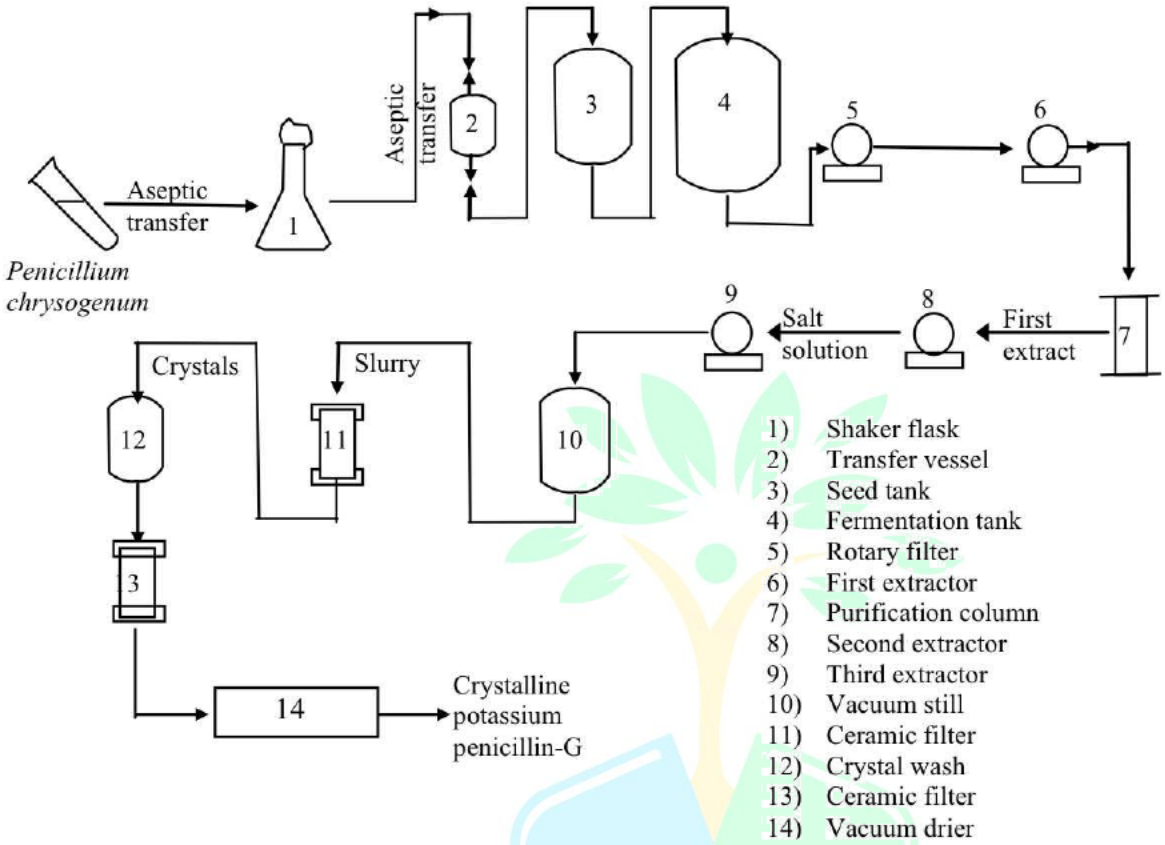


Figure 9.6: Flow Sheet for Large-Scale Production of Penicillin

4. Medium

The medium used in penicillin production should achieve:

1) An abundant growth of the mycelium,

2) Maximum accumulation of the antibiotic, and

3) Easy and economical extraction and purification of the antibiotic.

Mainly lactose is used as a source of carbon; however, glucose, sucrose, glycerol, and sorbitol can also be used. Sulphate, acetate or nitrate s alts of ammonium are used as sources of nitrogen. Corn-steep liquor is enriched with important amino acids desirable for mycelial growth, thus a medium containing corn steep liquor supports heavy formation of mycelium and spores.

Salts of potassium, phosp horus, magnesium, sulphur, zinc, and copper are used. Potassium and phosphorus are used in the form of potassium dihydrogen phosphate; magnesium, iron and copper are used in the form of sulphates. All these elements are present in corn steep liquor. Penicillin-F and penicillin -K are the naturally produced penicillins synthesized by P. notatum and P. chrysogenum, respectively, without using a precursor. In case phenylacetic acid is added in the medium, P. chrysogenum produces penicillin-G and not penicillin -K. In the same way, desired synthetic penicillins can be obtained by adding a suitable precursor in the medium.

Jackson designed a medium in 1958 in the following composition, and used it in fermentative production of penicillin (table 9.8).

Table 9.8: Composition of Jackson's Medium

Components	Concentration (%)
Corn steep liquor	3.5
Lactose	3.5
Glucose	1.0
Calcium carbonate	1.0
Potassium dihydrogen phosphate	0.4
Edible oil	0.25

Penicillin yields with time are linear from approximately 48 to 96 hours. The final penicillin yield ranging between 3 -5% depends on the amount of carbohydrate consumed during the fermentation process (1500 International Units per millilitre). Sylvester and Coghill in 1954 estimated that for producing 1000 gallons of ferm ented culture which can yield 2.2 -2.7kg of penicillin via submerged culture method, around 227kg of nutrients, 3400kg of steam, 45460It of water, 1000 kWh of electricity, and 7075m^3 of air is required.

Penicillin undergoes carboxylation under the influence of penicillinase enzyme to form the biologically inactive penicill anic acid. The penicillinase enzyme is widely distributed in different microorganisms, which may enter the fermenter at any stage and convert penicillin into penicillanic acid (figure 9.7).

In penicillin fermentation, contamination forms the major limitation, and thus should be prevented. The contamination risk was one of the main problems in the ancient times during penicillin production. This was because fermentation was carried out in bottles and contamination in one bottle may destroy penicillin in all the batches of bottles.

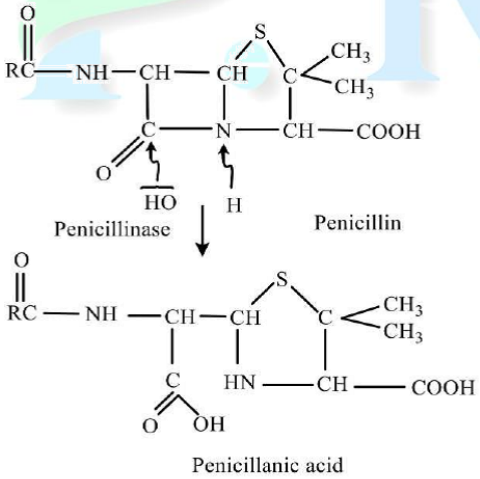


Figure 9.7: Carboxylation of Penicillin by the Action of Penicillinase

In a typical penicillin fermentation process, there is a growth of 10 hours duration with a doubling time of 6 hours and during this time the gr eater part of the cell mass is formed. Oxygen supply in the growing culture

is critical, as oxygen transfer is hindered by the increasing viscosity. After the growth phase, the culture proceeds to actual penicillin production. The growth is sharply reduced by feeding with various culture medium components.

The production phase can be extended to 120-180 hours. Penicillin production by continuous fermentation is difficult due to instability of the production strains. A batch fill and draw system is an alternative, in which 20-40% of the fermentation contents is replaced with fresh nutrient solution. This process is repeated around ten times without affecting the yield.

5. Extraction and Purification

When sufficient amount of penicillin has been produced during the fermentation, it is extracted and purified. This process is carried out in three steps:

1) Separation of Mycelium: The mycelium is separated from the medium using a rotatory vacuum filter. This process should be performed carefully to avoid contamination by the microorganisms which produce the penicillin -degrading enzyme, i.e., penicillinase.

2) Extraction of Penicillin: The penicillin is extracted into the medium with counter current extraction method and less than 1% remains as mycelium bound. After separating the mycelium, the liquid pH is adjusted to 2.0 to 2.5 by adding phosphoric or sulphuric acid. With this treatment penicillin converts into its anionic form.

The liquid is extracted using Podbielniak counter current extractor with an organic solvent (e.g., amyl acetate or butyl acetate or methyl isobutyl ketone) quickly because at low pH values penicillin is unstable. Then the penicillin is extracted back into water from the organic solvent by adding potassium or sodium hydroxide. This also elevates the pH to 7.0 to 7.5. The resulting aqueous solution is re -acidified and re -extracted with organic solvent. These shifts between the water and the solvent purify the penicillin, and it is obtained as sodium penicillin. The solvent consumed is recovered by distillation and used again.

3) Treatment of Crude Extract: The sodium penicillin obtained is treated with charcoal for removing the substances causing fever, i.e., pyrogens. It may also be sterilised using Seitz filter for removing bacteria. This treated sodium penicillin is converted into its crystalline form by crystallisation. It is either packed in sterile vials in the form of powders or is prepared as tablets or syrups for oral administration.

PRODUCTION OF CITRIC ACID

Citric acid was first discovered as a constituent of lemon. Citric acid at the current time is known as an intermediate of Krebs cycle, and therefore is present in every living organism. Previously, it was isolated from lemons (which contain 7-9% citric acid), and nowadays 99% of it is obtained by microbial fermentation.

1. Microbial Strains for Citric Acid Production

Citric acid can be obtained from various microorganisms. Aspergillus niger (fungus) is most commonly used for industrial production of citric acid.

Aspergillus clavatus, Aspergillus wentii, Penicillium luteum, Candida catenulata, Candida guilliermondii, and Corynebacterium sp. are some other organisms that may be used for the same purpose; however, these organisms are less important.

To improve the industrial production of citric acid, mutant strains of A. niger have been developed. These strains can tolerate high sugar concentration and low pH, and produce minimum undesirable by -products (oxalic acid, isocitric acid, and gluconic acid).

2. Medium

The yield of citric acid produced by A. niger is determined by the initial sugar concentration (15 -18%) and other organic acids produced in the medium. A concentration above or lower than 15 -18% is uneconomic and also amounts to lower yields. The concentration of other ingredients (i.e., nitrogen, phosphorous, and sulphur) and trace composition of basal medium for citric acid production are listed in the table 9.9:

Table 9.9: Production Media for Fermentation of Citric Acid by *A. niger*

Ingredients	Quantity (%)
Total reducing sugars (molasses, sucrose)	14-15
Nitrogen source	0.25
KH_2PO_4	0.10-0.15
$MgSO_4.7H_2O$	0.02-0.025
pH for Molasses pH for Sucrose	5.0-6.0 2.0-3.0
$CuSO_4$ (if molasses is used)	4.7mg/l
Octadecanol (anti-foaming agent)	0.75

3. Production Processes for Citric Acid

Industrial production of citric acid involves the following two processes:

1) Surface Process: In this process, the microorganisms are grown as a layer or a film on a surface in contact with the solid or liquid nutrient medium. Thus, the surface process has supported-growth systems.

2) Submerged Process: In this process, the microorganisms are either immersed in or dispersed throughout the nutrient medium. There are two types of submerged fermenters (bioreactors), i.e., stirred bioreactors and airlift bioreactors.

ctors.

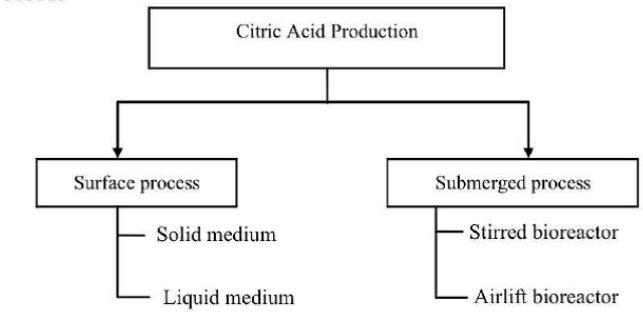


Figure 9.8: Industrial Processes for the Production of Citric Acid

4. Surface Processes

The surface process of fermentation of citric acid involves:

1) Solid Surface Fermentation: In this type of surface fermentation, the culture medium comprises of solid substrates, such as wheat bran or pulp from sweet potato starch. The medium pH is adjusted to4 = -5, followed by sterilisation. Thereafter, the inoculum (spores of A. niger) is spread as layers of 3-6cm thickness and incubated at 28°C temperature. The growth of organisms can be accelerated by adding a-amylase. Solid-state fermentation takes 80-100 hours for producing citric acid maximally. At the end, citric acid is extracted into hot water and isolated.

2) Liquid Surface Fermentation: In this type of surface fermentation, liquid is used as nutrient medium for citric acid production. This method is in use since the ancient times due to a simple technology, low energy costs, higher reproducibility, and minimal interference of trace metals and dissolved O_2 tension. However, the labour costs are higher since the manpower requirements for cleaning the systems are more.

In liquid surface fermentation, beet molasses are used as sources of nutrients. Fermentation is carried out in aluminium trays filled with sterile nutrient medium. The inoculum (spores) is sprayed over the medium, and sterile air is passed for supplying O_2 and also for cooling. Temperature throughout the process is maintained at 30°C. The spores start germinating within 24 hours of inoculation, and form a layer of mycelium over the medium. As the mycelium grows in size and forms a thick layer on the nutrient solution surface, the nutrient medium pH falls to less than 2. Fermentation is stopped after 7-15 days.

The mycelium and nutrient solution are separated. The mycelium is mechanically pressed and washed thoroughly to obtain maximum amount of citric acid. The nutrient solution is further processed for recovery of citric acid. The final yield of citric acid is in the range of 0.7. -0.9 per gram of sugar.

5. Submerged Processes

Submerged fermentation is the most preferred method due to its high efficiency and easy automation. It has disadvantages of adverse influence of trace metals and other impurities, variations in O_2 tension, and advanced control technology that requires highly trained personnel.

Stirred tanks and aerated towers are the two types of bioreactors used in submerged fermentation. Vessels of these bioreactors are made up of high - quality stainless steel. The sparging of air occurs from the base of the fermenter.

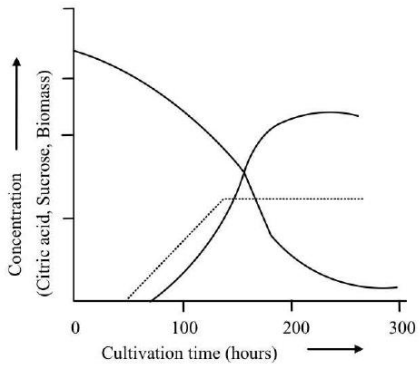


Figure 9.9: Diagrammatic Representation of Citric Acid (–) Production along with Sucrose (–) and biomass (···) Concentration in Relation to Time

The yield of citric acid production depends on the mycelium structure. Mycelium having forked and bulbous hyphae and branches aggregating into pellets are regarded as the most appropriate for citric acid formation; while, the loose and filamentous mycelium having limited branches results in no citric acid production. An adequate supply of O 2 (20-25% of saturation value) is required for good production of citric acid. The ideal aeration rate is inthe range of 0.2 to l vvm (volume/volume/minute).

In submerged fermenters, foam may be formed which may occupy about 1/3rd of the bioreactor. Formation of foam can be prevented by using antifoam agents (e.g., lard oil) and mechanical antifoam devices. Nutrient concentration is very important in the industrial production of citric acid.

Figure 9.9 gives a diagrammatic representation of sucrose, citric acid and biomass concentration with respect to cultivation time. It is estimated that under optimal conditions, in 250-280 hours, 100-110gm/L of citric acid is obtained from 140gm/L of sucrose with a biomass (dry weight) of 8-12gm/L.

6. Recovery of Citric Acid

The recovery steps of citric acid either from surface process or submerged process are comparable (figure 9.10). In the process of recovery, the culture roth is filtered and mycelium (which may contain about 10% of citric acid produced) is washed. Oxalic acid (an unwanted by -product) is removed by precipitation by adding lime at pH less than 3.

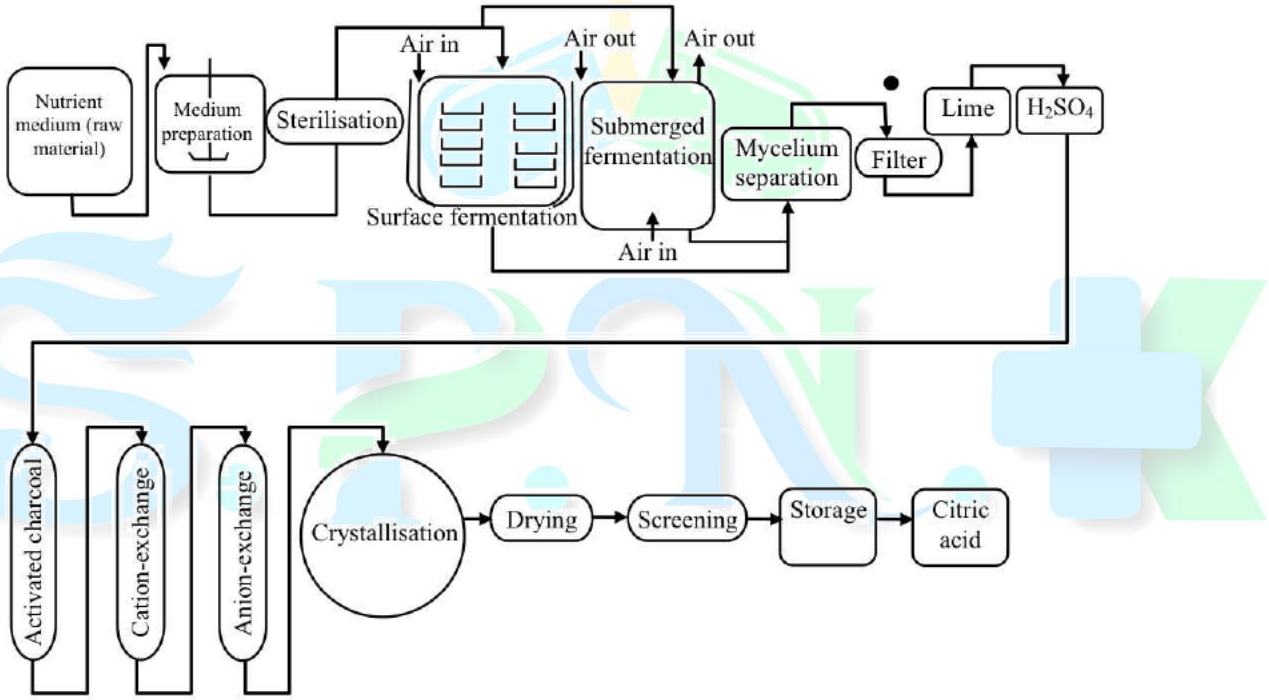


Figure 9.10: Flow Chart for Industrial Production of Citric Acid by Surface or Submerged Processes

The culture broth pH is adjusted to 7.2 and 1 ts temperature at 70 -90°C for precipitating citric acid. For further purification, citric acid is dissolved in sulphuric acid (calctum sulphate precipitate separates). In the final steps of citric acid recovery, treatment with activated charcoal, cationan d anion-exchangers, and crystallisation are carried out.

Citric acid monohydrate formed below 36°C temperature is the major commercial product. Above 40°C temperature, citric acid crystallizes out in an anhydrous form. The purity degree of citric acid produced depends on the purpose of its requirement. For instance, pure forms of citric acid are needed for use in food preparations, while for industrial use it can be in crude form.

Production of Vitamin B12

Vitamin B_{12} comes under a group of compounds known as cobalamins. Chemically, it has a central cobalt atom coordinated by corrin (a porphyrin –link group). Dimethylbenzimidazole (a base) and a cyano group occupy the axial coordination sites. B $_{12}$ analogues are the compou nds in which bases other than dimethylbenzimidazole are present.

Vitamin B_{12} was produced using many different microbes. In its commercial scale production, one organism from Pseudomonas (i.e., Pseudomonas denitrificans) and two from Propionibacteria (i.e., Propionibacterium shermanii and Propionibacterium freudenreichii) species are used. These have rapid growth and high productivity of selected mutants, thus were preferred over Streptomyces and other species.

Production of vitamin B12 on a laboratory -scale fermentation process using Pseudomonas denitrificans and pilot plant -scale fermentation process using Propionibacterium shermanii have been discussed below. Mutant strains of these organisms were utilised.

Fermentation of Pseudomonas denitrificans

Commercially producing mutants were developed from the original wild -type of P. denitrificans isolated by Miller and Rosenblum in 1960, and the process was described by Florent and Ninent in 1979. This process yielded about 60mg/L in the laboratory.

Sugar beet molasses cont aining 5-10% betaine (trimethyl glycine) serves as the carbohydrate source. Betaine stimulates vitamin B , production by promoting the synthesis of Ala -synthetase, responsible for the production of 6-aminolevulinic acid, which is the first intermediate of the pathway of cobalamin biosynthesis.

The growth of Pseudomonas and vitamin B ;, biosynthesis are carried out under aerated condition with agitation. Cobalamin biosynthesis by Pseudomonas denitrificans requires an external supply of 5, 6-dimethylbenzimidazole (5,6-DBI) and cobalt salt.

Laboratory-scale fermentation of vitamin B_{12} using Pseudomonas denitrificans occurs via following steps:

1) Maintenance Culture: The inoculated media is incubated for 4 days at 28°C temperature.

Table 9.10: Maintenance Culture

Ingredients	Quantity (gm/l)
Beet molasses	60
Brewer's yeast	1
Nz amine	1
$(NH_4)_2HPO_4$	2
$MgSO_4.7H_2O$	1.0
$MnSO_4.7H_2O$	0.2
$ZnSO_4.7H_2O$	0.02
$Na_2M_0O_4.2H_2O$	510-3
Agar	25.0
pH up to	7.4

2) Seed Culture: It is the same medium as above only — without agar and is incubated for 3 days at 28°C temperature on a rotary shaker.

3) Production Culture: The media inoculated with seed culture is incubated for 90 hours at 29°C temperature with 420 rpm agitation and 1v/v/m aeration.

Table 9.11: Production Media

Ingredients	Quantity (gm/l)
Beet molasses	100
Yeast	2
$(NH_4)_2HPO_4$	5
$MgSO_4.7H_2O$	3
$MnSO_4.H_2O$	0.2
$CO(NO_3)_2.6H_2O$	0.188
$ZnSO_4.7H_2O$	0.02
$Na_2M_0O_4.2H_2O$	5×10^{-3}
5,6-DBl	0.025
pH up to	7.4

Fermentation of Propionibacterium shermani

There are many propionic organisms grown on carbohydrate -based media under unaerated condition that produce cobaltocorrinoids. Production of cobalamin requires cobalt supplement, and also depends on the internal forma external supply of 5,6-dimethylbenzimidazole. The mutant strains of P. shermanii synthesises their own 5,6-DBI with an yield of around 65mg/L on pilot scale.

Aeration promotes 5,6 -DB1 formation, however represses one of the stages of vitamin B ;, biosynthesis. Hence, the first stage of fermentation (of 80 hours) should be performed under anaerobic condition without aeration and little agitation so that all the sugar in the media is utilised for the growth and the formation of cobinamide has no repressive effect. The following stage of fermentation (of the next 88 hours) is performed by agitation and little aeration to induce the biosynthesis of 5,6-DBI1 and converts the cobinamide to cobalamin.

Pilot plant -scale fermentation of vitamin B_{12} using P. shermanii occurs via following steps:

1) Maintenance Culture: The inoculated media is incubated for 4 days at 30°C temperature.

Table 9.12: Maintenance Culture

Ingredients	Quantity (gm/l)
Tryptone	10
Yeast extract	10
Filtered tomato juice	200
Agar	10
pH up to	7.2

2) Seed Culture (First Stage): It is the same medium as above only without agar, and is incubated for 2 days at 30°C temperature without agitation.

3) Seed Culture (Second Stage): The inoculated media composed of corn steep liquor (20gm/l) and glucose (90gm/L), is incubated for 24 hours at 30°C temperature without aeration, and is maintained at pH 6.5.

4) Main Culture: The production media composed of corn steep liquor (40gm/l), glucose (100gm/l), and $COCl_2.6H_2O$ (0.02gm/I) is incubated at 30°C temperature and is maintained at pH 7.0. The first 80 hou rsis performed without aeration with slight nitrogen pressure, followed by slow agitation. The next 88 hours is carried on with agitation, slight aeration (0.1 v/v/m), and at pH 7.0.

Isolation and Purification

Extraction begins with the release of an unstable cobalamin from the cells lysed, and treatment of this cobalamin with cyanide so that it converts into a more stable cyanocobalamin.

Cells are separated from the culture broth and lysed by heating at 80 -120°C temperature for 10 -30 minutes. The pH should be maintained at 6.5 -8.5. The lysed cells release various cobalamins, which are solubilised with potassium cyanide in the presence of sodium nitrite. All the cobalamins convert into cyanocobalamin. The ballast is then eliminated with zinc hydroxide.

The product is purified by adsorption on various substrates (_ e.g., amberlite IRC 50, Dowex 1 x 2, alumina, silanised silica gel, and Amberlite XAD 2), followed by elution with water-alcohol or water-phenol mixtures.

PRODUCTION OF GLUTAMIC ACID

The commercial production of glutamic acid(figure 9.11) by microbial fermentation offers 90% of world's total demand, and the remaining 10% is provided by chemical methods. For actual fermentation, microbial strains are grown in fermenters with volume of $500m^3$.

Medium

Hydrolysed starch solution, cane molasses, and beet molasses are the carbohydrates used as carbon source. The media containing cane molasses with high biotin content is also added with penicillin during the active growth of cell. Ammonia, ammonia gas, urea, etc. are used as nitrogen sources. Ammonium ions are included during fermentation, andthe other ions include K^+, Mg^{2+}, PO_4^{3-}, SO_4^{2-} and Cl^-. Biotin should be added in the fermenting media in concentration below 5ug/l. The media should be maintained at pH between 7-8 and temperature between 30-35°C.

The concentration of biotin in the fermentation medium significantly affects the glutamic acid yield. Fermentation takes 2 -4 days to complete, and at the end of the process, the ammonium salt of glutamic acid is present in the broth.

Process

In a downstream process, the bacterial cells are separated and the broth is passed through a basic anion exchange resin. Glutamic acid anions bind to the resin and release ammonia, which can be recovered by distillation and reused in fermentation. Elution is performed with NaOH to form Monosodium Glutamate (MSG) in the solution and to regenerate the basic anion exchanger. MSG is crystallised directly from the

elute followed by the conditioning steps like decolourisation and serving to yield a good-grade quality of MSG.

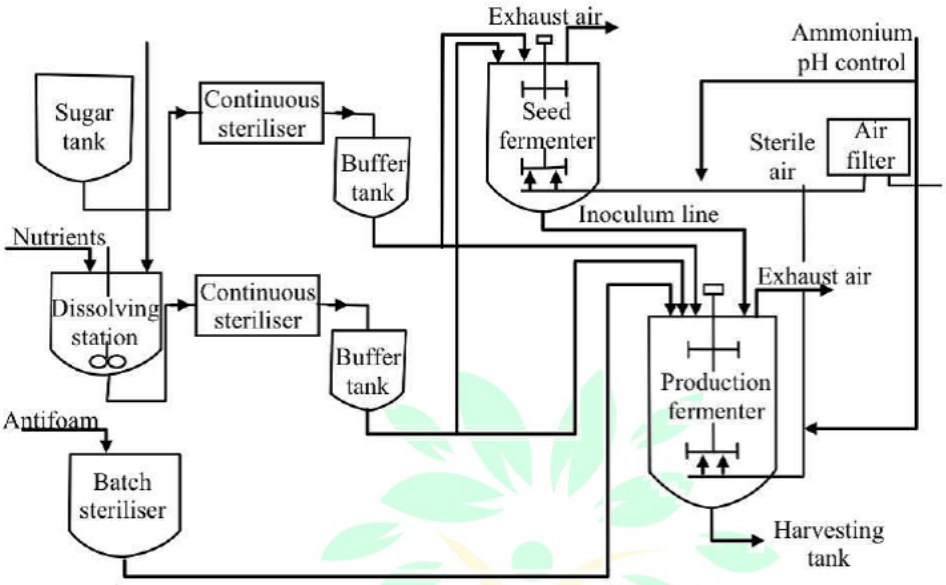
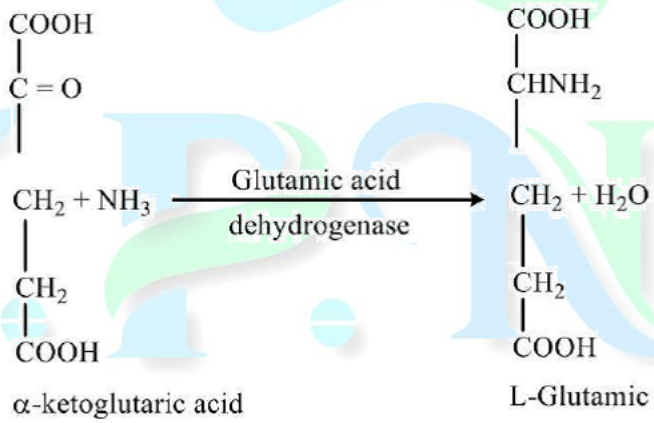


Figure 9.11: Flow Diagram of Commercial Production Method of Glutamic Acid (Glutamate)

The conversion of alpha-ketoglutaric acid (precursor of glutamic acid) into glutamic acid occurs in the presence of glutamic acid dehydrogenase enz yme. By adding penicillin in the medium, the production of glutamic acid can be increased.



Recovery

The product is extracted and purified from the concentrated broth by crystallisation of glutamic acid. Ion exchange resins are commonly used for this purpose.

PRODUCTION OF GRISEOFULVIN

Griseofulvin (a less toxic systemic antifungal antibiotic) is administered orally for treating and controlling fungal diseases of skin, hair, and nails caused by various dermat ophytes. This antibiotic can be produced by a large number of fungal organisms mainly including penicillin species . These species mostly belong to two main divisions of the genus Penicillium, i.e., Asymmetrica fasciculata and Asymmetrica divaricate . The st rains used for the commercial production of griseofulvin are mutants of Penicillium patulum, Penicillium raistrickii, or Penicillium urticae. The strains with conidia provides maximum product.

Inoculum Development

Spore suspension and vegetative cells are regarded suitable inocula for laboratory-scale fermentations. The sporulation medium (table 9.13) provides inoculum suitable for direct inoculation of shake-flask culture.

Table 9.13: Sporulation Medium for Preparation of Spore Inoculum

Ingredients	Quantity (gm/l)
Whey powder lactose	30.0
Whey powder nitrogen	50.0
KH_2PO_4	4.0
KCl	0.5
CSL solids	3.8

The germination medium (table 9.14) also provides inoculums for shake flask culture.

Table 9.14: Germination Medium for Vegetative Inoculum

Ingredients	Quantity (gm/l)
Protopeptone	20.0
Malted cereal extract	10.0
Glucose	40.0
Soluble starch	20.0
$NaNO_3$	3.0
KH_2PO_4	1.0
$MgSO_4.7H_2O$	0.5
$FeSO_4.7H_2O$	0.02

Table 9.15: Seed Stage (Vegetative) Medium

Ingredients	Quantity (gm/l)
C.S.L. nitrogen	3.0
Brown sugar	20.0
Chalk	10.0
Maize oil	10.0
Hodag M.F	0.3

Griseofulvin, similar to penicillin and streptomycin fermentation, requires early growth and then with the nutrient limitation strategy maintains a cell population within the aerating potent ial of the fermenter. The production level depends on good aeration, optimum pH, and availability of nitrogen. A high carbohydrate level (glucose, lactose, sucrose, upto 12%) generally results in a higher yield.

Phosphate also has a beneficial effect (0.4 -0.8%). Chlorine should be supplied through the entire fermentation process, or else dechloro-griseofulvin (lacking antifungal activity) will be obtained as the product.

Table 9.16: Typical Production Medium

Ingredients	Quantity (%)
Cornsteep liquor - N source	0.175
$CaCO_3$	0.4
KH_2PO_4	0.4
KCl	0.15

The carbohydrate glucose syrup (50%) is added stepwise after the first 16 -20 hours (10 -12%). The pH is maintained within 6.8 -7.2 by adding glucose continuously. Fermentation is carried at 25 °C temperature wi th an air flow of 1 vvm. After 10 days the yield will be 6 -8gm/l. The yield can be improved (12 - 15.5gm/l) by adding various methyl donors (choline salts, methyl xanthate, and folic acid) to the medium.

Extraction and Purification

Griseofulvin is stored intracellularly. The cells are removed by filtration and then extraction with organic solvent. The wet mycelium is treated with butyl acetate with subsequent evaporation. The crude griseofulvin is washed with chloroform and re -crystallised from aqueous met hanol to give a product with a 58% yield.

The mycelium is extracted with methylene chloride, evaporated, and then crystallised to obtain a pure product with 95% yield. The product can be improved (with a purity of 99%) by re-crystallisation with acetone.

BLOOD PRODUCTS AND PLASMA SUBSTITUTES

BLOOD PRODUCTS

INTRODUCTION

Blood is a thick and bright red coloured fluid connective tissue. It is alkaline (pH is 7.3 -7.45), salty, viscous, and heavier than water (specific gravity is 1.03-1.05). Blood is made up of aliquid portion (plasma) and acellular portion containing red blood cells (erythrocytes), white blood cells (leukocytes), and platelets (thrombocytes). The plasma contains water, electrolytes, different proteins such as clotting (coagulation) factors and immunoglobulins, and numerous metabolic substances. Blood plays various important roles in the human body, like transport of oxygen, nutrients and ions and serving as a buffer between the cells and environment. The major whole blood components are enlisted in table 10.1:

Table 10.1: Components of Whole Blood

Plasma	Cells
Water (97%)	Red blood cells
Ions	White blood cells
Organics	Platelets
Sugars	
Amino acids	
Lipids	
Trace elements	
Dissolved gases	

Blood transfusion is a lifesaving intervention with well-known benefits and risks.

It includes some infectious and non -infectious complications. Blood transfusion technique is used:

1) For maintaining adequate circulating blood volume,

2) For replacing specific blood components (erythrocytes, leukocytes, platelets, plasma, etc.), and

3) For removing harmful substances (such as bilirubin or excess red cells) by using various techniques of exchange transfusion.

Whole Human Blood

Whole blood is the simplest and most common type of blood donation. It can be transfused in its original form, or it can be separated into its specific components of red cells, white cells, plasma and platelets to be used in multiple patients.

Whole human blood is transfused for replacing the red blood cells, clotting factors, or other normal constituents missing from the patient's blood (either partially or wholly), or for restoring blood volume after acute haemorrhage. It is also utilised in various research applications, like drug target discovery, analysis of immune responses, and identification/validation or biomarkers.

Collection

Blood can be collected by the following steps:

1) An area of the donor's arm over the antecubital vein is cleaned and swabbed with an antiseptic solution.

2) A local anaesthetic is injected, and the sterile needle of a blood donor set is inserted in the vein.

3) The needle is connected to a bottle or plastic bag containing Acid-Citrate- Dextrose or ACD anti coagulant [disodium hydrogen citrate (2.0 -2.5gm), dextrose (3gm) and water (120ml)] via the tubing of the blood donor set.

4) The citrate binds to the calcium ions present in the blood and prevents clotting which would have occurred otherwise within a feminutes of colection.

5) Dextrose preserves the red cells, increase their life during storage from 4-7 days (in citrate alone) to 21 days (in ACD).

6) Volumes of 420ml of blood are collected and mixed appropriately with the solution in the container.

7) Two samples are withdrawn from the collected blood before being issued, one is checked for syphilis and blood group tests, and the other is checked for compatibility test made before the blood is given to a particular patient.

8) On standing, the cells settle down and form a dark red layer occupying about half the fluid volume, with a yellowish layer of plasma above.

9) The above layer of plasma is either clear or cloudy, which depends on the amount of lipid ingested by the donor in his meals before donating blood.

10) Blood which is contaminated or haemolysed through improper storage appear abnormal and should be carefully inspected before the blood is given to a patient.

Processing

Blood is drawn from a donor, consisting of erythrocytes, leukocytes, platelets, and plasma proteins with the anticoagulants -preservative solution. Whole blood is stored in a monitored refrig erator at 1 -6°C temperature for 21 days if collected in CPD (Citrate Phosphate Dextrose anticoagulant solution) or for 35 days if collected in CPDA -1 (Citrate Phosphate Dextrose Adenine Solution). Additive solutions cannot be added to whole blood to increase the storage period.

Before the technology of preparing blood component was developed, only whole blood was available as the blood product. When plastic was replaced with glass as the collection medium in the 1960s, whole blood can be easily separated into its components. From that time the availability of whole blood dropped, and it was replaced with red blood cells. In patients requiring only oxygen –carrying capacity from red blood cells, transfusion of whole blood leads to problems like circulatory overload, loss of viable platelets, and decrease in labile coagulation factors within the first 24 hours of storage. Thus, whole blood has a limited clinical use.

Whole blood is still believed to be used as a replacement in trauma and transplant cases where massive blood loss occurs. However practically instead of the whole blood, intravenous fluids and blood components are the standard therapy for massive resuscitation.

The processing of whole blood depends on the components to be produced from the whole blood unit. If plasma and RBCs are to be processed, the whole blood unit is transported on ice; while, if platelets are to be produced, the whole blood unit is maintained at room temperature till the plateletch plasma separates from the RBCs.

For production of plasma and RBCs , the whole blood unit is stored at 1 -6°C temperature or transported in a refrigerated system to maintain the optimal storage temperature for RBCs (i.e., 1 -10°C). For production of Fresh Frozen

Plasma (FFP), the whole blood unit is separated from the plasma within 8 hours of collection and stored in a freezer at temperature —18°C or less. A whole blood unit intended for plasma frozen within 24 hours of phlebotomy (it is the practice of drawing blood from patients and prepare the — blood samples for testing) is processed and frozen between 8-24 hours after collection.

For production of platelets refrigeration alters the platelet membrane, thus resulting in poor in vivo platelet recovery after transfusion. For production of a platelet concentrate the whole blood unit is transported in a container that can maintain the optimal temperature range for platelet viability (i.e.,22 -24°C). At the laboratory where components are prepared, the whole blood unit is processed to form a platelet concentrate within 8 hours of collection, and the remaining RBCs are refrigerated.

Storage

Given below are the storage requirements for whole human blood:

1) Soon after the blood is collected from the donor, it should be cooled to 4-6°C temperature and maintained at the same temperature for 21 days, except for short periods of 30 minutes (necessary during transportation or testing).

2) The concentration of haemoglobin in the blood -anticoagulant mixture should be not less than 9.7gm/dl.

3) The blood cells and the serum should be examined to determine the ABO blood group, and the cells alone should be examined for the Rh group.

4) The blood sterility should be checked before it is used for transfusion by testing a proportion of containers of blood older than 21 days as a check on the technique of collection and storage.

5) The label should bear the ABO and Rh groups, volumes of blood and anticoagulant solution present, date of collecting blood, expiry date, required storage conditions, a warning that the contents should not be used if any visible sign of deterioration is seen, and a number or code by which the history of the preparation can be traced back to the original donor.

Factors Affecting Storage

Blood should be stored at 4-10°C temperature (preferably at 4-6°C) for not more than 21 days. Given below are the factors that influence the storage of blood:

1) Deterioration during Storage: Red cells may get damaged or haemolysed on exposure to heat, freezing, excessive agitation, hypertonic or hypotonic solutions, incorrect environmental pH, or storage beyond the specified dating period. The blood may clot if incorrect collection procedures have been followed. Under normal conditions also, some small clots may be present, and thus blood should be administered always through a 'giving' or 'recipient' set, which includes a filter with a large surface area. This filter removes the clots, but does not get blocked during the transfusion.

2) Bacterial Contamination: If blood is sterilised by heat, filtration, or using chemical agents, the red cells and plasma proteins are irreversibly damaged. Thus, the only ways of preventing bacterial contamination of the whole blood to be transfused are the use of properly sterilised equipment, careful techniques of collection, and storage at 4 -6°C temperature at all times (even during transportation). Even if these measures are adopted, a few viable organisms (derived from the donor's skin) are _ still present in the collected blood. These organisms either die or their proliferation is prevented by storing the blood at desired temperature (4-6°C). In rare cases, the blood may get contaminated by cryophilic gram -negative organisms that grow at 4°C, and do not give rise to any macroscopic changes. Depending on the number of organisms present, blood tra nsfusion may cause a mild, severe or rapidly fatal reaction.

3) Transmission of Disease from the Donor: Syphilis, malaria and hepatitis are the diseases that can get transmitted by blood transfusion. A serological test for syphilis should always be done, however a negative test is not a complete precaution. Thus, the donor's medical history is checked to determine whether or not he/she is free of diseases that get transmitted by blood transfusion. The greatest risk is of transmitting hepatitis B (serum hepatitis). A high proportion of hepatitis carriers can be identified by immunological tests that detect the presence of hepatitis B surface antigen in their blood. Thus, all blood donations are screened by such tests.

4) Sensitisation to Blood Group Antigens: Blood transfusion is a kind of homograft (i.e., transfer of living tissue from one individual to another). Antibodies developed in the plasma reject the red cells of the type introduced by reacting with the antigens on the cell surfaces. In case of ABO blood group antigens, iso -antibodies are present in the blood even when the individual has not gone through any previous blood transfusions, and react with the cells of wrong group if these are transfused. The rejection reaction caused by the action of antibodie s on red cells results in intravascular agglutination, lysis or disruption of the transfused cells, and a fatal effect on the patient. Therefore before using the blood, it is tested and labelled according to its blood group; the patient is grouped and blood of appropriate group is selected; a compatibility test is performed in which the cells to be transfused are tested against the patient's serum to confirm that no antibodies which will agglutinate or lyse the cells are present. In order to avoid the transfusion of blood of wrong group, an elaborate system of labelling, record-keeping and cross-checks should be adopted.

Plasma, Serum and Plasma Fractions

During the storage period, the red cells lose their capacity to survive in the circulation of the transfused recipient. When 21 days old blood is transfused, around 70% of the cells remain in the patient's circulation after 24 hours. Therefore, blood older than 21 days is not considered suitable for transfusion.

The plasma proteins are relatively more stable, and the plasma should be aspirated from the sedimented red cells of time -expired blood to use it for therapeutic products. Freshly collected blood is subjected to centrifugation to obtain fresh plasma for prep aring certain labile components that cannot be recovered in active form from the time-expired blood. Even if the plasma and certain fractions derived from it are prepared from blood screened for hepatitis B surface antigen, they may still carry the hepatitis B virus. Thus, they should be used only when the benefits likely to accrue to the patient compensates the risk of transmitting the disease. These fractions should not be transfused as a vehicle for administering a drug or other sub stance. Dried plasma and serum are being replaced with albumin preparations, as they serve as volume expanders with no risk.

Proteins (complex macromolecules) can get easily damaged or denatured on exposure to heat, thus strict storage requirements should be maintained. Since proteins are instable in solution, some protein-containing products are issued as a freeze –dried powder, sealed in the presence of dry nitrogen. Such preparations are hygroscopic and if moisture enters t h rough a loose or faulty seal, they lose their stab.

In Pharmacopoeias, requirements are laid down with respect to sterility, identity, freedom from pyrogens, total protein content, electrolyte content, solubility, presence of preservatives and stabilisers, and residual moisture content for freeze-dried products. Apart from these special requirements for plasma proteins, required protein composition as determined by electrophoretic analysis or other procedures are also mentioned. The label of these products should include:

1) The product name,

2) The batch number,

3) Information on potency or concentration,

4) The name and address of manufacturer or authorised distributor,

5) The date of manufacture,

6) The date of expiry,

7) Th e required storage conditions, and

8) The nature and concentration of bacteriostatic agent (if added).

Products used in blood volume replacement therapy, including plasma, human albumin preparations and solutions of various substances are used as plasma substitutes.

Dried Human Plasma BP

Haemagglutinating antibodies of the ABO system are found in the plasma of some donors. When these antibodies are transfused into patients of certain blood groups, there is a risk of intravascular agglutination and haemolysis; however, this risk can be avoided if the haemagglutinins are neutralised with the soluble blood group substances present in the plasma of other donors of appropriate blood groups.

Plasma is selected from such pools of blood donated that the normal distribution of ABO groups is represen ted; thus a ten -donor pool contains plasma from equal numbers of A and O donations, along with atleast one B or AB donation. Many years back it was found in a research that on restricting plasma pools to ten donors, the risk of transmitting hepatitis B was low; thus, small-pool plasma was issued to be used with a risk of transmitting hepatitis in one out of 500 patients.

Attempts to inactivate the virus either with UV irradiation or with B-propiolactone proved to be ineffective; however combination of both inactivates the agent, but the so treated plasma shows electrophoretic and other abnormalities. Filtration of plasma is difficult and it is not practic ally possible to sterilise large numbers of small pools by filtration. As whole blood cannot be assumed to be sterile, it is necessary to perform sterility test on each ten -donor pool. The pools which pass these tests are redistributed in transfusion bottles in such quantities that they can be freeze dried. Dried plasma is a light to deep cream coloured powder that is free from streaks of red or pink indicative of red cells or haemoglobin. It is reconstituted to the original volume using water for injection at room temperature, and it dissolves in less than 10 minutes to give a cloudy solution. The protein content is not less than 45gm/l. Adding 17gm/I of calcium chloride to 1ml causes coagulation, thus indicating the presence of fibrinogen.

Storage

1) Dried human plasma should be stored under dry conditions.

2) It should be stored at temperature below 50°C.

3) It should be stored away from light.

Labelling

Apart from the general requirements, the label of dried human plasma should also state:

1) The names and percentages of anticoagulant and other added substances.

2) The quantity of water for injection required to recnstitute the original volume

3) The protein content of the reconstituted liquid.

4) Th e contents should not be used after 3 hours of reconstitution.

Uses

1) It is used for restoring the plasma volume in patients suffering from burns, scalds, or crush injury.

2) In cases of emergency, when whole blood is not available or the results of compatibility tests are still awaited, dried plasma can be used to restore blood volume.

3) In cases of emergency, when fibrinogen concentrate is not available, dried plasma is reconstituted to one third or one quarter of the original volume and used for treating acute fibrinogen deficiency.

Fresh Frozen Plasma

Fresh frozen plasma is prepared by centrifugation of whole human blood within a few hours of its collection from the donor, followed by storing it in the frozen state at temperature below —30°C. If itis not pooled it should be labelled and used according to the stat ed blood group. When the plasma is to be used, it is thawed for 45 minutes by immersion in a water bath at a temperature not more than 37°C.

Uses

1) It is used to preserve the labile clotting factors.

2) Itis asource of clotting factor VIII that is required for treating minor haemorrhage in mildly affected haemophiliacs.

Dried Human Serum, BP

Dried human serum is prepared by collecting blood without adding an anti - coagulant from donors who meet the requirements specified for whole human blood. The collected blood is then allowed to clot, and the separated fluid is pooled, bottled and freeze-dried.

The ratio of blood groups in the pool and the pool size for dried human serum are similar as that for dried human plasma. All the other requirements are also identical, with the exceptions that the protein content should not be less than 65gm/L, and the test for coagulation with calctum chloride does not apply. The uses of dried human serum are similar to those of dried human plasma, except for the fact that it is not a source of fibrinogen.

Plasma Substitutes

There are some limiting factors and disadvantages closely related to the use of human blood, plasma and its derivatives. Therefore, many attempts have been made to find substances (not of human origin) that can be used for the restoration of depleted blood volume. Such substances should be non _ -antigenic, non-toxic, get completely excreted or metabolised, and may exert a colloid osmotic pressure. Gum acacia, modified globin(— derived from human haemoglobin), synthetic polymers such as polyvinyl pyrrolidone, hydroxyethyl starch, modified gelatin and dextran are the substances which have been investigated. Dextran and gelatin (to a lesser extent) are the most widely used ones.

Gronwall and Ingelman suggested that dextrans (polysaccharides of bacterial origin) can be used as volume expanders, thus these substances have been examined comprehensively. Dextrans are long chain polymers of dextrose (D -glucose) with their sub -units linked through alpha-1,6-glycosidic linkages. As per the B.P., dextran to be used clinically is produced commercially by large -scale fermentation, using a strain of

Leuconostoc mesenteroides bacteria. Dextran that has been precipitated from the fermentation medium and purified to remove pyrogenic and antigenic substances is a clinically useful volume expander and possesses colloidal properties similar to plasma.

Ideal Requirements of Plasma Substitutes

The ideal requirements of a plasma substitute or the properties of an ideal plasma substitute are:

1) Its colloidal osmotic pressure should be similar to that of whole blood.

2) Its viscosity should be similar to that of plasma.

3) Its molecular weight should be such that the molecules do not easily diffuse through the capillary walls.

4) It should suffer a low rate of excretion or destruction by the body.

5) It should undergo a complete elimination from the body.

6) It should be free from toxicity, e.g., no impairment of renal function.

7) It should be free from antigenicity and pyrogenicity.

8) It should not produce confusing results on tests like blood grouping and erythrocyte sedimentation rate.

9) Its isotonicity in solution should be equal to that of blood plasma.

10) It should be highly stable in liquid form at normal and sterilizing temperatures and during transport and storage.

11) It should be easy to prepare and readily available at low cost.

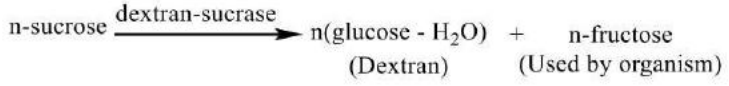
Polyvinylpyrrolidone (PVP)

The Germans in the Second World War introduced polyvinylpyrrolidone (a synthetic colloid) for shock treatment. It was marketed in the 1950s but was withdrawn later because of its suspected carcinogenicity.

Polyvinylpyrrolidone (PVP or povidone) is the generic name for the water - soluble homopolymer of N-Vinyl-2-Pyrrolidone (NVP). It is a synthetic polymer with average of molecular weight of 40 000. It is used as a 3.5% solution. It interferes with blood grouping and releases histamine. It mainly gets excreted via renal route; however small amounts of it are also excreted by liver through bile. Since it interacts with penicillin and insulin, its use as a plasma expander has become rare.

Dextran

Dextran, the most acceptable plasma substitute, is a polysaccharide produced when the bactertum Leuconostoc mesenteroides is grown in a medium containing sucrose. This organism secretes an enzyme that converts sucrose to dextran:



In sugar industries, dextran occurs as a slime that clogs the pipes and filters, and hinders crystallisation.

The two main groups of dextrans can be produced from different strains of Leuconostoc mesenteroides:

1) Long unbranched chains of glucose units linked via 1,6-glycosidic linkages.

2) Highly branched polymers of short chains of 1 -6 units linked via 1-4 and 1-3 linkages to branches.

Production of Dextran

Dextran is produced by laboratory culture followed by growth in seed tanks in the factory and then in fermenters of 4500dm^3 capacity. Enzyme synthesis and its actions on sucrose are rapid, thus it is not necessary to mai _ntain high degree of asepsis in antibiotic fermentation. Also, the process is inhibited by aeration, thus there is no need for a costly supply of sterile air.

Hydrolysis of sucrose into glucose and fructose during sterilisation of the culture media should also be prevented, or else dextran will not be obtained because the conversion does not involve invesion but a straight transglycosyhtion. Hydrolysis can be prevented by adjusting the media pH to neutral before sterilisation, and by avoiding overheating . On attaining maximum conversion to dextran, it is precipitated by adding a suitable organic solvent. Natural dextran consists of chains of around 2,00,000 glucose units with molecular weights of about 50 million.

Very large molecules having molecular weight above 2 ,50,000 have the following limitations:

1) They yield highly viscous solutions that are difficult to administer.

2) They cause renal damage and allergic reactions.

3) They form rouleaux (aggregates of red cells resembling piles of plates) that interfere with blood matching and sedimentation tests.

4) They produce colloidal osmotic pressures lower than those of small molecules.

The selected fraction still requires to be purified for removing:

1) Reducing sugars by solvent precipitation; the main contaminant is fructose (the by-product of fermentation).

2) Fractionation solvents by evaporation under reduced pressure.

3) Inorganic salts by demineralisation in a mixed bed ion exchanger; phosphates should be removed particularly as they cause precipitation during sterilization and storage.

4) Coloured substances by adsorption on activated charcoal.

5) Pyrogens by adsorption on asbestos or cellulose derivatives.

6) Microbes by filtration; between each treatment, the preparation is passed through a fibrous pad and before bottling also a membrane filter is used. The obtained solution is diluted to a concentration of 5% (either as 5% dextrose injection or sodium chloride injection), packed in sulphur-treated soda _ lime bottles, closed with lacquered rubber plugs, and finally sterilised by autoclaving.

Storage

Dextran 110 injections in 5gm/I dextrose should be stored at temperature below 25°C to retain its properties for 5 years. Sodium chloride injection of dextran 110 should be stored at temperature up to 40°C to retain its properties for 5 years.

Labelling

Apart from the general requirements, the label of dextran products should bear:

1) The dextran concentration,

2) The solvent name, and

3) The strain of Leuconostoc mesenteroides.

Uses

1) Dextran is used for emergency restoration of blood volume.

2) Large infusions of dextran interfere with blood coagulation; therefore dextran 110 should be carefully administered to patients with a bleeding tendency.

Dextran 40 Injection, IP.

This preparation has an average molecular weight of 40,000. Small molecular weight dextran of this size specifically diminishes red cell aggregation. Due to its small molecular size, it exerts a significant osmotic pressure but the resultant blood volume expansion is brief, 75% of the dextran gets excreted in 24 hours.

Dextran of small molecular weight promotes blood flow in endangered conditions, for example, as in thromboembolic conditions. The preparation method, the product appearance, and the required control tests are similar to those of Dextran 110. Dextran 40 injections should be stored at a temperature not more than 25°C; and temperature fluctuations should be avoided. Under these conditions it retains its properties for 5 years.