BPH 605T Pharmaceutical Biotechnology Unit: 4 Molecular Biology

4a.Immunoblotting Techniques

ELISA

ELISA (enzyme-linked immunosorbent assay) is a plate-based assay technique designed for detecting and quantifying peptides, proteins, antibodies and hormones. In an ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction.

ELISAs are typically performed in 96-well (or 384-well) polystyrene plates, which will passively bind antibodies and proteins. It is this binding and immobilization of reagents that makes ELISAs so easy to design and perform. Having the reactants of the ELISA immobilized to the microplate surface makes it easy to separate bound from non-bound material during the assay. This ability to wash away nonspecifically bound materials makes the ELISA a powerful tool for measuring specific analytes within a crude preparation.

Principle

Enzyme-linked immunosorbent assays (ELISA) principles are very similar to other immunoassay technologies. ELISA's rely on specific antibodies to bind the target antigen, and a detection system to indicate the presence and quantity of antigen binding. In order to maximize the sensitivity and precision of the assay, the plate must be carefully coated with high-affinity antibodies – a process that Boster Bio has mastered.

Procedure

An ELISA begins with a **coating** step, in which the first layer, consisting of a target antigen or antibody, is adsorbed onto a 96-well polystyrene plate. This is followed by a **blocking** step in which all unbound sites are coated with a blocking agent. Following a series of washes, the plate is **incubated with enzyme-conjugated antibody**. Another series of washes removes all unbound antibody. A **substrate** is then added, producing a calorimetric signal. Finally, the plate is **read**.

Because the assay uses surface binding for separation, several washes are repeated in each ELISA step to remove unbound material. During this process, it is essential that excess liquid is removed in order to prevent the dilution of the solutions added in the next assay step. To ensure uniformity, specialized plate washers are often used.

ELISAs can be quite complex and include multiple intervening steps, especially when measuring protein concentration in heterogeneous samples such as blood. The most complex and varying step in the overall process is detection, where multiple layers of antibodies can be used to amplify signal.

ELISA Types

ELISAs can be performed with a number of modifications to the basic procedure: direct, indirect, sandwich or competitive. The key step, immobilization of the antigen of interest, can be accomplished by direct adsorption to the assay plate or indirectly via a capture antibody that has been attached to the plate. The antigen is then detected either directly (enzyme-labeled primary antibody) or indirectly (enzyme-labeled secondary antibody). The detection antibodies are usually labeled with alkaline phosphatase (AP) or horseradish peroxidase (HRP). A large selection of substrates is available for performing the ELISA with an HRP or AP conjugate. The choice of substrate depends upon the required assay sensitivity and the instrumentation available for signal-detection (spectrophotometer, fluorometer or luminometer).

Among the standard assay formats, where differences in both capture and detection were the concern, it is important to differentiate between the particular strategies that exist specifically for the detection step. However an antigen is captured to the plate (by direct adsorption to the surface or through a pre-coated "capture" antibody, as in a sandwich ELISA), it is the detection step (as either direct or indirect detection) that largely determines the sensitivity of an ELISA.

Blotting Techniques

Introduction:

Blotting is used in molecular biology for the identification of proteins and nucleic acids and is widely used for diagnostic purposes. This technique immobilizes the molecule of interest on a support, which is a nitrocellulosic membrane or nylon. It uses hybridization techniques for the identification of the specific nucleic acids and genes. The blotting technique is a tool used in the identification of biomolecules such ad DNA, mRNA and protein during different stages of gene expression. Protein synthesis involves expression of a DNA segment which gets converted to mRNA to produce the respective protein. Molecules such as DNA, RNA and proteins are subjected to biochemistry analysis which are separated using blotting techniques. In the case of a cell, these molecules are present altogether and hence with the help of blotting scientists are able to recognise a specific molecule out of all others. Blotting is performed by allowing a mixture of molecules of interest pass through a block of gel which separates the molecules based on their molecular sizes. The hence processed molecules are required to be hard-pressed against a suitable membrane which will in turn transfer the molecules from the gel onto a suitable membrane (nylon, nitrocellulose or PVDF) via capillary action. After the molecules are transferred to the membrane their position does not change.

Southern blotting was introduced by Edwin Southern in 1975 as a method to detect specific sequences of DNA in DNA samples. The other blotting techniques emerged from this method have been termed as Northern (for RNA), Western (for proteins), Eastern (for post-translational protein modifications) and South-western (for DNA-protein interactions) blotting.

Subtypes of blotting such as northern, western & southern depend upon the target molecule that is being sought. When a DNA sequence is the foundation or code for a protein molecule, the particular DNA molecule of interest can be blotted using Southern Blotting technique. During gene expression,

when the DNA is expressed as mRNA for a protein production, this process can be identified by Northern blotting. Finally, the coded mRNA produces the concerned protein, this protein identification can be done by Western Blotting.

Western blot

A western blot is a laboratory method used to detect specific protein molecules from among a mixture of proteins. This mixture can include all of the proteins associated with a particular tissue or cell type. Western blots can also be used to evaluate the size of a protein of interest, and to measure the amount of protein expression. This procedure was named for its similarity to the previously invented method known as the Southern blot.

The first step in a western blot is to prepare the protein sample by mixing it with a detergent called sodium dodecyl sulfate, which makes the proteins unfold into linear chains and coats then with a negative charge. Next, the protein molecules are separated according to their sizes using a method called gel electrophoresis. Following separation, the proteins are transferred from the gel onto a blotting membrane. Although this step is what gives the technique the name "western blotting," the term is typically used to describe the entire procedure.

Once the transfer is complete, the membrane carries all of the protein bands originally on the gel. Next, the membrane goes through a treatment called blocking, which prevents any nonspecific reactions from occurring. The membrane is then incubated with an antibody called the primary antibody, which specifically binds to the protein of interest. Following incubation, any unbound primary antibody is washed away, and the membrane is incubated yet again, but this time with a secondary antibody that specifically recognizes and binds to the primary antibody. The secondary antibody is linked to a reporter enzyme that produces color or light, which allows it to be easily detected and imaged. These steps permit a specific protein to be detected from among a mixture of proteins.

Southern Blotting

The first of these techniques developed was the Southern blot, named after Dr. Edwin Southern who developed it to identify specific DNA sequences. Southern blotting is a detection technique used to find the target DNA sequences in the DNA sample in the field of molecular biology. The process starts from electrophoresis of DNA molecules which are hybridized in a blotting membrane followed by a transfer step where DNA from gel is transferred onto the blotting membrane.

Principle

Restriction endonucleases, which is an enzyme, is used to break the DNA into small fragments. These fragments are then separated using electrophoresis. The fragments achieved is then classified according to their size (kDa). Thus, DNA fragments are transferred to the blotting paper where it is incubated with probes. Probes used in the Southern blotting can be highly selective. They can selectively bind with a resolution of 1 in a million and the characteristics to bind to the intended target fragments.

4b. Organization of Genetic Material in Prokaryotes and Eukaryotes

The vast majority of an organism's genome is organized into the cell's **chromosomes**, which are discrete DNA structures within cells that control cellular activity. Recall that while eukaryotic chromosomes are housed in the membrane-bound nucleus, most prokaryotes contain a single, circular chromosome that is found in an area of the cytoplasm called the **nucleoid**. A chromosome may contain several thousand genes.

Organization of Eukaryotic Chromosome

Chromosome structure differs somewhat between eukaryotic and prokaryotic cells. Eukaryotic chromosomes are typically linear, and eukaryotic cells contain multiple distinct chromosomes. Many eukaryotic cells contain two copies of each chromosome and, therefore, are **diploid**.

The length of a chromosome greatly exceeds the length of the cell, so a chromosome needs to be packaged into a very small space to fit within the cell. For example, the combined length of all of the 3 billion base pairs[1] of DNA of the human genome would measure approximately 2 meters if completely stretched out, and some eukaryotic genomes are many times larger than the human genome. DNA **supercoiling** refers to the process by which DNA is twisted to fit inside the cell. Supercoiling may result in DNA that is either underwound (less than one turn of the helix per 10 base pairs) or overwound (more than one turn per 10 base pairs) from its normal relaxed state. Proteins known to be involved in supercoiling include **topoisomerases**; these enzymes help maintain the structure of supercoiled chromosomes, preventing overwinding of DNA during certain cellular processes like DNA replication.

During **DNA packaging**, DNA-binding proteins called **histones** perform various levels of DNA wrapping and attachment to scaffolding proteins. The combination of DNA with these attached proteins is referred to as **chromatin**. In eukaryotes, the packaging of DNA by histones may be influenced by environmental factors that affect the presence of methyl groups on certain cytosine nucleotides of DNA. The influence of environmental factors on DNA packaging is called **epigenetics**. Epigenetics is another mechanism for regulating gene expression without altering the sequence of nucleotides. Epigenetic changes can be maintained through multiple rounds of cell division and, therefore, can be heritable.

Organization of Prokaryotic Chromosomes

Chromosomes in bacteria and archaea are usually circular, and a prokaryotic cell typically contains only a single chromosome within the **nucleoid**. Because the chromosome contains only one copy of each gene, prokaryotes are **haploid**. As in eukaryotic cells, DNA **supercoiling** is necessary for the genome to fit within the prokaryotic cell. The DNA in the bacterial chromosome is arranged in several supercoiled domains. As with eukaryotes, topoisomerases are involved in supercoiling DNA. **DNA gyrase** is a type of topoisomerase, found in bacteria and some archaea, that helps prevent the overwinding of DNA. (Some antibiotics kill bacteria by targeting DNA gyrase.) In addition, **histone-like proteins** bind DNA and aid in DNA packaging. Other proteins bind to the origin of replication, the location in the chromosome where DNA replication initiates. Because different regions of DNA are packaged differently, some regions of chromosomal DNA are more accessible to enzymes and thus may be used more readily as templates for gene expression. Interestingly, several bacteria, including ***Helicobacter pylori*** and ***Shigella flexneri***, have been shown to induce epigenetic changes in their hosts upon infection, leading to chromatin remodeling that may cause long-term effects on host immunity.

4c. Microbial Genetics

Horizontal gene transfer (HGT) is the movement of genetic material between organisms. It plays a key role in bacterial evolution and is the primary mechanism by which bacteria have gained antibiotic resistance and virulence. Scientists have studied how HGT occurs in nature and have learned how to introduce genetic materials into cells in the lab.

The introduction of foreign DNA or RNA into bacteria or eukaryotic cells is a common technique in molecular biology and scientific research. There are multiple ways foreign DNA can be introduced into cells including transformation, transduction, conjugation, and transfection. Transformation, transduction, and conjugation occur in nature as forms of HGT, but transfection is unique to the lab. Let's take a look at these different methods of DNA insertion.

Transformation

Transformation is the **uptake of genetic material from the environment** by bacterial cells. In nature, this genetic material often comes from adjacent lysed bacteria and can include plasmid DNA or fragmented DNA released into the environment. Various factors promote natural transformation in different bacteria such as growth phase of the cells (Baltrus and Guillemin, 2006) or the presence of specific substances.

Though not all bacteria are naturally competent to take up DNA, they can be made competent through chemical manipulation in the lab. This is commonly done using calcium chloride which permeabilizes the cell membrane so the bacteria can easily uptake your plasmid of interest. Scientists can also use electroporation, the application of an electrical charge to cells, to increase cell membrane permeability and thus transformation efficiency. Check out Addgene's blog to learn about making your own competent cells and our protocols page to learn about bacterial transformation in the lab.

Transduction

Transduction occurs when foreign DNA or RNA is introduced into bacterial or eukaryotic cells **via a virus or viral vector**.

One example are bacteriophages that attach to bacterial membranes and inject their genetic material into the cell. Once inside, phages can follow one of two different life cycles: lytic or lysogenic. Lytic phages hijack the bacterial hosts machinery to make more viral particles. Eventually the cell lyses releasing the newly formed viral particles that can infect other bacteria. In the lysogenic cycle, the phage's genetic material is incorporated into the host's genome at a particular integration site. The integrated phage remains dormant until it is triggered to enter the lytic cycle.

During both of these life cycles bacterial DNA can be accidentally packaged into the newly created phages. Transfer of this DNA to another cell is referred to as transduction. Transferred DNA once inside the infected bacterium can either exist as transient extrachromosomal DNA, like a plasmid, or it can integrate into the host bacterium's genome through homologous or site directed recombination.

Transduction is a common tool used by scientists to introduce different DNA sequences of interest into a bacterial cell or a host's genome. To do this scientists commonly use phagemids, a DNA cloning vector that contains both bacteriophage and plasmid properties. The phagemids are packaged into replication-incompetent phage particles with assistance from a 'helper' phage prior to transduction.

Scientists also use transduction to introduce foreign DNA into eukaryotic cells, like mammalian cell lines. This can be done with lentiviral and Adeno Associated Viruses (AAV). Lentiviral and AAV can be used to create both transient cell lines, where a gene of interest is expressed but not integrated into the

genome and stable cell lines, where foreign DNA is incorporated into the cell's genome and is thus passed down through cell division.

Bacterial conjugation

Conjugation was the first extensively studied method of gene transfer and was discovered in 1946 by Joshua Lederberg and Edward Tatum when they observed genetic recombination between two nutritional deficient *E. coli* strains that resulted in a wild type *E. coli* .

During conjugation, genetic material is transferred from a donor bacterium to a recipient bacterium through **direct contact**. The donor bacterium contains a DNA sequence called the Fertility factor (F-factor). The F-factor is found on an episome, a piece of DNA that can replicate on its own or be integrated within a bacterial chromosome and allows the donor bacterium to make a small "bridge" or sex pilus that attaches to the recipient cell drawing it close. Once in contact the donor can transfer genetic material to the recipient bacterium. The genetic material transferred is commonly a plasmid and can infer genetic advantages such as antibiotic resistance.

Plasmids

In addition to the bacterial chromosome, many bacteria often contain small nonchromosomal DNA molecules called plasmids. Plasmids usually contain between 5 and 100 genes. Plasmids are not essential for normal bacterial growth and bacteria may lose or gain them without harm. They can, however, provide an advantage under certain environmental conditions. For example, under normal environmental growth conditions, bacteria are not usually exposed to antibiotics and having a plasmid coding for an enzyme capable of denaturing a particular antibiotic is of no value. However, if that bacterium finds itself in the body when the particular antibiotic that the plasmid-coded enzyme is able to degrade is being given to treat an infection, the bacterium containing the plasmid is able to survive and grow.

Structure and Composition

Plasmids are small molecules of double stranded, helical, non-chromosomal DNA. In most plasmids the two ends of the double-stranded DNA molecule that make up plasmids covalently bond together forming a physical circle. Some plasmids, however, have linear DNA. Plasmids replicate independently of the host chromosome, but some plasmids, called episomes, are able to insert or integrate into the host cell's chromosome where their replication is then regulated by the chromosome.

Although some plasmids can be transmitted from one bacterium to another by transformation and by generalized transduction, the most common mechanism of plasmid transfer is conjugation. Plasmids that can be transmitted by cell-to-cell contact are called conjugative plasmids. They contain genes coding for proteins involved in both DNA transfer and and the formation of mating pairs.

Functions

Plasmids code for synthesis of a few proteins not coded for by the bacterial chromosome. For example, R-plasmids, found in some Gram-negative bacteria, often have genes coding for both production of a conjugation pilus (discussed later in this unit) and multiple antibiotic resistance. Through a process called conjugation, the conjugation pilus enables the bacterium to transfer a copy of the R-plasmids to other bacteria, making them also multiple antibiotic resistant and able to produce a conjugation pilus. In addition, some exotoxins, such as the tetanus exotoxin, *Escherichia coli* enterotoxin, and *E. coli* shiga

toxin discussed later in Unit 2 under Bacterial Pathogenicity, are also coded for by plasmids. Thousands of different plasmids are known to exist.

Transposons

Transposons (transposable elements or "jumping genes") are small pieces of DNA that encode enzymes that transpose the transposon, that is, move it from one DNA location to another, either on the same molecule of DNA or on a different molecule. Transposons may be found as part of a bacterium's nucleoid (conjugative transposons) or in plasmids and are usually between one and twelve genes long. A transposon contains a number of genes, coding for antibiotic resistance or other traits, flanked at both ends by insertion sequences coding for an enzyme called transpoase. Transpoase is the enzyme that catalyzes the cutting and resealing of the DNA during transposition. Thus, such transposons are able to cut themselves out of a bacterial nucleoid or a plasmid and insert themselves into another nucleoid or plasmid and contribute in the transmission of antibiotic resistance among a population of bacteria.

Plasmids and conjugative transposons are very important in horizontal gene transfer in bacteria. Horizontal gene transfer , also known as lateral gene transfer, is a process in which an organism transfers genetic material to another organism that is not its offspring. The ability of *Bacteria* and *Archaea* to adapt to new environments as a part of bacterial evolution most frequently results from the acquisition of new genes through horizontal gene transfer rather than by the alteration of gene functions through mutations. (It is estimated that as much as 20% of the genome of *Escherichia coli* originated from horizontal gene transfer.)

4d.Microbial Biotransformation

Biotransformation is the process by which an organism or its enzyme bring out chemical changes on compounds that are not part of their metabolism and they result in the formation of novel or useful products that are often difficult or impossible to obtain by conventional chemical means. The total chemical transformation of one steroid to another not only requires many stages but an expensive process although provide only low yield.

The biotransformation is used for the preparation of products of defined chemical structure that are related to the substrate or starting material for the reaction by only a small number of chemical changes and in many cases the changes are brought about by the action of only a single enzyme. Biotransformation reactions reported in the chemical literature of nineteenth century was developed as part of the synthetic routes for the production of L-ascorbic acid (vitamin C) and ephedrine.

Oxidation of alcohol to acetic acid by bacterium *Acetobacer xylinum*; oxidation of glucose to gluconic acid by *Acetobacter acetii*, sorbitol to sorbose by Acetobacter but were not fully utilized. Biotransformation is realised beyond doubt in early part of 20^{th} century when the conversion of D-sorbitol to L-sorbose by *Acetobacter suboxidans* and benzaldehyde to phenyl (lactyl carbinol) by yeast. Mamoli and Vercellone (1937) were the first to demonstrate the oxidation of nuclear hydroxyl group of steroid and reduction of nuclear double bond of steroid by yeast.

Welsch and Hongshem (1948) have not only confirmed but also enlarged the above result by employing a Streptomces sp. Kramli and Horvath (1949) could oxidize cholesterol to hydroxyl cholesterol by Pencillium roseum and Azatobacter sp. Hench and his associates (1949) demonstrated the curative effect of cortisone on rheumatoid arthritis was possible only by introducing O_2 at its 11^{th} carbon atom

with the help of Rhizopus arrhizus which chemically was very difficult.Subsequently several people could accomplish this task by using different Fungi, Actinomycetes and Bacteria. Presently varieties of biocatalysts are in use for carrying biotransformation reaction.

Types of Biotransformation Reactions

(i) Oxidation:
Hydroxylation, epoxidation, dehydrogenation of C-C bonds, oxidation of alcohol and aldehydes, oxidative degradation of alkyl, carboxyalkyl or ketoalkyl chains, oxidative removal of substituents, oxidative deamination, oxidation of heterofunctions and oxidative ring fission.

(ii) Reductions:
Reduction of organic acids, aldehydes, ketones and hydrogenation of C-C bonds, reduction of heterofunctions, dehydroxylations and reductive elimination of substituents.

(iii) Hydrolysis:

Hydrolysis of esters, amines, amides, lactones, ethers, lactams etc.

(iv) Condensation:

Dehydration, O- and N-acylation, glycosidation, esterification, lactomization and amination.

(v) Isomerization:
Migration of double bonds or oxygen functions, racemization, rearrangements, formation of C-C bonds or hetero-atom bonds.

(vi) Mixed Reactions:
Hydroxylation with reduction; Hydroxylation with oxidation; hydroxylation with side chain degradation; rupture of C-C linkages with oxidation of side chain.

Methods of Biotransformation:
Transformation of organic compounds may be accomplished by use of microorganism, isolated enzyme, immobilization techniques and solvent selection. The submerged fermentation is carried out in a stainless steel tank with minimal nutritional quantities to allow maximum transformation and use of easy extraction and purification of transformation product.
The microorganisms are grown in a suitable medium for 12-72 hrs depending on bacterium and fungus at optimum temperature, pH, aeration and agitation.

The fermentation is carried out in two phases:

1. Growth phase

2. Product formation phase
At the end of suitable incubation period (growth phase), measured quantity of organic compound to be transformed is added to the growing culture. The enzyme produced by the microorganism act upon the organic compound and does the desired function (product formation phase). At the end of suitable incubation period, the microbial biomass is separated from the fermentation broth. The broth is subjected to separation of both added substratum and product formed by the transformation.
For analysis, if the product samples are obtained at regular intervals upto end of incubation period (1-5 days), which are analysed by using TLC, paper chromatography, gas chromatography or HPLC technique. The extraction of product is done by appropriate organic solvents such as methylenechloride, chloroform, ethylacetate and methyl isobutylketone. Product obtained from cell and substratum should be extracted separately. Different factors like pH, temperature, addition of steroid and mineral content are reported to influence biotransformations.

Biotransformations in a large scale are carried out under sterile conditions in aerated and stirred fermenter. The conversion process is being monitored chromatographically or spectroscopically. The process is terminated when a maximal titer is reached. Sterility is required because contamination can suppress the desired reaction, induce the formation of faulty conversion products or cause total substrate break down.

Advantages of microbial transformation

Many benefits can be obtained through microbial transformations studies. The process required in microbial transformation may most probably have the ability to operate at near neutral pH, ambient temperatures and atmospheric pressures . In contrast, chemistry often requires extremes of these conditions which are not exactly environmentally friendly and industrially undesired. Furthermore, extreme pH, temperature and pressure may provide harmful effects toward personnel operating the harsh procedures and may also affect community surrounding the areas.

More importantly biocatalysts are highly reaction specific, enantiomer-specific and regio-specific [6]. This is mainly and directly referring to the chemical structure of a compound one may want to obtain specifically. Many versatile microorganisms can be utilized to carry out extremely specific conversions using substrates of low cost.

4e.Mutation

A mutation is a change in a genetic sequence. Mutations include changes as small as the substitution of a single DNA building block, or nucleotide base, with another nucleotide base. Meanwhile, larger mutations can affect many genes on a chromosome. Along with substitutions, mutations can also be caused by insertions, deletions, or duplications of DNA sequences.

Some mutations are hereditary because they are passed down to an offspring from a parent carrying a mutation through the germ line, meaning through an egg or sperm cell carrying the mutation. There are also nonhereditary mutations that occur in cells outside of the germ line, which are called somatic mutations. Mutations can be introduced due to mistakes made during DNA replication or due to exposure to mutagens, which are chemical and environmental agents that can introduce mutations in the DNA sequence, such as ultraviolet light. Some mutations do not result in changes in the amino acid sequence of the encoded protein and can be described as silent mutations. Other mutations result in abnormal protein products. Mutations can introduce new alleles into a population of organisms and increase the population's genetic variation.

Types of Mutations

There are three types of DNA Mutations: base substitutions, deletions and insertions.

1. Base Substitutions

Single base substitutions are called point mutations, recall the point mutation Glu -----> Val which causes sickle-cell disease. Point mutations are the most common type of mutation and there are two types.

2. Transition: this occurs when a purine is substituted with another purine or when a pyrimidine is substituted with another pyrimidine.

3. Transversion: when a purine is substituted for a pyrimidine or a pyrimidine replaces a purine.

Mutant

A Mutant is an organism or a new genetic character arising or resulting from an instance of mutation, which is generally an alteration of the DNA sequence of the genome or chromosome of an organism..

BPH 605T Pharmaceutical Biotechnology Unit:5 Fermentation Technology

5a.Fermentation methods and general requirements

Fermentation is the process involving the biochemical activity of organisms, during their life span. Fermentation technology is the use of organisms to produce food, pharmaceuticals and alcoholic beverages on a large scale industrial basis. The basic principle involved in the industrial fermentation technology is that organisms are grown under suitable conditions, by providing raw materials meeting all the necessary requirements such as carbon, nitrogen, salts, trace elements and vitamins. The end products formed as a result of their metabolism during their life span are released into the media, which are extracted for use by human being and that have a high commercial value. The major products of fermentation technology produced economically on a large scale industrial basis are wine, beer, cider, vinegar, ethanol, cheese, hormones, antibiotics, complete proteins, enzymes and other useful products.

Types of Fermentation Processes:

(1) Batch fermentation
(2) Feb-batch fermentation and
(3) Continuous culture.

Batch Fermentation

In this method the organism s allowed to grow and multiply in fermentor or bioreactor, produce the desired product and allowed to die.Then the process is stopped and organism is separated from the culture medium and from the medium the product is extracted by adopting suitable techniques.

Feb-batch fermentation:

In this type of fermentation, freshly prepared culture media is added at regular intervals without removing the culture fluid. This increases the volume of the fermentation culture. This type of fermentation is used for production of proteins from recombinant microorganisms.

Continuous fermentation:

In this type of fermentation the products are removed continuously along with the cells and the same is replenished with the cell girth and addition of fresh culture media. This results in a steady or constant volume of the contents of the fermentor. This type of fermentation is used for the production of single cell protein (S.S.P), antibiotics and organic solvents.

Procedure of Fermentation:

(a) Depending upon the type of product required, a particular bioreactor is selected.
(b) A suitable substrate in liquid media is added at a specific temperature, pH and then diluted.
(c) The organism (microbe, animal/plant cell, sub-cellular organelle or enzyme) is added to it.
(d) Then it is incubated at a specific temperature for the specified time.
(e) The incubation may either be aerobic or anaerobic.
i. Aerobic conditions are created by bubbling oxygen through the medium.
ii. Anaerobic conditions are created by using closed vessels, wherein oxygen cannot diffuse into the media and the oxygen present just above is replaced by carbon dioxide released.
(f) After the specified time interval, the products are removed, as some of the products are toxic to the growing cell or at least inhibitory to their growth. The organisms are re-circulated. The process of removal of the products is called downstream processing.

5b. Large scale production Fermentor and its controls

A fermentor (bioreactor) is a closed vessel with adequate arrangement for aeration, agitation, temperature and pH control, and drain or overflow vent to remove the waste biomass of cultured microorganisms along-with their products.
A fermentor is used for commercial production in fermentation industries and is a device in which a substrate of low value is utilized by living cells or enzymes to generate a product of higher value. Fermentors are extensively used for food processing, fermentation, waste treatment, etc.

History of Fermentors

De Beeze and Liebmann (1944) used the first large scale (above 20 litre capacity) fermentor for the production of yeast. But it was during the first world war, a British scientist named Chain Weizmann (1914-1918) developed a fermentor for the production of acetone.
Since importance of aseptic conditions was recognised, hence steps were taken to design-and construct piping, joints and valves in which sterile conditions could be achieved and manufactured when required.
For the first time, large scale aerobic fermentors were used in central Europe in the year 1930's for the production of compressed yeast (de Becze and Leibmann, 1944). The fermentor consisted of a large cylindrical tank with air introduced at the base via network of perforated pipes.
In later modifications, mechanical impellers were used to increase the rate of mixing and to break up and disperse the air bubbles. This process led to the compressed air requirements. Baffles on the walls of the vessels prevented forming a vortex in the liquid. In the year 1934, Strauch and Schmidt patented a system in which the aeration tubes were introduced with water and steam for cleaning and sterilization
The decision to use submerged culture technique for penicillin production, where aseptic conditions, good aeration and agitation were essential, was probably a very important factor in forcing the development of carefully designed and purpose-built fermentation vessels.
In 1943, when the British Govt. decided that surface culture was inadequate, none of the fermentation plants were immediately suitable for deep fermentation. The first pilot fermentor was erected in India at Hindustan Antibiotic Ltd., Pimpri, Pune in the year 1950.

Design of Fermentors:

All bioreactors deal with heterogeneous systems dealing with two or more phases, e.g., liquid, gas, solid. Therefore, optimal conditions for fermentation necessitate efficient transfer of mass, heat and momentum from one phase to the other. Chemical engineering principles are employed for design and operation of bioreactors.

A bioreactor should provide for the following:

(i) Agitation (for mixing of cells and medium),
(ii) Aeration (aerobic fermentors); for O_2 supply,
(iii) Regulation of factors like temperature, pH, pressure, aeration, nutrient feeding, liquid level etc.,
(iv) Sterilization and maintenance of sterility, and
(v) Withdrawal of cells/medium (for continuous fermentors).
Modern fermentors are usually integrated with computers for efficient process monitoring, data acquisition, etc.
Generally, 20-25% of fermentor volume is left unfilled with medium as "head space" to allow for splashing, foaming and aeration. The fermentor design varies greatly depending on the type and the fermentation for which it is used. Bioreactors are so designed that they provide the best possible growth and biosynthesis for industrially important cultures and allow ease of manipulation for all operations.

Size of Fermentors:

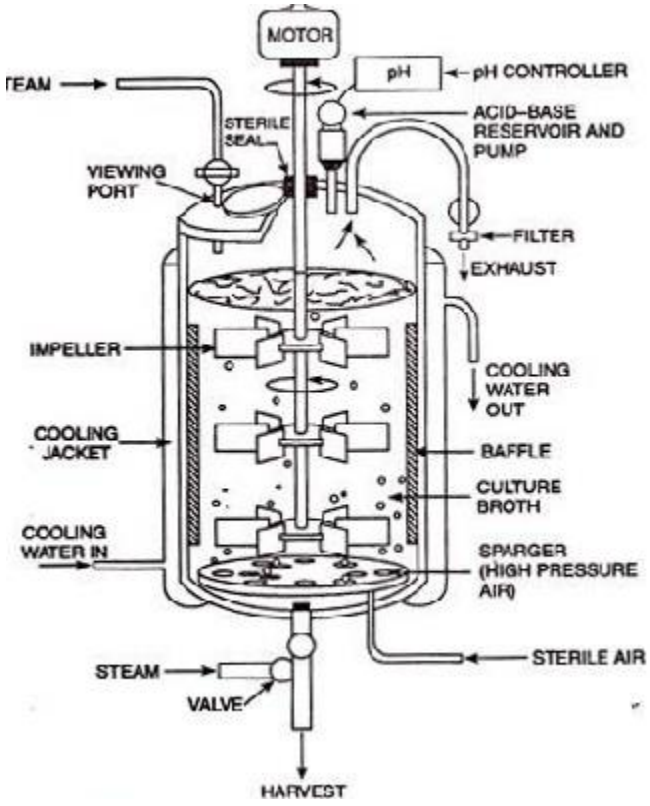
The size of fermentors ranges from 1-2 litre laboratory fementors to 5,00,000 litre or, occasionally, even more, fermentors of upto 1.2 million litres have been used. The size of the fermentor used depends on the process and how it is operated.

Construction of Fermentors:

Industrial fermentors can be divided into two major classes, anaerobic and aerobic. Anaerobic fermentors require little special equipment except for removal of heat generated during the fermentation process, whereas aerobic fermentors require much more elaborate equipment to ensure that mixing and adequate aeration are achieved.

Since most industrial fermentation process are aerobic, the construction of a typical aerobic fermentor is given below

DIAGRAMATIC DESIGN OF A FERMENTOR WITH CONTROLS



1. Cooling Jacket:

Large-scale industrial fermentors are almost always constructed of stainless steel. A fermentor is a large cylinder closed at the top and the bottom and various pipes and valves are fitted into it. The fermentor is fitted externally with a cooling jacket through which steam (for sterilization) or cooling water (for cooling) is run.

Cooling jacket is necessary because sterilization of the nutrient medium and removal of the heat generated are obligatory for successful completion of the fermentation in the fermentor. For very large fermentors, insufficient heat transfer takes place through the jacket and therefore, internal coils are provided through which either steam or cooling water is run.

2. Aeration System:

Aeration system is one of the most critical part of a fermentor. In a fermentor with a high microbial population density, there is a tremendous oxygen demand by the culture, but oxygen being poorly soluble in water hardly transfers rapidly throughout the growth medium.

It is necessary, therefore, that elaborate precautions are taken using a good aeration system to ensure proper aeration an oxygen availability throughout the culture. However, two separate aeration devices are used to ensure proper aeration in fermentor. These devices are sparger and impeller.

The sparger is typically just a series of holes in a metal ring or a nozzle through which filter-sterilized air (or oxygen-enriched air) passes into the fermentor under high pressure. The air enters the fermentor as a series of tiny bubbles from which the oxygen passes by diffusion into the liquid culture medium.

The impeller (also called agitator) is an agitating device necessary for stirring of the fermenter.

The stirring accomplishes two things:

(i) It mixes the gas bubbles through the liquid culture medium and

(ii) It mixes the microbial cells through the liquid culture medium. In this way, the stirring ensures uniform access of microbial cells to the nutrients.

The size and position of the impeller in the fermentor depends upon the size of the fermentor. In tall fermentors, more than one impeller is needed if adequate aeration and agitation is to be obtained. Ideally, the impeller should be 1/3 of the fermentors diameter fitted above the base of the fermentor. The number of impeller may vary from size to size to the fermentor.

3. Baffles:

The baffles are normally incorporated into fermentors of all sizes to prevent a vortex and to improve aeration efficiency. They are metal strips roughly one-tenth of the fermentors diameter and attached radially to the walls.

4. Controlling Devices for Environmental Factors:

In any microbial fermentation, it is necessary not only to measure growth and product formation but also to control the process by altering environmental parameters as the process proceeds. For this purpose, various devices are used in a fermentor. Environmental factors that are frequently controlled includes temperature, oxygen concentration, pH, cells mass, levels of key nutrients, and product concentration.

Use of Computer in Fermentor:

Computer technology has produced a remarkable impact in fermentation work in recent years and the computers are used to model fermentation processes in industrial fermentors. Integration of computers into fermentation systems is based on the computers capacity for process monitoring, data acquisition, data storage, and error-detection.

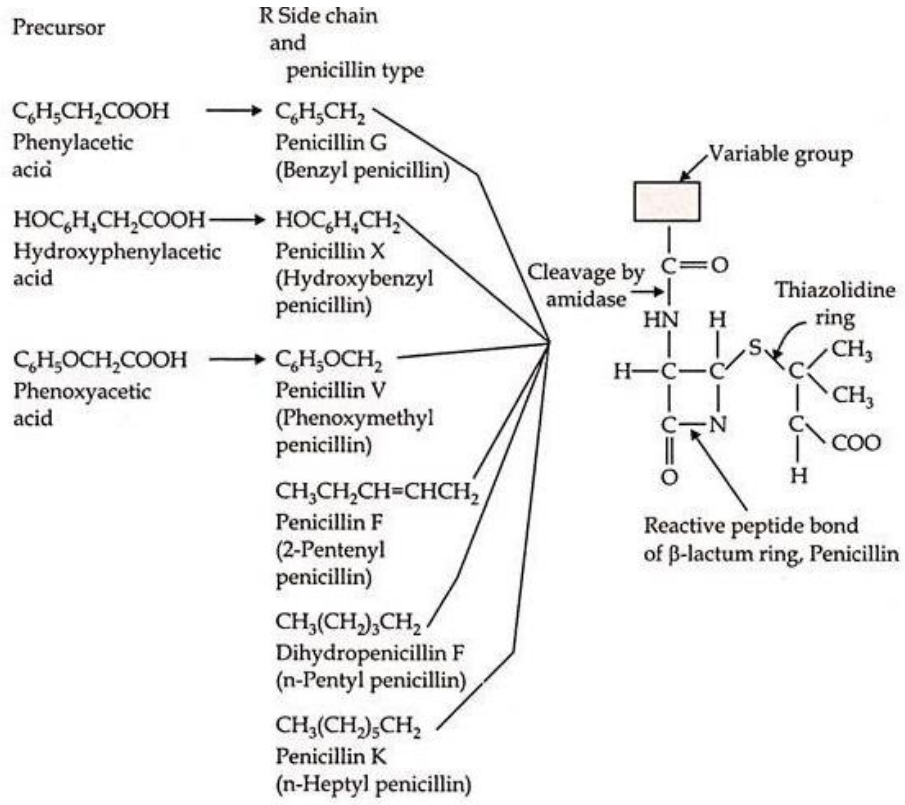
Some typical, on-line data analysis functions include the acquisition measurements, verification of data, filtering, unit conversion, calculations of indirect measurements, differential integration calculations of estimated variables, data reduction, tabulation of results, graphical presentation of results, process stimulation and storage of data.

5c. Fermentative Production of Penicillin,Citric acid,Vitamin.B12 and Glutamic acid.

Penicillin

Chemically the natural penicillin is 6-amino penicillanic acid (6 – APA), which consists of thiazolidine ring with a condensed β-lactum ring. The various penicillins differ primarily in the nature of R-side chain which are attached by an amido linkage to the chemical nucleus of the molecule. Fleming's original Penicillium notatum strain, when grown on his medium produced penicillin-F, which is known as 2-pentinyl penicillin.

Subsequently P. chrysogenum proved to be better fungus and more suitable for submerged fermentation. The basic structure of penicillin and different types of natural penicillin's differing in the composition of side chain are shown in Fig. below

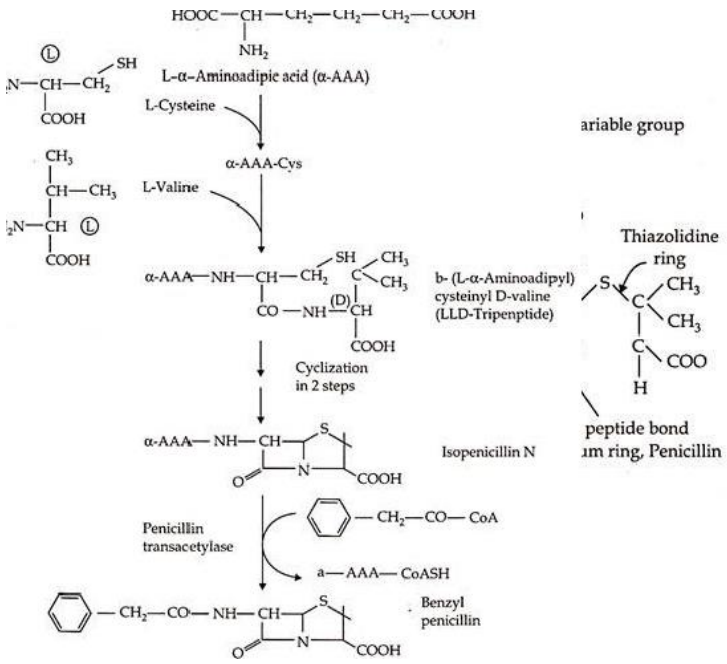


If penicillin fermentation is carried out without the addition of side chain precursor, the natural penicillins are formed from which only benzyl penicillin can be isolated. However, the desired penicillin can be obtained by adding suitable side chain precursor into the medium. Such penicillins are called as semi-synthetic penicillins.

Penicillin-G and Penicillin-V are generally produced commercially. When compared to natural penicillins, semisynthetic penicillins have improved characters viz, acid stability, resistance to plasmid or chromosomally coded β-lactamases, expanded antimicrobial effectiveness and are therefore, extensively used in therapy.

Biosynthesis of Penicillin:

The β-lactum thiazolidine ring of penicillin is formed by the condensation of L-cystine and L-valine. The biosynthesis occurs in a non-ribosomal process by means of dipeptide composed of (α – α – AAA) and α-cystine or a breakdown product of cystothiamine. Subsequently L-valine is connected via epimerization reaction resulting in the formation of tripeptide. The first product of cyclization of the tripeptide which can be isolated is isopenicillin N but the biochemical reactions leading to this intermediate is not understood. Benzyl penicillin is produced in exchange of α -α-AAA with activated phenylacetic acid



Organism

A high yielding strain of *Penicillium chrysogenum*. A strain of the fungus is sub-cultured from stock culture for inoculum development. Spores from primary source are suspended in water or in a dilute solution of a nontoxic wetting agent such as 1:10000 sodium lauryl sulfate. The spores are then added to flasks or bottles of wheat bran plus nutrient solution and these are incubated for five to seven days at 24°C so as to provide heavy sporulation. The entire process is repeated several times in order to have more sporulation. The resulting inoculum which is employed in a production tank is tested both by microscopic examination and by sub-culturing method. Many sporulation media have been designed to obtain large number of spores. The one developed by Moyer and Coghill (1946) is most extensively used and given below.

Composition of Moyer and Coghill (1946) sporulation medium

Component	Concentrating (gl^{-1})
Glycerol	7.5
Cane molasses	7.5
Corn steep liquor	2.5
Mg SO_4. $7H_2O$	2.5
KH_2PO_4	0.050
Peptone	0.060
NaCl	5.00
Fe-tartarate	0.005
$CuSO_4.5H_2O$	0.004
Agar	2.50
Distilled water	1.0

Inoculation:

Introduction of pure inoculum into the production tanks or fermenters is called as inoculation.

This is done by any one of the following three methods:

1. Dry Spores may be used as Inoculum:

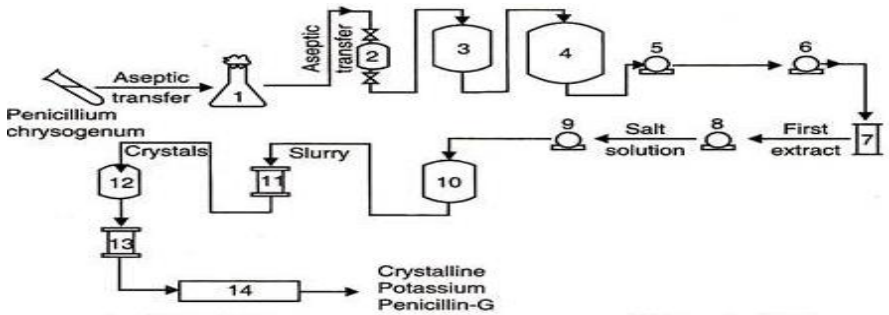
Since the spores of P. chrysogenum are hydrophobic, either spores are blown deep into the medium or a wetting agent such as sodium lauryl sulphate is used.

2. Suspension of Ungerminated Spores:

This suspension is made by using 1:10000 sodium lauryl sulfate solution. This suspension is fed to the fermenter by suitable techniques like spray guns or pipettes. This is followed by agitation and aeration of the fermentation medium in order to achieve equal and uniform distribution of the spores in the entire medium.

3. Feeding the fermentation tanks with pre-germinated spores or mycelial pellets which are prepared by the germination of spores. Pellets are generally fed to the fermentation medium after two or three days of spore inoculation.

Fermenters with a capacity of 40,000 to 2 lakhs liters are generally employed for the production of penicillin. Due to difficulties with the oxygen supply larger tanks are not employed. Some manufacturer's use of Waldh of fermenters or air lift fermenters, but this is only possible in mutants which generate low viscosity. Depending upon the production strain, the operational temperature is maintained between 25°-27°C. A typical flow chart for penicillin production is given below



1. Shaker flask
2. Transfer vessel
3. Seed tank
4. Fermentation tank
5. Rotary filter
6. First extractor
7. Purification column
8. Second extractor
9. Third extractor
10. Vacuum still
11. Ceramic filter
12. Crystal wash
13. Ceramic filter
14. Vacuum drier

(ii) Medium:

The medium employed for penicillin production should be suitable to achieve:

1. An abundant growth of the mycelium.
2. Maximum accumulation of the antibiotic.
3. Easy and inexpensive extraction and purification of the antibiotic.

A medium designed by Jackson (1958) which has the following composition, is generally used in fermentative production of penicillin

Component	Concentration (In Percentage)
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 is in the range of 3 to 5% which largely depends upon the amount of carbohydrate consumed during fermentation process, which is approximately equal to 1500 international units per milliliter. Sylvester and Coghill (1954) have estimated that to produce 1000 gallons of fermented culture, which is capable of yielding 2.2-2.7 kg of penicillin by the submerged culture method requires approximately 227 kg of nutrients, 3400 kg of steam, 45460 lt of water, 1000 kWh of electricity and 7075 m^3 of air.

In the typical penicillin fermentation there is a growth of 10 hrs duration with a doubling time of 6 hrs during which the greater part of the cell mass is formed. The oxygen supply in the growing culture is critical since the increasing viscosity hinders oxygen transfer. After 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 hrs. Penicillin production by continuous fermentation has been attempted but it has been difficult due to instability of the production strains. A batch fill and draw system has been suggested as an alternative. In this process 20-40% of the fermentation contents is drawn off and replaced with fresh nutrient solution. This process may be repeated up to 10 without affecting yield.

Extraction and Purification:

After it is assessed that sufficient amount of penicillin has been produced during fermentation process, it is extracted and then purified.

The entire process is carried out in three different stages.

They are:

(a) Separation of mycelium

(b) Extraction of penicillin and

(c) Treatment of crude extract

(a) Separation of Mycelium:

Mycelium is separated from the medium by employing rotatory vacuum filter. This process should be performed carefully in order to avoid contaminating microorganisms which produce penicillinase enzyme, degrading the penicillin.

(b) Extraction of Penicillin:

The penicillin is excreted into the medium and less than 1% remains as mycelium bound. Extraction of penicillin is carried out by employing counter current extraction method. The pH of the liquid after separation of the mycelium is adjusted to 2.0 to 2.5 by adding phosphoric or sulphuric acid. This treatment converts penicillin into anionic form.

The liquid is immediately extracted with an organic solvent such as amylacetate or butylacetate or methyl isobutyl ketone. This step has to be carried out quickly because penicillin is quite unstable at low pH values. Podbielniak counter current extractor is used for this purpose. The penicillin is then back extracted into water from the organic solvent by adding enough potassium or sodium hydroxide which also results in the elevation of pH to 7.0 to 7.5.

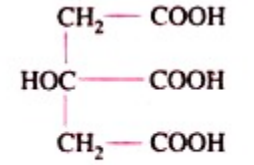
The resulting aqueous solution is again acidified and re-extracted with organic solvent. These shifts between the water and the solvent help in the purification of the penicillin. Finally, the penicillin is obtained in the form of sodium penicillin. The spent solvent is recovered by distillation for reuse.

(c) Treatment of Crude Extract:

The resulted sodium penicillin is treated with charcoal to remove pyrogens (fever causing substances). It is also, sometimes, sterilized to remove bacteria by using Seitz filter. Then, the sodium penicillin is prepared in crystalline form by crystallization. It may be packed as powder in sterile vials or prepared in the form of tablets or in the form of syrups for oral usage. The pharmaceutical grade may be used in the production of semi synthetic penicillin.

Citric Acid

Citric acid was first produced commercially by John and Edmund Storage Company in UK in the year 1826. Scheele (1789) reported the isolation and crystallization of the four constituents of lemon juice. Grimoux and Adams (1880) synthesized citric acid from glycerol. Wehmner (1893) observed the occurrence of citric acid as a microbial product by using Penicillium and Citromyces.It was Millard (1922) who recorded accumulation of citric acid in culture of Aspergillus niger under condition of nutrition deficiency. Meanwhile, Currie (1917) reported better yield while using A. niger. In 1923, Pfizer began operating fermentation based process in USA. The Chemical structure of citric acid is given below



Citric acid (2-hydroxy-1,2, 3 propane tricarboxylic acid)

Fermentation of Citric Acid

Aspergillus niger has been the choice for the production of this primary metabolite citric acid for several decades. A large number of other microorganisms (fungi and yeast) such as Aspergillus davatus, A. wentii, Penicillium luteum, P. citrinum, Mucor pyriforms, Candida lipolytica, C. oleophila, C. guillermondis, Hensenulaspp. Torulopsis spp., Pichiaspp., Debaromyces daussenii etc. have also been used for citric acid production in industries.

The advantages of using yeast, rather than A. niger are the possibility of using very high initial sugar concentration together with a much faster fermentations. This combination gives a high productivity run to which must be added the reported insensitivity of the fermentation to variations in the heavy metal content of the crude carbohydrates.

From 1965 onwards, yeasts are used for citric acid production using carbohydrate and n- alkanes. In all the processes, a variety of carbohydrates such as beet molasses, cane molasses, sucrose, commercial glucose, starch hydrolysate etc. used in fermentation medium.

The starchy raw material is diluted to obtain 20-25% sugar concentration and mixed with a nitrogen source (ammonium salts or urea) and other salts. The pH of the medium is kept around 5 when molasses is used and at pH 3 when sucrose used.

The fermentation is carried out by any of the processes:

(a) Kofi process or solid state fermentation:

It is a Japanese process in which special strains of *Aspergillus niger* are used with the solid substrate such as sweet potato starch.

(b) Liquid surface culture process:

In this case, *A. niger* floats on the surface of a solution.

(c) Submerged fermentation process:

It is the process in which the fungal mycelium grows throughout a solution in a deep tank.

(a) Koji process:

Mold is used in the preparation called Koji to which wheat bran was substituted in the sweet potato material. The pH of the bran is adjusted between 4 and 5, and additional moisture is picked up during steaming so as to get the water content of the mash around 70-80%.

After cooling the bran to 30-60°C, the mass is inoculated with a koji which was made by a special strain of *A. niger* which is probably not as possible to the presence of ions of iron as the culture strains used in other process.

Since bran contains starch which on saccharification by the amylase enzyme of A. niger induces citric acid production. The bran after inoculation, is spread in trays to a depth of 3-5 cm and kept for incubation at 25-30°C. After 5-8 days, the koji is harvested and citric acid is extracted with water.

(b) Liquid surface culture process:

In this case aluminium or stainless steel shallow pans (5-20 cms deep) or trays are used. The sterilized medium usually contains molasses and salts. The fermentation is carried out by blowing the spores of A. niger over the surface of the solution for 5-6 days, after which dry air is used.

Spore germination occurs within 24 hours and a white mycelium grows over the surface of the solution, eight or ten days after inoculation, the initial sugar concentration (20-25%) reduced to the range of 1-3%.

The liquid can be drained off and any portion of mycelial mat left becomes submerged and inactivated. The small quantity of citric acid is produced during the growth phase. This is called primary metabolite. The mycelium can also be reused.

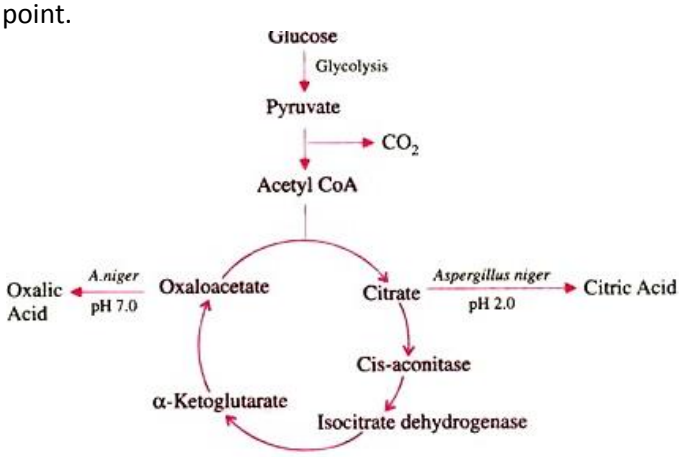
During the preparation of fermentable sugar from molasses, sucrose is the main carbohydrate along with some glucose as well as protein, peptide, amino acids, and inorganic ions. This is to be subjected to heat; so it contains saccharic acids and related compounds in traces.

The initial sugar concentration is about 20-25%. The removal of metallic ions or reduction in quantity of undesirable ions in sucrose syrup by adsorption with a combination of $CaCO_3$, colloidal silica, tricalcium phosphate and starch are other important steps, The iron is also precipitated by addition of calcium ferrocyanide.

Initially, the pH remains in the range of 5-6, but on spore germination, pH approaches the range of 1.5-2 as ammonium ions are removed from the solution. It is important to mention that at initial pH of 3-5 some oxalic acid is also produced.

The presence of iron also favours oxalic acid production, and of yellow or yellow green pigments in the mycelium sometimes secreted into the culture solution and is difficult to remove during product

recovery and purification. The Biosynthetic pathway of Citric acid is given below to understand above point.



(c) Submerged culture process

This process is quite economical. In this case, the organism (*Aspergillus japonicus*) which is a black Aspergillus is slowly bubbled in a steam of air through a culture solution of 15 cm depth. Since the organism shows subsurface growth and produces citric acid in the culture solution, the yields are inferior in comparison to liquid surface culture fermentation.

The earlier workers used shaking culture and extracted Mollard's phosphate deficiency concept to submerged fermentation. But they could not realize the role of metallic ions which are commonly occurring as impurities in phosphate salts. Aeration is required for the continuous fermentation.

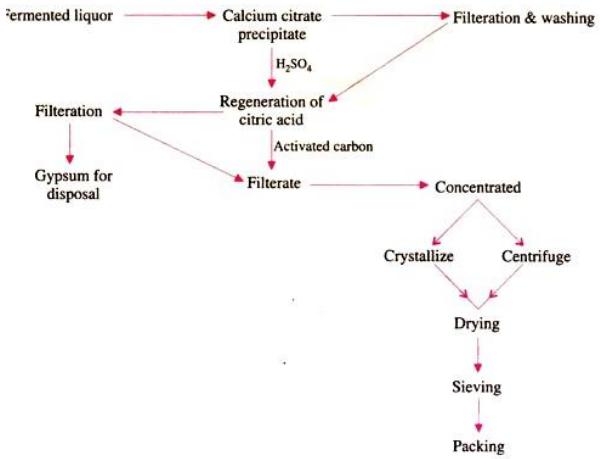
The addition of copper ion is must to ensure that the new growth is of the right biochemical type. The antifoam agents are necessary. Such agents must be free of iron, cobalt or nickel. Continuous culture techniques are not considered suitable for use in citric acid product.

Recovery of Citric Acid

The culture filtrate used to be hazy due to the presence of residual antifoam agents, mycelia and oxalate. The $Ca(OH_2)$ slurry is added to precipitate calcium citrate. After filtrations the filterate is transferred and treated with H_2SO_4 to precipitate Ca as $CaSO_4$.

This is subjected to the treatment with activated carbon. It is demineralized by successive passages through ion exchange beds and the purified solution is evaporated in a circulating granulator or in a circulating crystallizers.

The crystals are removed by centrifugation. The remaining mother liquor is returned to the recovery stream. The solvent extraction can also be performed by adding 100 parts tri-n-butyl phosphate and 5-30 parts n-butyl acetate or methyl isobutyl ketone which are to be mixed with the filterate. The solvent is then extracted with water at 70-90°C. Citric acid is further concentrated, decolorized and crystallized. The process can be better understood by the flowchart given below

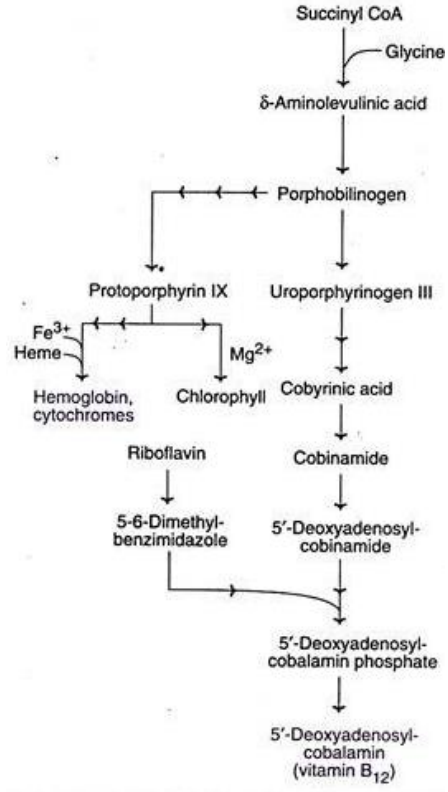


Vitamin B12

Vitamin B12 (cyanocobalamin) is a water soluble vitamin with complex structure. The empirical formula of cyanocobalamin is C63H90N14O14PCO. The structure of vitamin B12 consists of a corrin ring with a central cobalt atom.

Biosynthesis:

Vitamin B_{12} is exclusively synthesized in nature by microorganisms. An outline of the pathway is depicted in the dollowing figure. The biosynthesis of B12 is comparable with that of chlorophyll and hemoglobin. Many of the reactions in the synthesis of vitamin B12 are not yet fully understood.



Commercial Production of Vitamin B_{12}

Vitamin B_{12} is commercially produced by fermentation. It was first obtained as a byproduct of Streptomyces fermentation in the production of certain antibiotics (streptomycin, chloramphenicol, or neomycin). But the yield was very low. Later, high-yielding strains were developed. And at present, vitamin B_{12} is entirely produced by fermentation. It is estimated that the world's annual production of vitamin B_{12} is around 15,000 kg.

High concentrations of vitamin B_{12} are detected in sewage-sludge solids. This is produced by microorganisms. Recovery of vitamin B_{12} from sewage-sludge was carried out in some parts of United States. Unlike most other vitamins, the chemical synthesis of vitamin B_{12} is not practicable, since about 20 complicated reaction steps need to be carried out. Fermentation of vitamin B_{12} is the only choice.

Microorganisms and Yields of Vitamin B_{12}:

Several microorganisms can be employed for the production of vitamin B_{12}, with varying yields. The most commonly used microorganisms are — *Propionibacterium freudenreichii, Pseudomonas denitrificans, Bacillus megaterium and Streptomyces olivaceus.*

Carbon Sources for Vitamin B_{12} Production:

Glucose is the most commonly used carbon source for large scale manufacture of vitamin B_{12}. Other carbon sources like alcohols (methanol, ethanol, isopropanol) and hydrocarbons (alkanes, decane, hexadecane) with varying yields can also be used. A yield of 42 mg/l of vitamin B_{12} was reported using methanol as the carbon source by the microorganism Methanosarcina barkeri, in fed- batch culture system.

Production of Vitamin B_{12} Using Propionibacterium sp

Propionibacterium freudenreichii and P. shermanii, and their mutant strains are commonly used for vitamin B_{12} production. The process is carried out by adding cobalt in two phases.

Anaerobic phase:

This is a preliminary phase that may take 2-4 days. In the anaerobic phase 5'-deoxyadenosylcobinamide is predominantly produced.

Aerobic phase:

In this phase, 5, 6-dimethyl- Benz imidazole is produced from riboflavin which gets incorporated to finally form coenzyme of vitamin B-p namely 5'-deoxyadenosylcobalamin.

In recent years, some fermentation technologists have successfully clubbed both an anaerobic and aerobic phases to carry out the operation continuously in two reaction tanks.

The bulk production of vitamin B_{12} is mostly done by submerged bacterial fermentation with beet molasses medium supplemented with cobalt chloride. The specific details of the process are kept as a guarded secret by the companies.

Production of Vitamin B_{12} using Pseudomonas sp

Pseudomonas denitrificans is also used for large scale production of vitamin B_{12} in a cost-effective manner. Starting with a low yield (0.6 mg/l) two decades ago, several improvements have been made in the strains of P. denitrificans for a tremendous improvement in the yield (60 mg/l). Addition of cobalt and 5, 6-dimethyl Benz imidazole to the medium is essential. The yield of vitamin B_{12} increases when the medium is supplemented with betaine (usual source being sugar beet molasses).

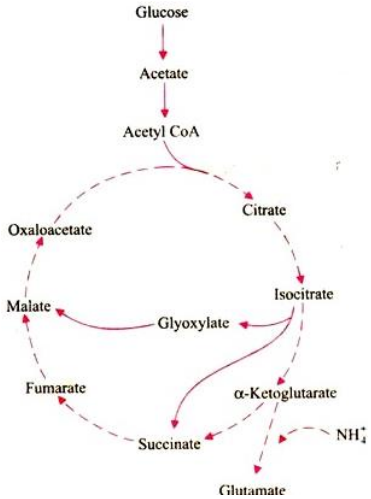
Recovery of vitamin B_{12}:

The cobalamins produced by fermentation are mostly bound to the cells. They can be solubilized by heat treatment at 80-120°C for about 30 minutes at pH 6.5-8.5. The solids and mycelium are filtered or centrifuged and the fermentation broth collected. The cobalamins can be converted to more stable cyanocobalamins. This vitamin B_{12} is around 80% purity and can be directly used as a feed additive. However, for medical use (particularly for treatment of pernicious anemia), vitamin B_{12} should be further purified (95-98% purity).

L-Glutamic Acid

L-glutamic acid ($C_5H_9O_4N$) is an amino acid and is used as a flavor enhancer in the form of Monosodium glutarate and also used for tenderization of meat. Kinosita et al. (1957) observed that L-glutamic acid ($C_5H_9O_4N$) is produced by using bacterial isolate, *Micrococcus glutamicus (syn: Corynebacterium glutamicum). Some microorganisms such as Corynebacterium herculis, C. lilium, Arthrobacter globiformis, Micro bacterium salicinovorum, Brevibacterium divaricatum, B. amino-genes, B.flavum, Bacillus megaterium* are other glutamic acid producing species.

Biosynthesis of L-Glutamic acid



(i) Fermentation

The medium contains glucose (121g), ammonium acetate (5g), molasses (6g), potassium hydrogen phosphate (1.2 g), potassium sulphate (6 µg), manganese sulphate (6 µg), and antifoam agent (0.1 ml) in one litre distilled water. The size of the inoculum remain 6 percent. The fermentative organism is Brevibacterium divaricatum (NRRL B-231).

The incubation was carried out for 16 hours at 35°C. At the beginning of the fermentations 0.65 ml per litre of olive oil is added. The pH is set at 8.5 with ammonia and is automatically maintained at 7.8 during fermentation. After growth of the culture (about 14 h), the temperature is increased from 32-33°C to 38°C. The glucose feeding is done until the fermentation is completed.

(ii) Recovery:

The glutamic acid content is analysed hourly. The fermentation is stopped after 30-35 hours with a yield of 100 g per litre. If molasses from starch saccharification is substituted for glucose, the glutamic acid yield is 94 g per litre after 36 hours.

5d. Collection Processing and Storage of Whole Human Blood, Dried Human Plasma and Plasma substitutes.

Whole Human Blood

Blood should be collected only by a licensed blood bank. Blood should be drawn from the donor by a qualified physician or under his/her supervision by assistants trained in the procedure. A physician should be present on the premises when the blood is being collected. Blood should be collected by single venepuncture and flow of blood should be continuous.The blood donor area should be clean, congenial, comfortable and conveniently approachable. As the temperatures vary widely in different seasons, it is mandatory to have air-conditioned rooms to make the donor comfortable and to minimise chances of contamination.

METHOD

A strict standardised procedure should be in use to achieve surgical cleanliness for preparing venepuncture site to provide maximum possible assurance of sterile product.

EQUIPMENT

The blood bags for collection of blood should be sterile, pyrogen free and disposable, with a closed system of collection as per standards provided by ISO / ISI. Multiple interconnected plastic bags should be used for blood component preparation (closed system). Venting of any container should be done under laminar airflow bench and such container should be used within 24 hours. To avoid venting in case of paediatric use, multiple inter-connected closed containers should be used.

ANTICOAGULANT SOLUTIONS

The anticoagulant solution should be sterile and pyrogen free. One of the following solutions should be used in the indicated volumes.

1 Citrate-Phosphate-Dextrose (CPD) Solution. 14 ml solution is required for 100 ml of blood.

2 Citrate-Phosphate-Dextrose-Adenine (CPD1) solution. 14 ml solution is required for 100 ml of blood.

3 100 ml SAM/ADSOL or any approved additive solution containing saline adenine and glucose (or with mannitol) is added to packed cells after separation of plasma for storage.

VOLUME OF BLOOD

Volume of blood collected should be proportionate to the volume of anti-coagulant, with ±10% variation and should not exceed 10 ml/kg body weight limited to a volume of 500 ml. Units of blood where volume collected is out of the permitted limits should not be used for transfusion. No attempt should be made to collect blood from such donor during the same session.

SAMPLES FOR LABORATORY TESTS

The blood samples in the pilot tubes (clotted and anticoagulated) should be collected at the time of collection of blood by the same person who collects blood. They should be marked before collection to be identified with the unit of blood. The integral donor tubing of plastic bag should be filled with anticoagulated blood and sealed in such a manner that it will be available with segment numbers for traceability for subsequent compatibility tests.

IDENTIFICATION

Each container of blood/blood components /pilot tubes should be identified by a numeric or alpha numeric at the time of collection of blood, so that it can be traced back to the donor and also to the recipient. The segment number printed on the integral donor tubing should be recorded.

STORAGE

Immediately after collection, the blood should be placed at 40C to 60C ± 2®C .

Dried Human Plasma

Preparation

Plasma is a Yellow coloured fluid and consists of 55% blood. This is used for Plasma transfusion. The preparation of dried human plasma starts with centrifugation of 400ml Whole Human blood collected from an eligible donor and packed in sterile, Bacteria proof container, at 18 degree Celsius to separate the serum. This is followed by **Primary drying** in chamber where the horizontally placed bottles are dried at 50 degree Celsius. Primary drying takes about 2 weeks. This is followed by **Secondary drying** in a Vacuum or in a desiccators for one day. After completion of this drying the residual moisture should be 0.5%.

Storage

The dried human Plasma should be stored at 28 degree Celsius,protected from moisture and Sunlight, where it is stable for 5 years.

Plasma substitutes

Plasma substitutes are already a part of Unit3g and their details their details are already presented in Unit 3g

BPH 605T Pharmaceutical Biotecnology Unit: Immunology No.: 3C,3D.3E.3F &3G

3C.TYPES OF HYPERSENTIVITY

Feature	Hypersensitivity Type			
	Type I	Type II	Type III	Type IV
Nature of antigen	Soluble	Cell surface-bound	Soluble	Soluble
Antibody (Ab) involved	IgE	IgG	IgM	Nil
Type of immune response	Humoral	Humoral	Humoral	Cellular
Effector molecules	Histamine and other biologically active molecules	Membrane-attack complex, complements C3a, C4a and C5a	Neutrophils	Various cytokines secreted by activated Tc cells
Mechanism	B cells ↓ Activated by allergen Secrete IgE ↓ IgE binds to Fc receptors on mast cells and blood basophils ↓ *Second exposure to allergen* Allergen cross-links IgE ↓ Activated mast cells and blood basophils secrete vasoactive amines ↓ • Smooth muscle contraction • Increased vascular permeability • Vasodialation	B cells ↓ Activated by antigen Secrete IgG ↓ IgG binds cell surface-bound antigen ↓ • Activation of Tc cells or • Activation of complement system ↓ • Tc cells secrete various cytokines leading ultimately to cell death • Activated complement system leads to cell death	B cells ↓ Antigen activation Secret IgM ↓ IgM interacts with antigen to form immune complex ↓ Activation of complement system ↓ Inflammatory response; massive infiltration by neutrophils	T_H1 cells ↓ Antigen activation Secrete cytokines ↓ Activation of macrophages and Tc cells ↓ • Phagocytosis by macrophages • Various cytokines secreted by Tc cells ↓ Cellular damage
Examples	Systemic anaphylaxis; localized anaphylaxis; *e.g.*, hay fever, asthma, hives, food allergies	Blood transfusion reactions, erythroblastosis foetalis, autoimmune haemolytic anaemia	Serum sickness, rheumatoid arthritis, systemic lupus erythmatus	Contact dermatitis, graft rejection, tubercular lesions

3D. IMMUNE STIMULATION AND IMMUNE SUPPRESSION

1.IMMUNE STIMULATION

Immune stimulation refers to stimulation of Immune system by an external source. The stimulation offers protection against infections and cancer. Our Immune system can be stimulated by administration of Antigens, Adjuvants and Endogenous substances like Female Sex Hormones. The Antigens are administered in the form of a Vaccine. Antigens produce specific Immune stimulation, while Adjuvants

and endogenous substances produce non specific Immune stimulation. Apart from these substances some chemicals (e.g. Deoxy Cholic Acid), Probiotics like certain Lactobacillus bacteria and some herbs possess Immuno stimulant properٹy.

2.IMMUNE SUPPRESSION

Immune suppression orImmunosuppression is a reduction of the activation or efficacy of the immune system. Some portions of the immune system itself have immunosuppressive effects on other parts of the immune system, and immunosuppression may occur as an adverse reaction to treatment of other conditions.

In general, deliberately induced immunosuppression is performed to prevent the body from rejecting an organ transplant. Additionally, it is used for treating graft-versus-host disease after a bone marrow transplant, or for the treatment of auto-immune diseases such as systemic lupus erythematosus, rheumatoid arthritis, Sjögren's syndrome, or Crohn's disease. This is typically done using medications, but may involve surgery plasmapheresis or radiation. A person who is undergoing immunosuppression, or whose immune system is weak for some other reasons (chemotherapy or HIV), is said to be immunocompromised.

3E.Preparation of Vaccines,Antitoxins,Toxoids

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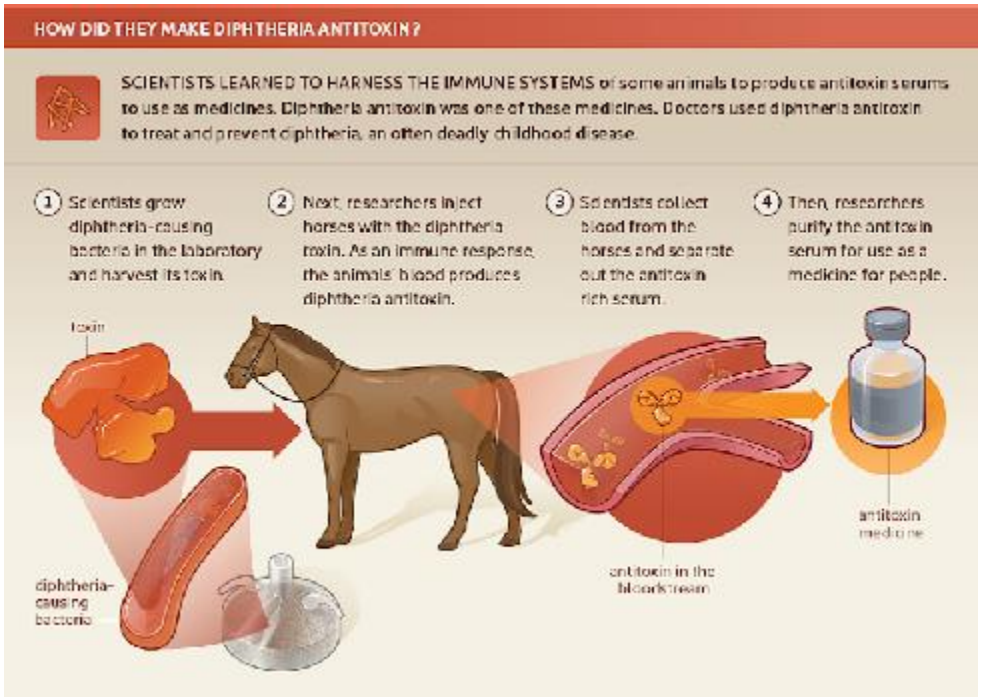
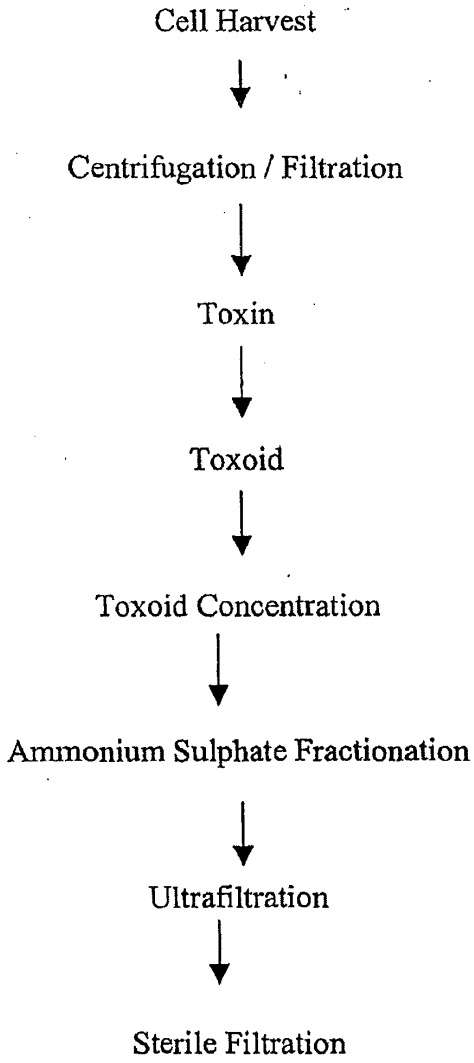


The above material is the pictorial presentation of preparation of Antitoxin for your better understanding.

Antitoxins are Antibodies. Antitoxin. as an immulogical product contains antibodies for toxins. Antitoxins were used against Diptheria, Tetanus toxins. Antitoxins are produced by injection of sub lethal doses of toxins to animals like horse,so as to develop antibodies . The blood is collected and processed to separate antibodies. The separated antibodies are then packed suitably. Antitoxins offer passive immunity, which has shorter life span than Active immnity offered by Vaccines. Antitoxins were also. Used in Scarlet fever.
For more details,refer Cooper and Gunn's Tutorial Pharmacy,Edited by S. J. Carter

METHOD OF PREPARATION OF TOXOIDS

A **toxoid** is an inactivated toxin (usually an exotoxin) whose toxicity has been suppressed either by chemical (formalin) or heat treatment, while other properties, typically immunogenicity, are maintained. Toxins are secreted by bacteria, whereas toxoids are altered form of toxins; toxoids are *not* secreted by bacteria. Thus, when used during vaccination, an immune response is mounted and immunological memory is formed against the molecular markers of the toxoid without resulting in toxin-induced illness. Such a preparation is also known as an **anatoxin**. There are toxoids for prevention of diphtheria, tetanus and botulism. The following flow chart outlines the preparation of Toxoids.



3E. Storage conditions and stability of official vaccines

the words "official vaccines" means the Vaccines which have monographs in various Pharmacopoeias. Generally the storage conditions are prescribed in the monograph of the vaccine. Some vaccines are processed as a Lyophilised (Freeze dried) powder,while many are processed as liquids. Both forms of vaccines are packed in small volume containers(e.g. Ampoules,Vials) and stored in refrigerator at a temperature range 2-8 degree Celsius,for their stability. While exposure outside this temperature range may destabilize the vaccine,which results in loss of desired activity.

3F.HYBRIDOMA TECHNOLOGY

Introduction

In **1975** George Kohler and Cesar Milstein, working at the MRC Laboratory of Molecular Biology in Cambridge, found a way of mimicking the effect to produce monoclonal antibodies "to order". They did it by merging myeloma cells – cancerous cells resulting from the uncontrolled cells division resulting from a lymphocyte dividing to form a clone of identical cells – with antibody-producing B cells. By fusing the B cell with the myeloma cell, it acquires the ability to divide rapidly, allowing large numbers of identical antibody producing cells to be grown in cell culture.

The system is known as **Hybridoma technology** because it involves cell hybrids to produce sets of identical monoclonal antibodies directed against specific antigens. Kohler and Milstein started out working independently. Milstein had developed cancerous forms of antibody-producing cells that grew and multiplied forever but which churned out antibodies of unknown specificity, while Kohler managed to get antibody-producing cells to make specific antibodies, but these cells didn't survive for very long. By combining their discoveries, they came up with a way of making monoclonal antibodies of exquisite precision from cells that divided and divided and effectively lived forever.

Monoclonal antibodies, or **"MAbs",** have revolutionised immunology in terms of analytic tests and diagnostics, and are now a standard treatment for certain forms of cancer with drugs such as Herceptin (trastuzumab), Avastin (bevacizumab) and Campath (alemtuzumab).

Kohler and Milstein were awarded a share of the 1984 Nobel prize in physiology or medicine for their breakthrough.

Steps involved in Hybridoma Technology

The manufacturing process of MAbs can be done in vivo or in vitro or even it can be a complex of both the process. In hybridoma method hybrid cells are made prior to the manufacture that will give the antibodies of interest.

Step 1: Immunization of Mice and Selection of Mouse Donors for Generation of Hybridoma Cells:

At first the specific antigen was injected into a healthy mice and such antigen was made by the process of emulsification with Freund's adjuvant or it was homogenized with a gel slice. Some other sources of immunogens can be intact cells, entire membranes, and even microorganisms are used times. It a common practice for the researchers to use mice in their respective labs in order to obtain their antibodies of interest. All it takes 14-21 days for the mice to be immunized which may fluctuate from researcher to researcher. When the time comes and the mice serum attains the optimum level of antibody titer, then the spleen is taken out and cells are collected which will be fused with myeloma cells.

Step 2: Screening of Mice for Antibody Production:

At this stage, when few weeks have passed by sample of blood are collected from the immunized mice and the antibody quantity is recorded. Multiple methods can be used to identify the antibody titer and among these methods the ELISA and flow cytometry are the most popular. It is necessary for the titer to be in maximum amount in order for the fusion to be done. A boosting dose may be required in the case of insufficient titer amount. After the boosting dose blood was collected respectively until the enough repercussion is obtained. The boosting dose is also given even if the titer prevails in adequate amount. In this case the animals are treated solely by the antigen but this time no adjuvant will be used. This is done by either through intraperitonial route or IV route and must be performed 3 days prior to fusion but a couple of week followed by the initial antigen introduction. Then lastly the spleens from the immunized mice were collected in order to manufacture hybridoma cells in vitro.

Step 3: Preparation of Myeloma Cells:

The cells that were collected from the spleen having the ability to produce antibody survives for a definite period of time. In stage 3 these cells are fused with immortal tumor of lymphocytes. The resulting product is known as hybridoma and its core characteristic is that it shows indefinite growth. Such tumor cells are known as myeloma cells and such cells are grown with 8-azaguanine and thus it make sure its susceptibility to the hypoxanthine-aminopterin-thymidine(HAT) medium. HAT medium is the culture medium that will be used following the fusion step. The myeloma cells are developed in 8-azaguanine one week prior to fusion. Elevated durability and enhanced development is highly desired from the cells.

Step 4: Fusion of Myeloma Cells with Immune Spleen Cells:

For the production of MAbs by hybridoma technology this step is believed to be the most hardiest and vital of all. It is found that three methods are available for the fusion of cells in other words creating hybridomas. They are 1) using PEG, 2) fusogenic viruses, 3) electrical cytofusion where no. 3 is found to be the most feasible most popular one.

Step 5: Cloning of Hybridoma Cell Lines by "Limiting Dilution" or Expansion and Stabilization of Clones by Ascites Production:

This is the final step in hybridoma method and the purpose of this step is to collect the end product that was obtained by fusion method and also known as biological cloning. In order to obtain hybridoma clones limiting dilution cloning is used. To start this step firstly the cells that were put into the 96 well plates are made to be grown into fresh small groups. After this, sorting for the antigen attachment were done or it can be further cloned by mouse ascites method. The use of "limiting dilution" guarantees that each of the wells is accompanied solely by a single clone. There are some complications too that the resulting antibodies might show toxicity. In order to prevent such phenomenon mouse ascites expansion method can be applied.

Soft agar method:

In this technique, the hybridoma cells are cultured in soft agar. It is possible to simultaneously grow many cells in semisolid medium to form colonies. These colonies will be monoclonal in nature. In actual practice, both the above techniques are combined and used for maximal production of MAbs.

6. Characterization and Storage:

The monoclonal antibody has to be subjected to biochemical and biophysical characterization for the desired specificity. It is also important to elucidate the MAb for the immunoglobulin class or sub-class, the epitope for which it is specific and the number of binding sites it possesses.The stability of the cell lines and the MAbs are important. The cells (and MAbs) must be characterized for their ability to withstand freezing, and thawing. The desired cell lines are frozen in liquid nitrogen at several stages of cloning and culture.

3G. Blood products and plasma substitutes

Plasma substitutes

Dextran 70 and **polygeline** are macromolecular substances which are metabolized slowly; they may be used to expand and maintain blood volume in shock arising from conditions such as burns or septicaemia. They are rarely needed when shock is due to sodium and water depletion as, in these circumstances, the shock responds to water and electrolyte repletion.

Plasma substitutes should not be used to maintain plasma volume in conditions such as burns or peritonitis where there is loss of plasma protein, water and electrolytes over periods of several days. In these situations, plasma or plasma protein fractions containing large amounts of albumin should be given.

Plasma substitutes may be used as an immediate short-term measure to treat massive haemorrhage until blood is available, but large volumes of some plasma substitutes can increase the risk of bleeding by depleting coagulation factors. Dextran may interfere with blood group cross-matching or biochemical measurements and these should be carried out before the infusion is started.

Dextran 70

Dextran is a representative plasma substitute. Various preparations can serve as alternatives

Infusion (Solution for infusion), dextran 70 6% in glucose intravenous infusion 5% or sodium chloride intravenous infusion 0.9%

Uses:

short-term blood volume expansion

Contraindications:

severe congestive heart failure, renal failure; bleeding disorders such as thrombocytopenia and hypofibrinogenaemia

Precautions:

cardiac disease or renal impairment; monitor urine output; avoid haematocrit falling below 25–30%; where possible, monitor central venous pressure; can interfere with blood group cross-matching and biochemical tests—take samples before start of infusion; monitor for hypersensitivity reactions; pregnancy

Dosage:

Short-term blood volume expansion, *by rapid intravenous infusion* , adult 500–1000 ml initially, followed by 500 ml if necessary; total dosage should not exceed 20 ml/kg during the initial 24 hours; if required 10 ml/kg daily may be given for a further 2 days (treatment should not continue for longer than 3 days); child total dosage should

not exceed 20 ml/kg

Adverse effects:

hypersensitivity reactions including fever, nasal congestion, joint pains, urticaria, hypotension, bronchospasm—rarely severe anaphylactoid reactions; transient increase in bleeding time

Polygeline

Polygeline is a representative partially degraded gelatin. Various preparations can serve as alternatives

Infusion (Solution for infusion), polygeline 3.5% with electrolytes, 500-ml bottle

Uses:

correction of low blood volume

Contraindications:

severe congestive heart failure; renal failure

Precautions:

blood samples for cross-matching should be taken before infusion; haemorrhagic diasthesis; congestive heart failure, renal impairment, hypertension, oesophageal varices; **interactions:** Appendix 1

Dosage:

Correction of low blood volume, *by intravenous infusion* , initially 500–1000 ml of a 3.5% solution

Adverse effects:

hypersensitivity reactions including urticaria—rarely severe anaphylactoid reactions; transient increase in bleeding time

Plasma fractions for specific use

Factor VIII is essential for blood clotting and the maintenance of effective haemostasis; von Willebrand factor is a mediator in platelet aggregation and also acts as a carrier for factor VIII. Blood coagulation factors VII, IX, and X are essential for the conversion of factor II (prothrombin) to thrombin. Deficiency in any of these factors results in haemophilia. Bleeding episodes in haemophilia require prompt treatment with replacement therapy. **Factor VIII** , used for the treatment of haemophilia A, is a sterile freeze-dried powder containing the blood coagulation factor VIII fraction prepared from pooled human venous plasma. Standard factor VIII preparations also contain von Willebrand factor and may be used to treat von Willebrand disease. Highly purified preparations, including recombinant factor VIII, are available; they are indicated for the treatment of haemophilia A but do not contain sufficient von Willebrand factor for use in the management of von Willebrand disease.

Factor IX Complex is a sterile freeze-dried concentrate of blood coagulation factors II, VII, IX and X derived from fresh venous plasma. Factor IX complex which is used for the treatment of haemophilia B may also be used for the treatment of bleeding due to deficencies of factor II, VII, and X. High purity preparations of factor IX which do not contain clinically effective amounts of factor II, VII, and X are available. A recombinant factor IX preparation is also available.

Factor VIII concentrate

Plasma fractions should comply with the WHO Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives (Revised 1992). WHO Technical Report Series, No. 840, 1994, Annex 2

Factor VIII concentrate is a complementary preparation and a representative

coagulation factor preparation. Various preparations can serve as alternatives
Infusion (Powder for solution for infusion), factor VIII 250–1500 units
Uses:
control of haemorrhage in haemophilia A
Precautions:
intravascular haemolysis after large or frequently repeated doses in patients with blood groups A, B, or AB (less likely with high potency, highly purified concentrates)
Dosage:
Haemophilia A, *by slow intravenous infusion* , ADULT and CHILD according to patient's needs
Adverse effects:
allergic reactions including chills, fever

Factor IX complex (coagulation factors II, VII, IX, X) concentrate

Plasma fractions should comply with the WHO Requirements for the Collection, Processing and Quality Control of Blood, Blood Components and Plasma Derivatives (Revised 1992). WHO Technical Report Series, No. 840, 1994, Annex 2
Factor IX complex concentrate is a complementary preparation and a representative coagulation factor preparation. Various preparations can serve as alternatives
Infusion (Powder for solution for infusion), factor II, VII, IX, and X 500–1500 units
Uses:
replacement therapy for factor IX deficiency in haemophilia; bleeding due to deficiencies of factors II, VII or X
Contraindications:
disseminated intravascular coagulation
Precautions:
risk of thrombosis (probably less risk with highly purified preparations)
Dosage:
Haemophilia B, *by slow intravenous infusion* , ADULT and CHILD according to patient's needs and specific preparation used
Treatment of bleeding due to deficiencies in factor II, VII or X as well as IX, *by slow intravenous infusion* , ADULT and CHILD according to patient's needs
Adverse effects:
allergic reactions including chills, fever