Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Introduction to biotechnology, scope, potential and achievements

Biotechnology is "the integrated use of biochemistry, microbiology, and engineering sciences in order to achieve technological (industrial) application of the capabilities of microorganisms, cultured tissue cells and parts thereof".(European Federation of Biotechnology)

Biotechnology is "the application of biological organisms, system or processes to manufacturing and service industries".

Scope & Potential:

Biotechnology was being used even in ancient times although in it's more primitive and nascent form. Ex. - Making of curd, bread, wine and beer.

Genetic engineering in biotechnology stimulated hopes for both therapeutic proteins, drugs and biological organisms themselves, such as seeds, pesticides, engineered yeasts, and modified human cells for treating genetic diseases.

The field of genetic engineering remains a heated topic of discussion in today's society with the advent of gene therapy, stem cell research, cloning, and genetically-modified food.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

GENE TRANSFER

Gene transfer refers to movement of genetic information between organisms. In the most of the eukaryotes it is the essential part of life cycle and usually occurs by sexual reproduction.

Mechanisms of gene transfer

Gene transfer in bacteria can occur by following three different methods: Transformation, Transduction, Conjugation

Transformation

Definition – It is a transfer of cell free or naked DNA from one bacteria to other. Transformation was discovered by Frederik Griffith in 1928. Transformation was observed in organisms of following genera - Bacillus, Acinetobacter, Neisseria, Staphylococcus

Mechanism of transformation

It is a process whereby small piece of cell free or naked DNA is transferred from one bacterial cell to other.

1. Uptake of DNA occurs only at a certain stage (competence stage) in cell's growth cycle.

2. Once DNA reaches the entry sites endonucleases cut the double stranded DNA into units of 1000 to 10,000 nucleotides.

3. The two stands of DNA separate and only one strand enter the cell.

4. Inside the cell donor single stranded DNA must combine with the portion of recipient's chromosome immediately otherwise it may be destroyed.

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Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Transduction

Definition - Transduction is the method of transferring genetic material from one bacterium to another by viruses.

Types of transduction

There are two types of transduction –

1. **Specialized transduction** - in this only specific genes are transferred. Several lysogenic phages are known to carry out specialized transduction. Lamda phage in E. coli is extensively studied.

2. **Generalised transduction** – In generalised transduction any bacterial gene can be transferred by virus. Ex. – P22 phage of Salmonella typhimurium

Significance of transduction

1. It transfers genetic material from one bacterial cell to another and alters genetic characteristics of recipient cell.

2. Incorporation of page DNA into bacterial cell demonstrates close evolutionary relationship between prophage and bacteria.

3. The discovery that prophage can exists in a cell for a long period of time suggests a similar possible mechanism for the viral origin of cancer. Viral genes inserted into human chromosome could disrupt regulation of some genes.

4. Transduction provides a way to study gene linkage.

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Conjugation

In conjugation like transformation and transduction genetic material is transferred from one bacterial cell to another.

Conjugation differs from other mechanisms in two ways: It requires direct contact between donor and recipient cell. It transfers larger quantities of DNA

Mechanism of conjugation

A. The transfer of plasmids

Two types of cells called F^+ and F^- were found to exist in any population of E. coli capable of conjugating. F^+ cells contain extra chromosomal DNA called F plasmid (fertility). F^- cells lack F plasmid. F^+ cells make an F pilus. F pilus forms a bridge by which it attaches to F^- cells, when conjugation takes place between F^+ and F^- cells. The copy of F plasmid is then transferred from F^+ to F^- cell .

B. High frequency recombination

In F^+ cell population some of the cells are found to induce recombination more frequently than F^+ cells (1000 times than F^+ cells).

Hfr strains arise from F^+ strains, when F plasmid is incorporated into bacterial chromosome at one of the several possible sites.

When Hfr cells serve as a donor in conjugation the F plasmid initiates transfer of chromosomal DNA. Only a part of plasmid called as initiating segment is transferred along with some adjacent chromosomal genes.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Isolation of DNA

Many of the techniques in gene manipulations require large quantities of purified DNA. Lysis of the cell wall will result in the nuclear material spilling out from the broken cells. This material can be harvested, but the DNA will be contaminated with both RNA and with proteins. Methods are therefore required to remove these contaminants to yield a purified DNA fraction.The host cell wall lysis is carried out by lysozyme, EDTA, or detergents – SDS

Methods of purification of DNA

A. Purification by removing cellular components

B. Direct purification of DNA:

1. Isopycnic centrifugation - The DNA sample is mixed with caesium chloride and ethidium bromide and centrifuged at high speed for approximately 48 h.

2. Use of silica particles – This technique is based on the principle of tight binding between DNA and silica particles in presence of denaturating agents such as guanidium thiocynate.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Genomic and cDNA library

Genomic library: It is a collection of the total genomic DNA from a single organism. The DNA is stored in a population of identical vectors, each containing a different insert of DNA.

In order to construct a genomic library, the organism's DNA is extracted from cells and then digested with a restriction enzyme to cut the DNA into fragments of a specific size. The fragments are then inserted into the vector using DNA ligase. Next, the vector DNA can be taken up by a host organism - commonly a population of Escherichia coli or yeast - with each cell containing only one vector molecule. Using a host cell to carry the vector allows for easy amplification and retrieval of specific clones from the library for analysis.

Genomic libraries are commonly used for sequencing applications..

cDNA library: is a combination of cloned cDNA (complementary DNA) fragments inserted into a collection of host cells, which together constitute some portion of the transcriptome of the organism and are stored as a "library". cDNA is produced from fully transcribed mRNA found in the nucleus and therefore contains only the expressed genes of an organism.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Gel electrophoresis

Electrophoresis is a separation technique based on the principle that a charged particle in solution will migrate towards one of the electrodes when placed in an electrical field.

Technique is used in clinical laboratories to separate proteins from each other:

– Proteins in body fluids: serum, urine, CSF

– Proteins in erythrocytes: hemoglobin

– Nucleic acids: DNA, RNA

Basic Procedure

1. Sample is applied to an agarose gel

2. Gel is placed into electrophoresis cell containing barbital buffer at pH 8.6

3. Power is applied creating an electrical field and the proteins are separated

4. Proteins are fixed to the gel and stained

5. Separated proteins on gel are scanned

6. Gel and densitometer scan

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Blotting techniques

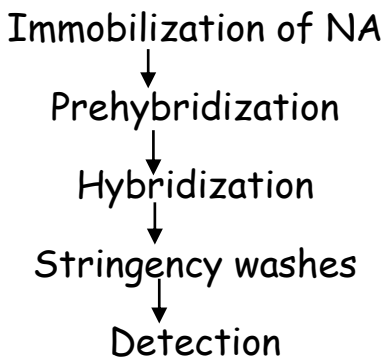
Blotting techniques are widely used analytic tools for specific identification of desired DNA or RNA fragments from thousands of molecules.

Blotting refers to process of immobilization of sample nucleic acid on solid support (nitrocellulose or nylone membranes) the blotted nucleic acids are then used as targets in the hybridization experiments for their specific detection.

Types of blotting techniques:

1. Southern blot : Identification of DNA
2. Northern blot : Identification of RNA
3. Dot blot: identification of DNA/RNA
4. Colony or plaque blot (cells from colony)

An outline of NA blotting technique:



Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

DNA hybridization

The presence of target nucleotide sequence in a DNA sample can be determined by DNA hybridization using DNA probe. DNA hybridization depends upon formation of stable base pairs between probe and target DNA.

DNA hybridization is possible because ds DNA can be converted to ss DNA by heat or alkali treatment. It breaks hydrogen bonds that holds the bases together - denaturation. It does not affect phosphodiester bonds of the DNA backbone. If heated solution is rapidly cooled, the stands remain single stranded.

If temperature of heated DNA solution is lowered slowly the ds DNA can be reestablished because of base pairing of complementary nucleotides – renaturation The process of heating and slowly cooling ds-DNA is called annealing.

If DNAs from two different sources with some shared sequence (homologous) are mixed, heated to 100°C and slowly cooled, there will be some hybrid DNA molecules among annealed products i.e. DNA with strands from different Sources

Applications:

1. Detection of gene
2. Screening specific clone from cDNA or genomic library
3. Determining the location of a gene in chromosome and
4. Diagnosis of diseases.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Site directed mutagenesis

The site directed mutagenesis is the technique for generating amino acid coding changes in DNA (gene). By this approach specific (site directed) changes (mutagenesis) can be made in the base of gene to produce a desired enzyme/ protein. The net result in site directed mutagenesis is incorporation of desired amino acid (of one's choice) in place of specific amino acid in a protein or polypeptide.

By using this technique enzymes that are more efficient and more suitable than natural enzymes can be created for industrial applications.

But site directed mutagenesis is a trial and error method that may or may not result in a better protein. The detailed information about structure and function are desirable to undertake site directed mutagenesis.

Methods of site directed mutagenesis:

1. **Oligonucleotide directed mutagenesis** –it is done by Creating Specific DNA Changes Using Primer Extension Mutagenesis. Bacteriophage M13 is used in this method
2. **Casettee mutagenesis** – It relies on the presence of two restriction enzyme recognition sites flanking the DNA that is to be mutated.
3. **PCR employed oligonucleotide directed mutagenesis** - In this method mutations are introduced within the PCR primers themselves

Examples of proteins modified by site directed mutagenesis:

1. α_1 antitrypsin
2. tPA
3. DHFR

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

RFLP –Restriction Fragment Length Polymorphism

Restriction analysis of DNA by its digestion with restriction endonucleases (RE) in specific restriction sites.

In the case the sequence difference (polymorphism) creates or disturbs a specific site for RE, after restriction, fragments with different sizes are formed

Principle:

1) Starting DNA (genomic DNA, PCR product)
2) With a restriction enzyme into fragments with different sizes
3) Fragments electrophoresis separation

Restriction endonucleases (RE):

a) Known about 2100 bacterial RE
b) RE recognize variously short nucleotides sequences (4,6,8) in which then they digest covalent phosphodiester bonds.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

DNA fingerprinting

Fingerprints have played a central role in human identity cases for decades. No two individuals, except for identical twins, will have genomes with the same nucleotide sequences.

The human genome contains DNA polymorphisms. Polymorphisms that can provide invaluable evidence in cases of uncertain identity.

DNA markers in fingerprinting and disease diagnosis

1. **Single nucleotide polymorphism (SNPs)** - In this a single base pair is different between one individual and another. result in the alteration of restriction enzyme recognition sites. Base changes at these sites results in different length DNA fragments being produced upon restriction digestion.

2. **Minisatellites / variable number tandem repeats / VNTRs)** - The number of copies of variable number tandem repeats (VNTRs) at a specific genomic location can vary widely between individuals, and is described as being highly polymorphic.

3. **Microsatellites (STRs)** - Microsatellites are short, 2 – 6 bp, tandemly repeated sequences that occur in a seemingly random fashion distributed throughout the genome of all higher organisms.

Applications - It is a valuable tool in cases of uncertain identity, such as paternity, rape, murder, and the identification of mutilated bodies after explosions, crashes, or other tragedies.

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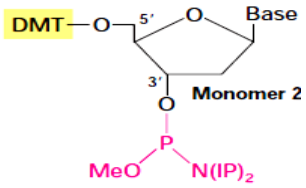
Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Gene synthesis and gene machine

The oligonucleotide of about 100 bases can be produced in about 10 hours.

Method used for synthesis is **phosphoramidite method.**

The building blocks used for synthesis are DNA phosphoramidite nucleosides (sometimes called monomers). These are modified to prevent branching or other undesirable side reactions from occurring during synthesis. They are modified at the 5-end (with a dimethoxytrityl group) and at the 3-end (with diisopropylamine)



Automated synthesis is performed on solid supports, usually polystyrene /CPG beads. A loaded column is attached to reagent delivery lines on a DNA synthesizer and the chemical reactions proceed under computer control. Bases are added to the growing chain in a 3' to 5' direction (opposite to enzymatic synthesis by DNA polymerases).

The procedure involves following steps:

a. Nucleoside attachment to solid support
b. Preparation of phosphoramidite
c. Coupling
d. Capping
e. Oxidation and deprotection

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Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Gene Sequencing Methods

Objectives: Compare and contrast the chemical (Maxam/Gilbert) and chain termination (Sanger) sequencing methods.

- List the components and molecular reactions that occur in chain termination sequencing.
- Discuss the advantages of dye primer and dye terminator sequencing.
- Derive a text DNA sequence from raw sequencing data.
- Describe examples of alternative sequencing methods, such as bisulfite sequencing and pyrosequencing.

Determining the Sequence of DNA Methods:

1. Chain termination or dideoxy method
    - F. Sanger
2. Shotgun sequence method
3. 2nd generation sequence methods
    - Pyrosequencing
4. Maxam/Gilbert chemical sequencing
5. Array sequencing

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Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Principle of Recombinant DNA Technology

The principle of recombinant DNA technology involved four steps.

a. Gene cloning and development of recombinant DNA:
The foreign DNA (gene of interest) from the source is enzymatically cleaved and ligated (joined) to other DNA molecule i.e. cloning vector (plasmid, phagemid etc.) to form recombinant DNA.

b. Transfer of vector into the host:
This cloning vector with recombinant DNA is transferred into and maintained within a host cell. The introduction of rDNA into a bacterial host cell is called transformation.

c. Selection of transformed cells (host):
Those host cells that take up the rDNA are identified and selected from the pool.

d. Transcription and translation of inserted gene:
If required, an rDNA construct can be prepared to ensure that the protein product that is encoded by the cloned DNA sequence is produced by the host cell.

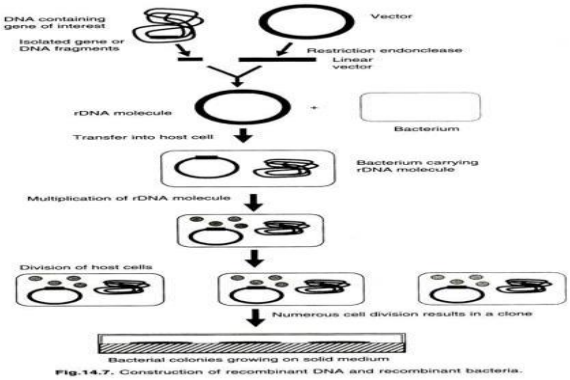

Fig.14.7. Construction of recombinant DNA and recombinant bacteria.

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Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Gene cloning

The foreign DNA (gene of interest) from the source is enzymatically cleaved and ligated (joined) to other DNA molecule i.e. cloning vector (plasmid, phagemid etc.) to form recombinant DNA.

Any gene to be cloned must be inserted in a cloning vector (plasmid).

A foreign gene (DNA fragment) introduced (by transformation) into a bacterium cell will not be replicated with bacterium.

The reason for this is that the enzyme DNA polymerase, which is responsible for copying DNA, does not initiate the process at random. It is initiated at selected sites known as "origin of replication". Generally, small fragments of DNA do not possess an origin of replication.

Using rDNA technology, it is possible to insert the gene into a 'cloning vector', which in turn will make copies of the fragment (inserted DNA). A cloning vector is simply a DNA molecule possessing an 'origin of replication' and which can replicate in the host cell of choice.

Cutting and insertion of desired foreign gene into the plasmid require special enzymes known as restriction endonucleases or restriction enzymes.

Both vector DNA and foreign DNA to be inserted is cut by the same restriction enzyme, generating complementary ends. and join to make again a circular molecule.

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Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Enzymes acting on DNA – restriction endonuclease,

A restriction enzyme (or restriction endonuclease) is an enzyme that cuts double-stranded or single stranded DNA at specific recognition nucleotide sequences known as restriction sites.

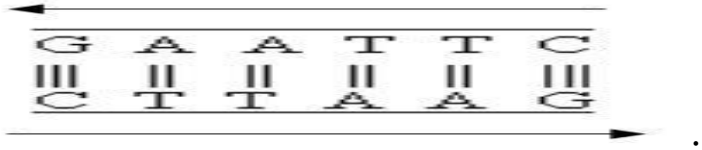
They break the phosphodiester bonds that link adjacent nucleotides in DNA molecules.

Restriction enzymes recognize a specific sequence of nucleotides, and produce a double-stranded cut in the DNA, these cuts are of two types:

1. Blunt ends: These blunt ended fragments can be joined to any other DNA fragment with blunt ends.

2. Sticky ends: DNA fragments with complimentary sticky ends can be combined to create new molecules which allows the creation and manipulation of DNA sequences from different sources

While recognition sequences vary widely , with lengths between 4 and 8 nucleotides, many of them are palindromic.



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A palindromic sequence in DNA is one in which the 5' to 3' base pair sequence is identical on both strands. These are the most commonly available and used restriction enzymes

Examples:

*Eco*R1 (E. coli)

*Bam*HI (Bacillus amyloliquefacience)

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Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Alkaline phosphatase, nucleases

Alkaline phosphatase

It is an enzyme involved in removal of phosphate group. This enzyme is useful to prevent unwanted ligation of DNA molecule which is a frequent problem encountered in cloning experiments. When linear DNA plasmid is treated with alkaline phosphatase the 5'-terminal phosphate is removed. This prevents both recircularization as well as plasmid DNA dimer formation. With this now it is possible to insert foreign DNA.

Nucleases –

Nucleases break the phosphodiester bonds (that hold the nucleotides together)

i. **Endonucleases** - Act on internal phosphodiester bond

- Restriction endonucleases are example of endonuclease. They cut double standed DNA molecules at specific site.
- Deoxyribonuclease I (Dnase I) – cuts either single or double stranded DNA molecules at random sites
- Nuclease S1 – specifically act on single stranded DNA or RNA molecule

ii. **Exonucleases** – Degrade DNA from terminal ends. Exonuclease III – It removes nucleotides from 3'– end of DNA. It cuts DNA and generates molecule with protruding 5' end.

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Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

DNA polymerases, Ligase

DNA polymerases

These enzymes synthesize nucleic acids by joining together nucleotides whose bases are complementary to the template strand bases. The synthesis proceeds in a 5′→3′ direction, as each subsequent nucleotide addition requires a free 3′-OH group for the formation of the phosphodiester bond. These enzymes are template-dependent and can be used to copy long stretches of DNA or RNA.

Different polymerases:

1. DNA Polymerase I
2. RNA dependent DNA polymerase / reverse transcriptase – synthesizes DNA from RNA
3. DNA dependent RNA polymerase – produces RNA from DNA

DNA ligases

The cut DNA fragments are covalently joined together by DNA ligases. The action of DNA ligases is absolutely required to permanently hold DNA pieces. This is essential because hydrogen bonds formed between complementary bases of two DNA strands are not strong enough to hold the strands together. In genetic engineering it is used to seal discontinuities in the sugar—phosphate chains that arise when recombinant DNA is made by joining DNA molecules from different sources.

It can therefore be thought of as molecular glue, which is used to stick pieces of DNA together. DNA ligase joins (seals) the DNA fragments by forming phosphodiester bond between phosphate groups of 5′ carbon of one deoxyribose with hydroxyl group 3′ carbon of other deoxyribose.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Cloning vectors

Def - Vectors are the DNA molecules, which can carry a foreign DNA fragment to be cloned. They are self replicating in an appropriate host cell. These vectors they replicate themselves and any attached passenger DNA so that the passenger is amplified and can be eventually isolated. The most important vectors are – plasmids, bacteriophages, cosmids and phasmids.

pBR 322 plasmid:

It has sequence of 4361 bp. It carries gene for ampicillin resistance. (Amp^r)and tetracycline resistance (tet^r), that serve as markers for identification of clones containing plasmid. It has unique recognition site for action of restriction endonuclease such as *EcoRI, HindIII*, *BamHI*, *SalI*, and *PstI*. *HindIII*, *BamHI*, *SalI* sites are present in region of tetracycline resistance gene. *PstI* site is present in ampicillin resistance gene. A unique *EcoRI* site is not within any coding region of DNA. It has origin of replication that functions only in *E. coli*.

pUC plasmids:

Multiple cloning site of the pUC plasmids is special because it also codes for a small peptide.(alpha peptide of beta galctosidase) It is 2686 bp long. It contains: Ampicillin resistance gene, Regulatable segment of ß-galactosidase gene: lacZ of lactose operon of E.coli and lac I gene produces repressor protein that regulates the expression of lacZ gene. It contains a short sequence with many unique cloning sites (EcoRI, sacI, KpnI, XmaI, SmaI, SalI, HindII, AccI, BspMI, PstI ,SphI, HindIII) which is called a multiple cloning site. (multiple cloning sequence, polylinker) and Origin of replication from pBR322

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Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Cloning vectors - YAC, Cosmid, Ti

Cosmids: These are vectors possessing characteristic of both plasmid and bacteriophage.They are constructed by adding a fragment of phage lambda DNA including *cos* site to plasmid. A foreign DNA (about 40 kb) can be inserted into plasmid DNA. Recombinant DNA can be packed as phages and injected into host cell (E. coli). Once inside the host cell, cosmid DNA behave just like plasmid and replicate. It contains ,An origin of replication (ori) of plasmid ,Ampicillin resistance gene (*amp*), of plasmid., a cos site of phage (a sequence yield cohesive end), restriction sites for cloning.

YAC;It is a synthetic DNA that can accept large fragment of foreign DNA (200-2000 kb).Yeast artificial chromosome (YAC) vectors allow the cloning, within yeast cells. The YAC plasmid has following composition: E. coli selectable marker gene (Amp^r), Origin of replication that functions in E. coli (Ori^E) and Yeast sequences include URA3, CEN, TRP1 and ARS.

Ti plasmid: It is a large-sized tumor inducing plasmid found in *Agrobacterium tumefaciens*. It directs crown gall formation in certain plant species. The strains of *A. tumefacience* that do not possess Ti plasmid can not induce crown gall tumours. Ti plasmid possesses a T-DNA region. The length of T-DNA region can vary from approximately 12-24 kilo base pairs (kb). T-DNA is defined by its left and right borders and include genes for the biosynthesis of auxine, cytokinine and an opine. These genes are transcribed and translated only in plant cells.

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Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Shuttle Vector

A shuttle vector is a vector (usually a plasmid) constructed so that it can propagate in two different host species. Therefore DNA inserted into shuttle vector can be tested or manipulated in two different cell types. It can be manipulated in E. coli and then used in a system which is more difficult or slower to use e.g. Yeast

They are frequently used to quickly make multiple copies of gene in E. coli (amplification) They can also be used for in vitro experiments and modifications- mutagenesis, PCR.

One of the most common types of shuttle vector is yeast shuttle vector. Yeast shuttle vectors have components that allow for replication and selection in both *E.coli* cells and yeast cells.

The E. coli component of yeast shuttle vector includes: Origin of replication and Selectable marker. Ex. Antibiotic resistance and beta-lactamase gene

The yeast component of yeast shuttle vector includes: An autonomously replicating sequence (ars), A yeast centromere (cen) and A yeast selectable marker e.g. Ura3 – a gene that encodes gene for uracil synthesis.

Other examples of other shuttle vectors: pHV14, pEB10, pHp3 - They replicate both in *B. subtilis* and *E. coli.* There are adenovirus shuttle vectors which can propagate in *E. coli* and mammals

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Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Expression vectors

It is usually a plasmid or virus designed for protein expression in cell. The vector is used to introduce a specific gene into a target cell and can commandeer the cells mechanism for protein synthesis to produce protein encoded by the gene. The plasmid is engineered to contain regulatory sequences that act as enhancer and promoter regions.

An expression vector has: Origin of replication, A selectable marker, and Suitable site for insertion of a gene such as multiple cloning site, Elements for expression, Protein tags

Elements for expression: These are: A strong promoter, A transcription termination sequence.The correct translation initiation sequence:- ribosomal binding site and start codon, A strong termination codon and Protein tags

pGEX vectors: These are expression vectors used to synthesize desired proteins tagged with GST. 13 pGEX vectors are available. Nine have expanded MCS that contain 6 recognition sites. pGEX-3X vector: Copy number – 15-20 . Size - 4952 base pairs.

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Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Expression vectors pPIC and CHO cell lines

Expression vectors pPIC

It is a plasmid vector. Size – 4006 bp The pCI is a mammalian expression vectors designed to promote constitutive expression of cloned DNA inserts in mammalian cells. The pCI expression Vector contains the human cytomegalovirus (CMV) major immediate-early gene enhancer/promoter region. It controls the expression of inserted gene.

Vector Components: Enhancer/Promoter Regions, Chimeric Intron, T7 Promoter, Multiple Cloning Region, SV40 Late Polyadenylation Signal, f1 Origin of Replication and Plasmid Replicon

Chinese hamster ovary cell (CHO)

For protein therapeutics to be effective, they must be synthesized in biologically active forms, requiring proper folding and post translational modifications.

CHO cell line derived from ovary of Chinese hamster. They have found use in studies of genetics, toxicity screening, and nutrition and gene expression, particularly to express recombinant proteins. Today CHO are most commonly used mammalian host for industrial production of recombinant protein therapeutics.

Properties:All CHO cell lines are deficient in proline synthesis.

CHO cells do not express the epidermal growth factor receptor (EGFR)

Application: CHO cell lines are the most common mammalian cell lines used for mass production of therapeutic proteins

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Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Recombinant insulin production

Insulin hormone is produced by β-cells of islets of Langerhans of pancreas. Insulin is required for treatment of insulin dependant type diabetes mellitus. Diabetes mellitus affects 2- 3% of general population. It is the 3rd leading cause of death.

Technique :

- A and B chains of insulin are seperately synthesized.
- The genes for insulin A chain and B chain are separately inserted to plasmids of two different E. coli cultures.
- The lac operon system is used for expression of both the genes.
- Lac operon system consists of inducer gene, promoter gene, operator gene and structural gene Z for β-galactosidase
- Presence of lactose induces culture to synthesize A and B chains.
- The protein which is formed consists partly of B-galactosidase, joined to either the A or B chain of insulin. The A and B chains are then extracted from the B-galactosidase fragment and purified.
- These insulin chains are isolated, purified and joined together to form human insulin.

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Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Recombinant Somatotropin / human growth hormone

Human growth hormone is produced by pituitary gland. It regulates growth and development. Insufficient production of growth hormone causes disorder in children. It results in pituitary dwarfism. Only human GH is effective for treatment of pituitary dwarfism.

Limitations in recombinant hGH production

hGH is a protein containing 191 amino acids. During natural synthesis in body it is tagged with signal peptide (containing 26 amino acids). Signal peptide is removed during secretion to release active hGH. Signal peptide interrupts production of hGh by recombinant technology

A novel approach for hGH production

- The nucleotide base sequence in cDNA encoding signal peptide (26 amino acids) plus neighbouring 24 amino acids. i.e. total 50 amino acids is cut by restriction endonuclease ECoRI
- A gene for 24 amino acids (cDNA) is synthesized and ligated to remaining hGH cDNA.
- This constituted cDNA is attached to a vector.
- This vector is inserted into bacterium such as E. coli for production of hGH
- This approach produces biologically active hGH

Recombinant hGH is approved for human use in 1985. It is marketed as protropin by Genetech Company and Humatrope by Eli Lilly company

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Recombinant interferon production

Interferon is an antiviral substance and is a 1st line of defence against viral infections. It was discovered in 1957 by Isaacs and Jean Lindeman and was considered as single substance. It is now known that interferon actually consists of a group of more than 20 substances with molecular weight between 20000-30000. All interferons are proteinic in nature and many of them are glycoproteins.

Production of interferon by yeasts

The yeast *Saccharomyces cerevisiae* is more suitable for production of recombinant interferon. This is mainly because yeast possess mechanism fro glycosylation of proteins similar to that occurs in mammalian cells

DNA sequence coding for specific human interferon can be attached to yeast alcohol dehydrogenase gene in a plasmid and introduced into yeast cell.

The yield of interferon is several folds higher than E. coli.

Therapeutic applications of interferon

INF-α, INF-β, INF-γ were respectively approved for therapeutic use in human in years 1986, 1993, and 1990. Interferons are used for treatment of a large number of viral diseases and cancers. Viral diseases for which interferon therapy used are AIDs, multiple sclerosis, genital warts, hepatitis C, herpes zoster etc.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Gene therapy

More than 4000 genetic diseases have been characterized and studied. These diseases are caused by lack of production of single gene product or production of mutated gene product. Theoretically gene therapy is the permanent solution for genetic diseases.

It is the process of inserting genes into cells to treat diseases. The newly inserted genes will encode proteins and correct the deficiencies that occur in genetic diseases.

Approaches to gene therapy:

1. **Somatic gene therapy:** It involves insertion of fully functional and expressible gene into target somatic cell to correct the genetic disease permanently. Genetic alterations in somatic cells are not carried to the next generations and therefore preferred.
2. **Germ cell gene therapy:** This gene therapy involves introduction of DNA into germ cells. Thus the introduced gene is passed onto successive generations. For safety, ethical and technical reasons germ cell gene therapy is not being attempted at present.

There are two types gene therapy

1. ***Ex vivo* gene therapy:** it involves transfer of genes to cultured cells (Ex. – bone marrow cells) which are then reintroduced into patient.
2. ***In vivo* gene therapy:** it is the direct delivery of genes into the cells of a particular tissue.

Applications: It is used for treatment of Severe combined immunodeficiency, Cystic fibrosis, Emphysema, Hemophilia - B , Sickle cell anemia , Head and neck cancer

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Transgenic Animals

With advent of modern biotechnology it is now possible to carry out improvement in genetic characteristics by making manipulations at genetic level to get the desired characteristics in animals.

Transgenesis refers to phenomenon of introduction of exogenous DNA into genome to create and maintain a stable heritable character. The foreign DNA that is introduced is called transgene. The animal whose genome is altered by adding one or more transgenes is said to be transgenic.

The transgenes behave like other genes present in the anima' genome and are passed on to the offsprings. Thus transgenic animals are genetically engineered or genetically modified organisms (GMOs) with new heritable character. It was in 1980s the genetic manipulation of animal by introducing gene into fertilized egg became a reality.

Commonly used animals for transgenesis

1st animal used for transgenesis was a mouse. The super mouse was created by inserting a rat gene for growth hormone into the mouse genome. The offspring was much larger than parents. Mouse is the animal of choice for most transgenic experiments. The other animals used for transgenesis are rat, rabbit, pig, cow, goat, sheep and fish.

Methods of transgenesis:

1. Retroviral method
2. Microinjection method
3. Embryonic stem cell method

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Applications of transgenic animals

1. **Transgenic mice** are extensively used as model for understanding human diseases and production of therapeutic agents.
Mouse models for several human diseases such as cancer, muscular dystrophy, arthritis, alzheimer's have been developed.

2. **Transgenic cattle** – mammary gland of dairy cattle is a bioreactor for producing several new proteins of pharmaceutical importance. Ex. - Lactoferrin, Interferon, a1- interferon, CFTR

3. **Transgenic sheep and goats** – transgenesis experiments in sheep and goat mostly involve development of mammary glands as bioreactors for production of proteins of pharmaceutical use.

4. Transgenic chicken – Transgenesis in chicken can be used to develop disease resistance, low fat and cholesterol and high protein containing eggs.

5. Transgenic fish - several transgenic fish have been developed with increase in their growth and size.

6. **Pig in organ farm** – Human organs (heart, liver, pancreas, kidney and lungs) for transplantation can be developed in pigs. This is still in experimental stage.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

GERMPLASM STORAGE & CRYOPRESERVATIONS

In Vitro Plant Germplasm Conservation

Germplasm refers to the sum total of all the genes present in a crop and its related pecies. A global organization- International Board of Plant Genetic Resources (IBPGR) has been established for germplasm conservation and provides necessary support for collection, conservation and utilization of plant generic resources throughout the world.

The Germplasm Is Preserved By The Following Two Ways:

1. **In-situ conservation**-

   The germplasm is conserved in natural environment by establishing biosphere reserves such as national parks, sanctuaries. This is used in the preservation of land plants in a near natural habitat along with several wild types.

2. **Ex-situ conservation**-

   This method is used for the preservation of germplasm obtained from cultivated and wild plant materials. The genetic material in the form of seeds or in vitro cultures are preserved and stored as gene banks for long term use.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Production of monoclonal antibody

Monoclonal antibodies are defined as antibodies produced by single clone of cell. They are produced by hybridoma technology

Hybridoma technology is a technology of forming hybrid cell lines by fusing a specific antibody-producing B cell with a myeloma (B cell cancer) cell that is selected for its ability to grow in tissue culture and for an absence of antibody chain synthesis.

Hybridoma creates Monoclonal antibodies:

Monoclonal antibodies are typically made by fusing myeloma cells with the spleen cells from a mouse that has been immunized with the desired antigen. However, recent advances have allowed the use of rabbit B-cells.

Steps in Hybridoma Technology:

1. Inject the protein Ag into a mouse.
2. Remove the spleen.
3. Identify which spleen cells are producing antibodies.
4. Separate these cells and grow in tissue culture tubes.
5. Screen each Ab for cross reactivity.
6. Select the Ab which doesn't cross react with any other protein.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Applications of monoclonal antibodies

As MAbs are highly pure and highly specific, they have wide range of applications.

1. **Diagnostic applications**

   A. Biochemical analysis for diagnosis of pregnancy, cancer, hormonal disorder, infectious diseases
   B. Diagnostic imaging – (Immunoscitigraphy) – for detection of myocardial infarction, deep vein thrombosis, atherosclerosis, cancers and bacterial infections.

2. **Therapeutic uses**

   A. Direct use as therapeutic agent - to destroy disease caausiing organisms, in the treatment of cancer, in immunosupression of cancers, in treatment of AIDs and autoimmune diseases.
   B. As a targeting agents in therapy – as immunotoxins (in treatment of cancers), in drug delivery, for dissolving blood clots, in radioimmunotherapy (of tumors)

3. **Protein purification** – by immunoaffinity technique

4. **Miscellaneous applications** – as catalytic agents (abzymes), in autoantibody fingerprinting

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

INTRODUCTION TO ENZYME TECHNOLOGY - IMMOBILIZATION

Biotechnology offers an increasing potential for the production of goods to meet various human needs. In enzyme technology – a sub-field of biotechnology – new processes have been and are being developed to manufacture both bulk and high added-value products utilizing enzymes as biocatalysts, in order to meet needs such as food (e.g., bread, cheese, beer, vinegar), fine chemicals (e.g., amino acids, vitamins), and pharmaceuticals. Enzymes are also used to provide services, as in washing and environmental processes, or for analytical and diagnostic purposes. The driving force in the development of enzyme technology, both in academia and industry, has been and will continue to be:

• The development of new and better products, processes and services to meet these needs; and/or

• The improvement of processes to produce existing products from new raw materials as biomass.

The goal of these approaches is to design innovative products and processes that are not only competitive but also meet criteria of sustainability. The concept of sustainability was introduced by the World Commission on Environment and Development (WCED, 1987) with the aim to promote a necessary development that meets the needs of the present without compromising the ability of future generations to meet their own needs". To determine the sustainability of a process, criteria that evaluate its economic, environmental and social impact must be used.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

IMMOBILIZATION OF ENZYME – METHODS OF IMMOBILIZATION

The costs associated with the use of enzymes for industrial purposes can also be reduced by immobilising the enzymes. Enzymes for industrial processes are more valuable when they are able to act in an insolubilised state rather than in solution. Enzymes are immobilised by binding them to, or trapping them in a solid support.

Methods for Immobilising Enzymes:

1. Entrapment, 2. Surface Immobilization 3. Covalent bonding

4. Cross-linking

1. **Surface adsorption:** Enzymes are held on to a solid support (matrix) by weak forces such as hydrogen bonding.

2. **Entrapment**: Enzymes are trapped within the structure of a solid polymer (usually in the form of beads) – the enzyme is trapped rather than bound.

3. **Covalent bonding:** Enzymes are covalently bonded to a matrix such as cellulose or collagen

4. **Cross linking**: Cross-linking and covalent bonding may cause some enzymes to lose their catalytic activity especially if the active site is involved in forming the linkages.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

IMMOBILIZATION OF ENZYME

APPLICATIONS

1. Compared with free enzymes in solution, immobilised enzymes have a number of advantages for use in industrial processes

2. The stability of many enzymes is increased when they are in an immobilised state; they are less susceptible to changes in environmental conditions such as temperature and pH fluctuations.

3. Immobilised enzymes can be recovered and re-used, reducing overall costs

4. The products of the reaction are not contaminated with enzyme eliminating the need to undertake costly separation of the enzyme from the product.

5. Immobilising enzymes allows for continuous production of a substance with greater automation.

6. The ability to stop the reaction rapidly by removing the enzyme from the reaction solution (or vice versa)

7. The ability to stop the reaction rapidly by removing the enzyme from the reaction solution (or vice versa)

9. Product is not contaminated with the enzyme (especially useful in the food and pharmaceutical industries)

10. Reduces effluent disposal problems.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

FERMENTER DESIGN

A fermenter can be defined as a large, for growing organisms such as bacteria or yeast that are used in the biotechnological manufacture of substances such as pharmaceuticals, antibodies, or vaccines, or for the bioconversion of organic waste.

Fermentor maintains optimal conditions for the growth of microorganisms, and organisms are stimulated to produce desired products

CHARACHETERS OF STIRRED TANK FERMENTER

Size – variable - 20 liters - 250 million liters

Cylindrical vessel with doomed top and bottom

Aspect ratio - Height / diameter ratio = 3 - 5

Motor driven shaft – with one or more agitators / impellers

Diameter of impeller – 1/3rd of vessel diameter

Distance between impellers – 1.2 x impeller diameter

Sparger – introduces air under pressure in fermenter

Reaction vessel is surrounded by jacket – for circulation of hot / cold water

It has side ports for pH, temperature and DO sensors, Connections for acid, alkali, antifoam, inoculum addition above the level of liquid in reaction vessel

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

TYPES OF FERMENTORS

Industrial Biotechnology: the process by which large quantities of cells are grown under aerobic or anaerobic conditions.

The industrial microorganisms are grown under controlled conditions with an aim of optimizing the growth of the organism for production of a target microbial product.

Types of Bioreactors

Depending on the design of the reactor, the bioreactors are of following types:

a) Continuous stirred tank bioreactors -

b) Bubble column bioreactors -

c) Airlift bioreactors -

Further there are divided as

(1) Internal loop bioreactor

(2) External loop airlift bioreactor

(3) Two stage airlift bioreactors

d) Fluidized bed bioreactors -

e) Packed bed bioreactors-

f) Photobioreactors -

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

OPERATION OF CONVENTIONAL BIOREACTOR

1.**Sterilization** - Aseptic conditions are required for successful fermentation. Bioreactor, its accessories, growth medium, and air should be sterile. Method for sterilization of bioreactor and medium - 1. In situ sterilization 2. Continuous heat sterilization

2. **Inoculation and sampling** - Inoculum added to growth medium in fermentor under aseptic conditions. Inoculum – 1-10%

3. **Systems of aeration** – stirred system of aeration and air lift system of aeration

4. **Adequate mixing** – It is essential to ensure: optimum supply of nutrients and oxygen, prevent accumulation of toxic metabolites.
Various parameters are needed to be maintain optimum conditions in fermentor for maximum product formation

1. pH – maintained by addition of acid or alkali

2. Temperature – heating and cooling systems

3. Dissolved oxygen – oxygen is sparingly soluble. Continuous supply of air is essential

4. Foam formation – growth medium is rich in protein – aeration and agitation results in foam. Antifoams are added to break foam

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

DOWNSTREAM PROCESSING

It comprises all operations required for extraction and purification of a product produced by a biotechnological process such as microbial fermentation, plant and tissue culture, transgenic plants and animals.

The various stages of processing that occur after the completion of the fermentation or bioconversion stage, are separation, purification, and packaging of the product.

Selection of process: Different processes are available for isolation and purification of product.The selection of specific process depends on: Product location – extracellular / intracellular, Product sensitivity, Concentration of product, Properties / use of product, Acceptable standards of purity, Possible impurities, Economy of process and market price of product

Stages in Downstream Processing

1. Removal of Insolubles
2. Product Isolation
3. Product Purification
4. Product Polishing

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Different methods of downstream processing

1. **Cell separation:** cells are separated from fermentation broth by flocculation, centrifugation or filtration of harvest

2. **Cell disruption**; required when product is intracellular. It is done by using homogenizers or hydrolytic enzymes. Cell disruption is done by agitation with **abrasives** – glass beads. Cells can also be disrupted by ultrasonication.

3. **Clarification** : it is remval of insolubles like cell debris or particulate matter. It is done by centrifugation or filtration. Continuous Rotary Vacuum filter is the most commonly used type of filtration. centrifugation is done by Tubular bowl centrifuge, Disc bowl centrifuge, Multi-chamber centrifuge, Scroll centrifuge.

4.**Concentration**: of product is done by various methods such as evaporation, Centrifugation, precipitation, filtration, chromatography, ultrafiltration , partitioning, distillation

5. **High resolution techniques**: used in purification of products are - chromatography , electrophoresis

6. **Finishing/packaging** – crystallization, filtration, , gel electrophoresis, drying

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Manufacturing of penicillin: media and inoculums

Outline of penicillin fermentation

1. Media preparation
2. Inoculum preparation
3. Fermentation process and control parameters (pH, aeration, agitation, DO. etc)
4. Downstream processing (recovery and purification)

Medium used for penicillin production: Medium contains corn steep liquor – 4-5% dry wt. (CSL), Lactose is added as carbon source. Addition of yeast extract, soya meal or whey is done for good supply of nitrogen. Sometimes ammonium sulfate is added to supply nitrogen. Phenylacetic acid (pen G) or phenoxyacetic acid (pen V) serve as precursor for penicillin biosynthesis are continuously fed during fermentation

Inoculum development: Organism used for production of penicillin is *Penicillium crysogenum* . Improved mutant Strain Q176 of *Penicillium crysogenum* is preferred. The lyophilized culture of spore is cultivated for inoculum development which is transferred to prefermenter and then to fermenter. Inculum is added at 1- 10% concentration

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Penicillin fermentation: process control and recovery

Fermentation: Penicillin production is carried out by submerged process. Penicillin production is an aerobic process and therefore continuous supply of O_2 to the growing culture is essential. Required aeration rate: 0.5 – 1 vvm. pH is maintained around 6.5. Optimum temperature for production: 25-27^{0}C. Antifoam agents added are tributyl citrate, octadecanol, lard oil to prevent foam formation. Growth phase lasts for 40 hrs. Penicillin is a secondary metabolite. After growth phase is stabilized penicillin production starts and increases exponentially. Penicillin production stage can be extended to 150-180 hrs. (6-7 days)

Recovery of penicillin

After fermentation is complete, broth containing 1% penicillin is processed for extraction. Following steps are used:

Filtration – rotary vacuum filter - removal of mycelium

Solvent extraction: Filtrate cooled - < 10°C, pH of filtrate is adjusted to acidic level - < 3. This is done to minimize chemical and enzymatic degradation of penicillin. Extraction of penicillin is carried out by using organic solvent - n-butyl acetate or methylketone

Crystallization and Drying of crystals - by fluid bed drying

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Manufacturing of vitamin B12

Vitamin B12_has role in functioning of brain and nervous system, formation of blood. Deficiency causes pernicious anemia which is an causes low Hb, less RBCs.Dietary reference intake for an adult ranges from 2 to 3 µg per day

Vitamin B12 is exclusively synthesized in nature by microorganisms. Chemical synthesis of vitamin B12 is not practical – 20 steps . Production by fermentation is the only choice

Microorganisms used: *Streptomyces olivaceous, S. gresius, Pseudomonas denitrificans, Propionibacterum shemanii, Clostridium butylicum*

Production vitamin B12 by using Propionibacterium shermanii

Medium – CSL, beet molasses or soyabean meal can be usedas C source, ammonium phosphate as N 2source

Anaerobic fermentation – 3 days, Aerobic fermentation 4 days

Centrifugation of harvest to collect cells.

Cells are treated with acid and heated to release vitamin B12.

Cynide solution is added to convert B12 to cyanocobalamine

Purification by adsorption on IRC – 5o resin.

Evaporation is done to get Vitamin B12 crystals.

Class- T. Y. B.Pharm **Subject-Pharmaceutical Biotechnology**

Subject Incharge-Mrs. V. A. Warad **Lecture Synopsis no:**

Vit B12 production - *Streptomyces olivaceous*

Inoculum preparation – Pure culture of *Streptomyces olivaceous* is inoculated into flask containing Bennett's broth. 5% inoculum is required for addition in production medium for fermentation

Fermentation medium : Distiller's soluble - 4%, dextrose 1%, CaCO3 – 0.5%, COCl2 -2-5ppm, pH -7-7.5

Fermentation: Medium is sterilized and inoculum is added. Aeration rate – 0.5 vvm . Antifoams used – soyabean oil, corn oil, lard oil. In first 24 hrs. pH of medium is reduced due to rapid sugar consumption and it rises again after 48-96 hrs due to lysis of mycelium. Fermentation is carried out for 3 - 4 days

Recovery of vitamin B12: Vitamin is mainly present in mycelium. Fermented broth is filtrated / centrifuged for collecting mycelium

Concentration of B12 – Reduction of pH to 5 by sulfuric acid, and boiling of mixture is done to release B12 from cells. Filtration of broth to remove cell debris. Treatment of broth with cynide to convert cobalamine to cyanocobalamine. Adsorption of cyanocobalamine on is done charcoal or bentonite. Purification can be done by Solvent extraction – with organic solvents, Precipitation , Chromatography on alumina ,Crystallization from ethanol-acetone, or drying by drum drying / spray drying.