



ANIMAL BIOTECHNOLOGY

VPB-321



MODULE-1: ANIMAL BIOTECHNOLOGY

Learning objectives

The learner will learn about

- Definitions and scope of Animal biotechnology
- Activities of Animal biotechnology

BIOTECHNOLOGY

- **Biotechnology** is defined as “the application of biological organisms systems or processes to manufacturing and service industries”(Coombs, 1984).
- It is also defined as “the application of scientific and engineering principles to the processing of materials by biological agents to produce goods and services” (Coleman, 1986). In this, the word “agent” denotes a wide range of biological things such as enzymes, whole cells or multicellular organisms. Services and goods mean such processes as waste-water treatment.
- The scientific and engineering principles are chiefly microbiology, biochemistry, genetics, biochemical and chemical engineering.
- The term biotechnology was coined by Karl Ereky (1917)

BRANCHES OF BIOTECHNOLOGY

- Animal Biotechnology
 - **Animal biotechnology** includes manipulation of genome of animals to produce more milk, meat and wool and other desirable characteristics, improved utilization of animal feed, embryo transfer, developing vaccines etc.
- Healthcare Biotechnology
 - **Healthcare biotechnology** involves development of genetically engineered vaccines, early disease diagnostic kits, gene profiling, development of newer antibiotics, production of artificial skin and organs, stem cells etc.
- Agricultural Biotechnology
 - **Agricultural biotechnology** has contributed in development of crops and fruit varieties with higher yield, great resistance to diseases and stresses and higher nutritional value, increasing efficiency of nitrogen fixation in plants and vaccine production from plants.
- Industrial Biotechnology
 - **Industrial biotechnology** has a wide range of applications such as production of antibiotics, extraction of metals by microbial methods, enrichment of ores etc. It plays a vital role in food processing and beverage industries.
- Aquaculture and Marine Biotechnology
 - **Aquaculture and Marine biotechnology** helpful in transgenic fish production, bioremediation, conservation, sea weeds culture etc.
- Environmental Biotechnology
 - **Environmental biotechnology** helps in biodegradation of xenobiotic compounds, bio-mining, restoration of denuded areas, bio-sensors, processing of wastes, composting, anaerobic processing, etc.

BRANCHES OF BIOTECHNOLOGY

- **Blue biotechnology** is used to refer application of biotechnology in animal , marine and aquatic improvements.
- **Red biotechnology** is used for medical processes, like finding genetic cures by manipulating genome and creating organisms for production of antibiotics.
- **Green biotechnology** is used to refer agricultural processes that use biotechnology for better production such as development of transgenic plants that are produced to survive under specific environmental conditions, developing disease resistant transgenic plants.

- **White biotechnology** is used to refer industrial biotechnology. It is used to develop an organism that could able to produce certain useful chemicals by natural processes rather than using the industrial way to produce it.

ANIMAL BIOTECHNOLOGY

- Animal biotechnology is the application of scientific and engineering principles to the processing or production of materials by animals to provide goods and services to human kind.
- Examples of animal biotechnology include generation of transgenic animals, using gene knockout technology to generate animals in which a specific gene has been inactivated, and production of nearly identical animals by somatic cell nuclear transfer.

OBJECTIVES OF ANIMAL BIOTECHNOLOGY

- Identification and characterization of animal breeds,
- Developing DNA - based diagnostics and genetically engineered vaccines for animals,
- Studying animal genomics and its varied applications
- Developing embryo - transfer technology, cloning, transgenic animals
- DNA forensics, molecular diagnostics, cloning, wildlife conservation, stem cell research and bio - processing technologies are other import areas of animal biotechnology.

APPLICATIONS OF ANIMAL BIOTECHNOLOGY

- Animal biotechnology has diverse and widespread applications in the areas of food quality control, analyses of milk and milk products and other animal products, besides development of disease – free animals.
 - Transgenic technology
 - Gene knockout technology
 - Molecular genetics
 - Embryo transfer technique
 - *In vitro* embryo production
 - Modern vaccines
 - Molecular diagnostics
 - Nutritional biotechnology

TRANSGENIC TECHNOLOGY

- Transgenic livestock have been produced with increased growth rates, enhanced lean muscle mass, enhanced resistance to disease.
- Transgenic poultry, swine, goats, and cattle also have been produced that provide large quantities of human proteins in eggs, milk, blood, or urine.
- Examples of human pharmaceutical proteins include enzymes clotting factors, albumin and antibodies.
- The major limiting factor for widespread use of transgenic animals in agricultural production systems is the relatively less success rate (less than 10 percent) of production of transgenic animals and high production cost.

KNOCKOUT TECHNOLOGY

- The gene knockout technology creates a possible source of replacement of organs for humans.
- The process of transplanting cells, tissues, or organs from one species to another is called "xenotransplantation".
- At present, the pig is the major animal considered as a xenotransplant donor to humans.

MOLECULAR GENETICS

- In livestock populations with a high degree of genetic variation, molecular markers are being increasingly used to study the distribution and patterns of genetic diversity.
- Global surveys indicate that 40% of domestic livestock breeds are at risk of extinction.
- Most of these breeds are found only in developing countries, and often little is known about them or their potential.

- Rapid progress is being made in the preparation of dense microsatellite linkage maps to assist in the search for genetic traits of economic importance.
- Molecular markers have been widely used in the identification of genotypes and the 'genetic fingerprinting' of organisms.
- Genotype verification is used intensively to determine the parentage of domestic animals and to trace livestock products in the food chain back to the farm and animal of origin.

EMBRYO TRANSFER TECHNIQUE

- One of the major reproductive technologies that can facilitate genetic improvement in cattle is ET.
- Multiple ovulation and embryo transfer (MOET) takes AI one step further, in terms of both the possible genetic gains and the level of technical expertise and organisation required.
- The main potential advantage of MOET is that the elite females of local breeds can be identified, and bulls can be produced from them for use in a field programme of breed improvement in developing countries.

IN VITRO EMBRYO PRODUCTION

- High rates of oocyte maturation (70% to 90%), fertilisation (60% to 70%) and embryo cleavage (40% to 50%), and moderate to low rates of blastocyst formation (15% to 30%) and calf production (10.5%) have been reported.
- The practical use of IVEP is limited by high production costs and the low overall efficiency under field conditions.

MODERN VACCINES

- Two main approaches are being used to develop vaccines using recombinant DNA technology.
 - The first involves deleting genes that determine the virulence of the pathogen, thus producing attenuated organisms (non-pathogens) that can be used as live vaccines. Currently, this strategy is more effective against viral and bacterial diseases than against parasites. Attenuated live vaccines have been developed against the herpes viruses that cause pseudorabies in pigs and infectious bovine rhinotracheitis in cattle. A number of candidate *Salmonella* vaccines have also been produced.
 - The second approach is to identify protein subunits of pathogens that can stimulate immunity. The International Livestock Research Institute (ILRI) used this approach to develop a vaccine against *Theileria parva*, the parasite that causes East Coast fever in African cattle.
- A recent approach has been to use vaccines based on DNA. The use of DNA in vaccines is based on the discovery that injecting genes in the form of plasmid DNA can stimulate an immune response to the respective gene products. This immune response is a result of the genes being taken up and expressed by cells in the animal after injection. The live-vector and DNA vaccination systems could be manipulated further to enhance the immunity conferred by the gene products.

MOLECULAR DIAGNOSTICS

- Molecular diagnostic technologies that are either already in use or being include polymerase chain reaction (PCR), monoclonal antibodies and recombinant antigens.
- Enzyme-linked immunosorbent assays have become the standard means of diagnosing and monitoring many animal and fish diseases worldwide, and the PCR technique is especially useful in diagnosing livestock disease.
- Molecular characterisation of the virus serotypes causing foot and mouth disease has helped in the vaccination and control programmes in Asia.

NUTRITIONAL BIOTECHNOLOGY

- The shortage of feed in most developing countries and the increasing cost of feed ingredients mean that there is a need to improve feed utilisation.
- Aids to animal nutrition, such as enzymes, probiotics, single-cell proteins and antibiotics in feed, are already widely used in intensive production systems worldwide to improve the nutrient availability of feeds and the productivity of livestock.
- Gene-based technologies are being increasingly used to improve animal nutrition, either through modifying the feeds to make them more digestible or through modifying the digestive and metabolic systems of the animals to enable them to make better use of the available feeds.

- Seeds derived from genetically modified (GM) plants, such as grain, silage and hay, have contributed to increases in growth rates and milk yield.
- Genetically modified crops with improved amino acid profiles can be used to decrease nitrogen excretion in pigs and poultry.
- Increasing the levels of amino acids in grain means that the essential amino acid requirements of pigs and poultry can be met by diets that are lower in protein.

MODULE-2: CELLULAR CLASSIFICATION

Learning objectives

The learner will be able to learn about

- Classes of cells
- Differences between Prokaryotes and Eukaryotes
- Macromolecules and their importance

PROKARYOTES

- Prokaryotes are the simplest living cells. They are bounded by a *Cell membrane (Plasma membrane)* comprising a lipid bilayer in which are embedded proteins that allow the exit and entry of small molecules.
- The cell interior (*Cytoplasm or cytosol*) contains a single, circular chromosome compacted into a *nucleoid*.
- The surface of a prokaryote may carry *pili*, which allow it to attach to other cells and surfaces and *flagella*, whose rotating motion allows the cell to swim.

EUKARYOTES

- Eukaryotes are taxonomically classified into *4 kingdoms* comprising
 - Animals,
 - Plants,
 - Fungi and
 - Protists.
- Structurally eukaryotes are defined by their possession of membrane enclosed organelles with specialized metabolite function.
- Eukaryotic cells tend to be larger than Prokaryotes.
- They are surrounded by a plasma membrane, which can have a highly convoluted shape to increase its surface area.
- The cytoplasm is a highly organized gel that contains, in addition to organelles and ribosomes, an array of protein fibers called the *cytoskeleton* which controls the shape and movement of the cell and which organizes many of its metabolic functions.
- Eukaryotic cytoskeleton consists *microtubules*, made of *tubulin* and *microfilaments* made of *actin*.
- Many Eukaryotes are multicellular.

DIFFERENCE BETWEEN PROKARYOTES AND EUKARYOTES

S. No.	Components	Prokaryote	Eukaryote
1	Nuclear envelope	Absent	Present
2	DNA	Naked	Combined with proteins
3	Chromosomes	Single	Multiple
4	Nucleolus	Absent	Present
5	Division	Amitosis	Mitosis
6	Ribosomes	70 S (50S + 30 S)	80 S (60 S + 40 S)

7	Endomembranes	Absent	Present
8	Mitochondria	Respiratory and Photosynthetic Enzymes in the plasma membrane	Present
9	Chloroplast	Absent	Present in plant cells
10	Cell wall	Non-cellulosic	Cellulosic only in plants
11	Exocytosis & Endocytosis	Absent	Present
12	Locomotion	Single, fibril, flagellum	Cilia and Flagella

- Macromolecules**

GENERAL ORGANISATION OF EUKARYOTES

Main components or compartments	Subcomponents or subcompartment	Main function
Cell membrane	- Cell wall (Plant) - Cell coat - Plasma membrane	- Protection - Cell interaction - Permeability, Endo and Exocytosis
Nucleus	- Chromatin and chromosomes - Nucleolus - Nucleoplasm	- Genetic information system - Ribosomes synthesis
Cytoplasm Matrix, Cytosol, Cytoskeleton	- Soluble enzymes - Microfilaments - Microtubules - Ribosomes	- Glycolysis - Cell motility - Cell shape and motility - Protein synthesis
Endomembranes	- Nuclear envelope - Endoplasmic reticulum – Rough and smooth - Golgi complex	- Nuclear permeability - Synthesis and transport of materials, secretions - Secretion
Membrane organelles	- Mitochondria - Chloroplast - Lysosomes - Peroxisomes	- Cell respiration - Photosynthesis - Digestion - Peroxisation
Microtubular organelles	- Centrioles and spindle - Basal bodies, cilia and flagella	- Cell division - Cell motility

PROTEINS AND NUCLEIC ACID

- Proteins are polymers of aminoacids linked together by peptide bonds. The proteins have both structural and functional roles.
- The nucleic acids, DNA and RNA are polymers of nucleotides, which consist of a nitrogenous base, a pentose sugar and phosphoric acid.

- Nucleic acids are involved in the storage and processing of genetic information, but the expression of this information requires proteins.

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LIPIDS

- While individual lipids are not strictly macromolecules, many are built up from smaller monomeric units and they are involved in many macromolecular assemblies.
- Large lipid molecules are predominantly hydrocarbon in nature and are poorly soluble in water.
- Some are involved in the storage and transport of energy while others are key components of membranes, protective coats and other cell structures.
- Glycerides have one, two or three long chain fatty acids esterified to a molecule of glycerol.
- In animal triglycerides, the fatty acids have no double bonds (saturated) so the chains are linear, the molecules can pack tightly and the resulting fats are solid.
- Plant oils contain unsaturated fatty acids with one or more double bonds. The angled structures of these chains prevent close packing so they tend to be liquids at room temperature.
- Membranes contain phospholipids, (which consists of glycerol esterified to two fatty acids and phosphoric acid) spingolipids.

COMPLEX MACROMOLECULES

- Many macromolecules contain covalent or non-covalent associations of more than one of the major classes of large biomolecules. For example, nearly all enzymes are proteins, but some have noncovalently attached RNA component which is essential for catalytic activity. Association of nucleic acid and protein are known as nucleoproteins.
- Glycoproteins contain both protein and carbohydrate components. Glycosylation is the commonest form of post-translational modification of proteins.
- Proteoglycans (mucoproteins) are large complexes ($> 10^7$ Da) of protein and mucopolysaccharide found in bacterial cell walls and in the extracellular space in connective tissue. Proteoglycans act as lubricants and shock absorbers in extracellular spaces.
- Lipid-linked proteins have a covalently attached lipid component. This is usually a fatty acyl (e.g. myristoyl or palmitoyl) or isoprenoid (e.g. farnesyl or geranyl group).

LIPOPROTEINS

- The lipids and proteins are linked noncovalently. Because lipids are poorly soluble in water, they are transported in the blood as lipoproteins.

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MODULE-3: NUCLEIC ACID

Learning objectives

The learner will be able to learn about

- Composition of nucleic acids

- Structures of purines and pyrimidines
- Base pairing and
- Importance of Nucleic acids

COMPOSITION OF NUCLEIC ACIDS

- Nucleic acids consist of a chemically linked sequence of nucleotides. Each nucleotide contains a heterocyclic ring of carbon and nitrogen atoms (the nitrogenous base), a five carbon sugar in ring form (a pentose) and a phosphate group.
- Two types of pentose are found in nucleic acids. These distinguish DNA and RNA and give rise to the general names for the two types of nucleic acid. In DNA the pentose is 2-deoxy ribose whereas in RNA it is ribose.
- View the animation of Nucleotides and Pentose sugars

PURINES AND PYRIMIDINES

- The nitrogenous bases fall in to two types.
- Purines
 - Any of a group of organic compounds containing two fused rings of carbon and nitrogen atoms. One ring has six members, the other has five, and each has two nitrogens.
- Pyrimidines
 - Heterocyclic organic compound ($C_4H_4N_2$). It including uracil, cytosine, and thymine

STRUCTURE OF PURINES AND PYRIMIDINES

- Pyrimidines have a six member ring. Purines have fused five and six member ring
- Each nucleic acid is synthesized from only four types of bases. The two types purines, Adenine and Guanine are present in both DNA and RNA. The two pyrimidines in DNA are Cytosine and Thymine, in RNA, Uracil is found instead of Thymine.
- The only difference between Uracil and Thymine is the presence of methyl substituent at C5. The bases are usually referred by their initial letters. So DNA contains A,G,C,T, while RNA contains A,G,C,U.
- In nucleic acids, the bases are covalently attached to the 1'-position of a pentose sugar ring, to form a *nucleoside*. The point of attachment of the base to sugar is the 1-position (N-1) of the pyrimidines and the 9-position of purines. The bond between the bases and the sugar is the *glycosylic (or glycosidic) bond*. If the sugar is ribose (RNA), the nucleosides are adenosine, guanosine, cytidine and uridine. If the sugar is deoxyribose (DNA), the nucleosides are deoxyadenosine, deoxyguanosine, deoxycytidine and deoxythymidine.
- A *nucleotide* is a nucleoside with one or more phosphate groups bound covalently to the 3', 5'-or the 2' position (in RNA only). If the sugar is dexoyribose, then the compounds are termed as *deoxynucleotides*. The phosphate group attached to the 5'-carbon of the sugar and upto three phosphate groups can attach at that position.
- During the synthesis of DNA or RNA, two phosphates are split off as pyrophosphate to leave one phosphate per nucleotide incorporated into the nucleic acid chain. The repeat unit of a DNA or RNA chain is hence a nucleotide.
- Nucleotides are linked together into a polynucleotide chain by a back bone consisting of an alternating series of sugar and phosphate residues. The 5' position of one pentose ring is connected to the 3' position of next pentose ring via phosphate group. Thus the phosphodiester sugar back bone is said to consists of 5'---- 3' linkages. The terminal nucleotide at one end of the chain has a 5' group, the terminal nucleotide at the other end has a free 3' group. It is conventional to write nucleotide sequences from the 5' direction towards the 3' terminus. A base sugar moiety is a nucleoside; a base sugar-phosphate moiety is nucleotide.
- View animation for Purines and Pyrimidines

BASE PAIRING BETWEEN NITROGENOUS BASES

Chargaff's Rule of Base Pairing between nucleotides of two strands

- The rules of base pairing (or nucleotide pairing) are:
 - **A with T:** the purine *adenine* (A) always pairs with the pyrimidine *thymine* (T).
 - **C with G:** the pyrimidine *cytosine* (C) always pairs with the purine *guanine* (G).
- This is consistent with there not being enough space (20 Å) for two purines to fit within the helix and too much space for two pyrimidines to get close enough to each other to form hydrogen bonds between them.

But why not A with C and G with T?

- The answer: only with A & T and with C & G are there opportunities to establish *hydrogen bonds* (shown here as dotted lines) between them (*two between A & T; three between C & G*). The ability to form hydrogen bonds makes the base pairs *more stable* structurally.
- These base pair relationships are often called *Chargaff's rules of DNA base pairing*, named after the Columbia University scientists who observed that there are equal molar concentration of A & T, as well as G & C in most DNA molecules.
- The rules of base pairing tell us that if we can "read" the sequence of nucleotides on one strand of DNA, we can immediately deduce the complementary sequence on the other strand.
- The rules of base pairing explain the phenomenon that whatever the amount of adenine (A) in the DNA of an organism, the amount of thymine (T) is the same (*Chargaff's rule*). Similarly, whatever the amount of guanine (G), the amount of cytosine (C) is the same.
- The **C+G : A+T** ratio varies from organism to organism among the prokaryotes, (but within particularly the limits of experimental error), **A = T** and **C = G**.

Relative Proportions (%) of Bases in DNA				
Organism	A	T	G	C
Human	30.9	29.4	19.9	19.8
Chicken	28.8	27.3	20.5	21.5
Grasshopper	29.3	29.3	20.5	20.7
Sea Urchin	32.8	32.1	17.7	17.3
Wheat	27.3	27.1	22.7	22.8
Yeast	31.3	32.9	18.7	17.1
E. coli	24.7	23.6	26.0	25.7

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DNA STRUCTURE

- Double helix model requires the two polynucleotide chains to run in opposite directions (antiparallel). Therefore one strand runs in the 5'---- 3' direction while the other strand runs in 3'----- 5'. The two polynucleotide strands are joined only by hydrogen bonds.
- Four different structural forms are present. They are A form, B form, and Z form. The B form was constructed by *Watson and Crick* and naturally present.

DNA REPLICATION

- **DNA replication** is to copy their strands. Each strand of original strand act as template to copy the information to synthesize the new strands. It is a fundamental process of all living organisms.

Steps in DNA replication

1. Separation of the two parental strands is the starting point of DNA replication.
2. Separation of strands happens in places of the chains which are rich in A-T.
3. **Helicase** is the enzyme that separates the two strands.
4. The initiation point where the separation starts is called "origin of replication".
5. The structure that is created is known as "**Replication Fork**".
6. Once the DNA strands separated, then, **RNA Primase** binds in the the initiation point of the 3'-5' parent chain. **RNA Primase** can attract RNA nucleotides which bind to the DNA nucleotides of the 3'-5' strand due to the hydrogen bonds between the bases. RNA nucleotides are the primers (starters) for the binding of DNA nucleotides.
7. The **elongation** process is different for the 5'-3' and 3'-5' template.
8. **5'-3' Template:** The 3'-5' proceeding daughter strand -that uses a **5'-3' template-** is called **leading strand** because **DNA Polymerase** can "read" the template and continuously adds nucleotides.
9. **3'-5' Template:** The **3'-5' template** cannot be "read" by DNA Polymerase. The replication of this template is complicated and the new strand is called **lagging strand**. In the lagging strand the RNA Primase adds more RNA Primers. **DNA polymerase** reads the template and lengthens. The gap between two RNA primers is called "**Okazaki Fragments**".
10. In the lagging strand the **DNA Pol I -exonuclease-** reads the fragments and removes the RNA Primers. The gaps are closed with the action of DNA Polymerase (adds complementary nucleotides to the gaps) and DNA Ligase (adds phosphate in the remaining gaps of the phosphate - sugar backbone).

RNA STRUCTURE

RNA structure

- Primary structure is the same as that of DNA, a polynucleotide chain with 5'----- 3' sugar phosphate linked.
- But generally it exists as a single polynucleotide chain rather than a double helix of antiparallel strands. However base pairing can take place within and between RNA molecule.
- RNA occurs as a single stranded molecule and in RNA, thymine is replaced by uracil. There are three types of RNA,
 - mRNA,
 - tRNA and
 - rRNA
 - Details of RNAs

mRNA (messenger RNA)

- mRNA has the same function in all the cells but there are difference in the synthesis and structure of prokaryotic and Eukaryotic mRNA
- In prokaryotes, mRNA is translated and transcribed in the single cellular compartment and the two processes occur simultaneously. Bacterial mRNA is unstable and therefore translated into proteins.
- In a eukaryotic cell, synthesis and maturation of mRNA occur in the nucleus. Only after maturation, mRNA is exported to the cytoplasm, where it is translated by ribosomes. Eukaryotic mRNA is more stable and continues to be translated for several hours.
- *Monocistronic*: Some mRNAs code for a single gene.
- *Polycistronic*: Some mRNAs which carry sequences coding for several proteins tRNA structure and function.

TOP

tRNA (transfer RNA)

- tRNAs are the adaptor molecules that deliver amino acids to the ribosome and decode the information in mRNA. The clover leaf structure is a common secondary structure of tRNA molecule. tRNA is a small molecule whose polynucleotide chain is only 75- 85 bases long.
 - Acceptor arm.
 - T Y C arm is named for the presence of a base Pseudouridine (Y stands for pseudouridine – modified base).
 - Anticodon arm always contains the anticodon triplet in the center of the loop.
- D – arm is named for its content of the base dihydrouridine.
- When tRNA is charged with the amino acid corresponding to its anticodon, it is called aminoacyl tRNA. The process of charging a tRNA is catalyzed by a specific enzyme, aminoacyl tRNA synthetase.

TOP

rRNA (ribosomal RNA)

- Ribosomal RNA (rRNA) is a component of the ribosomes, the protein synthetic factories in the cell. Eukaryotic ribosomes contain four different rRNA molecules: 18 s, 5.8 s, 28 s, and 5 s rRNA.
- Three of the rRNA molecules are synthesized in the nucleolus. rRNA molecules are extremely abundant. They make up at least 80% of the RNA molecules found in a typical eukaryotic cell.
- Synthesis of the three nucleolar rRNA molecules is unusual because they are made on one primary transcript that is chopped up into three mature rRNA molecules. These rRNA molecules and the 5 s rRNA combine with the ribosomal proteins in the nucleolus to form pre 40 s and pre 60 s ribosomal subunits. These pre-subunits are exported to the nucleus where they mature and assume their role in protein synthesis.
- The rRNA molecules have several roles in protein synthesis. First, the 28 s rRNA has a catalytic role, it forms part of the peptidyl transferase activity of the 60 s subunit. Second, 18s rRNA has a recognition role, involved in correct positioning of the mRNA and the peptidyl tRNA. Finally, the rRNA molecules have a structural role. They fold into three-dimensional shapes that form the scaffold on which the ribosomal proteins assemble.

TRANSCRIPTION

- Transcription generates a single stranded RNA identical in sequence with one of the strands of the duplex DNA.
- Several different types of RNA are generated by transcription: the three principal classes involved in the synthesis of proteins are
 - Messenger RNA (mRNA)
 - Transfer RNA (tRNA)
 - Ribosomal RNA (rRNA)
- Transcription is the synthesis of a single stranded RNA from a double stranded DNA template. RNA synthesis occurs in the 5' 3' direction and its sequence corresponds to that of the DNA strand which is known as the sense strand. The strand of DNA that directs the synthesis of mRNA via complementary base pairing is called the template strand or antisense strand. The other strand bears the same sequence as the mRNA is called the coding strand or sense strand. View image...

- There are 3 steps involved in transcription.
 - Initiation
 - RNA polymerase is the enzyme responsible for transcription. It binds to specific DNA sequences called promoters to initiate RNA synthesis. These sequences are upstream (to the 5'-end) of the region that codes for protein, and they contain short, conserved DNA sequences which are common to different promoters.
 - The RNA polymerase bind to the ds DNA at a promoter sequence, resulting in local DNA unwinding.
 - The position of the first synthesized base of the RNA is called the start site and is designated as position +1.
 - Elongation
 - RNA polymerase moves along the DNA and sequentially synthesizes the RNA chain.
 - DNA is unwound ahead of the moving polymerase, and the helix is reformed behind it.
 - Termination
 - RNA polymerase recognises the terminator which causes no further ribonucleotides to be incorporated. This sequence is commonly a hairpin structure. Some terminators require an accessory factor called rho for termination.

***E.coli* RNA Polymerase**

- RNA Polymerase is responsible for RNA synthesis (Transcription). A single type of RNA Polymerase is responsible for all synthesis of mRNA, rRNA and tRNA.
- The core enzyme consisting of 2 α , 1 β , 1 β' and 1 ϵ subunits, is responsible for transcription elongation. The sigma factor (σ) is also required for correct transcription initiation.
- The complete enzyme, consisting of the core enzyme plus the σ factor, is called the holoenzyme. Two alpha (α) subunits and one beta (β) subunit present in the RNA polymerase are involved in promoter binding.
- One beta prime (β') subunit present in the RNA polymerase is involved in template DNA binding.
- Sigma (σ) factor is a separate component from the core enzyme and is required for initiation at the correct promoter site. View image...

The *E.coli* promoter

- Promoters contain conserved sequences which are required for specific binding of RNA polymerase and transcription initiation. The promoter region extends for around 40 bp.
- The -10 sequence is a 6 bp region present in almost all promoters. This hexamer is generally 10bp upstream from the start site. The consensus -10 sequence is TATAAT and is important for unwinding. This is referred to as Pribnow box.
- The -35 sequence is a further 6 bp region recognizable in most promoters. This hexamer is typically 35 bp upstream from the start site. The consensus -35 sequence is TTGACA.

TRANSCRIPTION IN EUKARYOTES

- Three eukaryotic polymerases transcribe different sets of genes.
 - *RNA polymerase I (RNA Pol I)* is located in the nucleoli. It is responsible for the synthesis of the precursors of most rRNAs. The pre rRNA transcription units contain three sequences that encode the 18S, 5.8S, 28SrRNAs.
 - *RNA polymerase II (RNA Pol II)* is located in the nucleoplasm and is responsible for the synthesis of mRNA precursors and some small nuclear RNAs. Many RNA polymerase II promoters contain a sequence called **TATA box** which is situated 25-30bp upstream from the start site of transcription. The pre mRNAs are processed after synthesis by cap formation at the 5' end of RNA and poly(A) addition at the 3' end, as well as removal of introns by splicing.
 - *RNA polymerase III (RNA Pol III)* is located in the nucleoplasm. It is responsible for the synthesis of the precursors of 5S rRNA, tRNAs and small nuclear and cytosolic RNAs.
 - Each RNA polymerase has 12 or more subunits.
 - In Eukaryotes, transcription factors have a modular structure consisting of DNA binding and transcription activation domains. Some transcription factors have dimerization domains.

MODULE-5: GENETIC CODE

Learning objectives

The learner will learn about

- Genetic codes
- Translation
- Steps in translation
- Post-translational modifications

THE GENETIC CODE

- The genetic code is the correspondence between the sequence of four bases in nucleic acids and the sequence of the 20 amino acids in protein. The code is a triplet code where three nucleotides encode one amino acid. Since there are only 20 amino acids to be specified and potentially 64 triplets, most amino acids are specified by more than one triplet and the genetic code is said to be degenerate or to have redundancy.
- The genetic code is degenerate, this is because 18 out of 20 amino acids have more than one codon to specify them, called *synonymous* codons. Only methionine and tryptophan have single codons.
- Among the 64 triplet codons, only the 61 codons represent amino acids and 3 codons cause termination. The three codons UAA, UAG and UGA that do not represent amino acids are used specifically to terminate protein synthesis. These three codons are called stop codons or termination codons (non sense codons). UAG is known as amber, UAA as ochre and UGA is sometimes called the opal codon.
- The genetic code is universal and exceptions to the universal genetic code also occur in the mitochondria from several species.

First position (5' end)	Second position				Third position (3' end)
	U	C	A	G	
U	UUU Phe	UCU Ser	UAU Tyr	UGU Cys	U
	UUC Phe	UCC Ser	UAC Tyr	UGC Cys	C
	UUA Leu	UCA Ser	UAA Stop	UGA Stop	A
	UUG Leu	UCG Ser	UAG Stop	UGG Trp	G
C	CUU Leu	CCU Pro	CAU His	CGU Arg	U
	CUC Leu	CCC Pro	CAC His	CGC Arg	C
	CUA Leu	CCA Pro	CAA Gln	CGA Arg	A
	CUG Leu	CCU Pro	CAG Gln	CGG Arg	G
A	AUU Ile	ACU Thr	AAU Asn	AGU Ser	U
	AUC Ile	ACC Thr	AAC Asn	AGC Ser	C
	AUA Ile	ACA Thr	AAA Lys	AGA Arg	A
	AUG Met	ACG Thr	AAG Lys	AGG Arg	G
	Start				
G	GUU Val	GCU Ala	GAU Asp	GGU Gly	U
	GUC Val	GCC Ala	GAC Asp	GGC Gly	C
	GUA Val	GCA Ala	GAA Glu	GGA Gly	A
	GUG Val	GCG Ala	GAG Glu	GGG Gly	G

CODON- ANTI CODON INTERACTION

- The anticodon at one end of the tRNA interacts with the complementary triplet bases on the mRNA, the codon, when both are brought together in the cleft of the ribosome.

INITIATOR tRNA

- A special tRNA (initiator tRNA), recognizing the AUG start codon, is used to initiate protein synthesis in both prokaryotes and eukaryotes.
- In prokaryotes, the initiator tRNA is first charged with methionine by methionyl - tRNA synthetase.

MECHANISM OF PROTEIN SYNTHESIS

- The actual mechanism of protein synthesis can be divided into three stages.
 - Initiation - assembly of a ribosome on an mRNA molecule.
 - Elongation - repeated cycles of aminoacid addition.
 - Termination - the release of the new protein chain.
- In prokaryotes, the factors are abbreviated as IF or EF for initiation and elongation factors respectively, whereas in eukaryotes they are called eIF and eEF.

INITIATION

- The purpose of initiation step is to assemble a complete ribosome on to an mRNA molecule at the start point, the initiation codon.
- The components involved are the large and small subunits, the mRNA, the initiator tRNA in its charged form, the three initiation factors (IF1, IF2 and IF3) and GTP.
- IF1 and IF2 bind to a free 30S subunit. This helps to prevent a large subunit binding to it without an mRNA molecule and forming an inactive ribosome.
- IF3 complexed with GTP then binds to the small subunit. It will assist the charged initiator tRNA to bind.
- The assembled ribosome has two tRNA binding sites. These are called A-site (or acceptor) for aminoacyl and P-site (or donor) and peptidyl sites. The A-site is where incoming aminoacyl - tRNA molecules bind and the P-site where the growing polypeptide chain is usually found.
- One major outcome of initiation is the placement of the initiator tRNA in the P-site.

ELONGATION

- With the formation of 70S initiation complex, the elongation cycle can begin. It can be subdivided into *three steps*,
 - Aminoacyl-tRNA delivery
 - Peptide bond formation
 - Translocation (movement)
- It involves three elongation factors. Ef-Tu, Ef-Ts and Ef-G which will bind GTP or GDP.

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TRANSLATION IN EUKARYOTES

Initiation in Eukaryotes

- Most of the differences in the mechanism of protein synthesis between prokaryotes and eukaryotes occur in the initiation stage. Eukaryotes have atleast 9 eIF.

- Eukaryotic initiator tRNA does not become formylated as in prokaryotes.
- Since there is no Shine-Dalgarno sequence in Eukaryotic mRNA, the 40S subunit containing the initiator tRNA attaches to the 5' end of the mRNA and scans along the mRNA until it finds an appropriate AUG. (Kozak's scanning mechanism)

Elongation

- The factors eEF1 a ,eEF1 b g and eEF2 are involved in elongation.

Termination

- Eukaryotes have only one release factor eRF which recognise all the stop codons.

POST- TRANSLATIONAL MODIFICATIONS

- A translated polypeptide does not always immediately generate a functional protein (inactive).
- Apart from correct folding and the possible formation of disulfide bonds, there are number of other alterations that may be required for activity. These include cleavage and various covalent modifications.
- Cleavage is very common, especially trimming by amino – and carboxy peptidases, but the removal of internal peptides also occurs.
- Chemical modifications are many and varied and have been shown to take place on the N and C termini, as well as on most of the 20 aminoacid side chains with the exception of Alanine, Glycine. Isoleucine, Leucine, Methionine and Valine.
- The modifications include acetylation, hydroxylation, Phosphorylation, methylation, Glycosylation and even the addition of nucleotides. Often phosphorylation controls the activity of protein.

WOBBLE SYNTHESIS

- The wobble hypothesis which states that the pairing between codon and anticodon at the first two codon at the two codon position always follows the usual rule but that exceptional wobble occur at the third position. tRNAs can recognise either one, two or three codons excluding stop codons depending on their wobble base (the 5' anticodon base).

Base in first position	Bases recognized in third of anticodon position of codon
U	A or G
G only	C
A	U only
G	C or U

MODULE-6: RECOMBINANT DNA TECHNOLOGY

Learning objectives

The learner will learn about

- Definition of recombinant DNA
- Recombinant DNA technique
- Purposes and applications of recombinant DNA technique

RECOMBINANT DNA

- *Recombinant DNA* refers to DNA which has been altered by joining genetic material from two different sources.

- It usually involves joining a gene sequences from one organism into the genome of a different organism, generally of a different species.

RECOMBINANT DNA TECHNIQUE

- *Recombinant DNA techniques* otherwise called as *gene cloning* is the technique which facilitate the formation of chimeric DNA by fusing DNAs from different sources.
- This is achieved by using specific restriction enzymes for cutting the DNA into suitable fragments and then joining together the appropriate fragments using DNA ligase enzyme.

PURPOSE OF RECOMBINANT DNA TECHNIQUE

- The purposes of recombinant DNA technique includes :
 - To obtain a large number of copies of specific DNA fragments
 - To recover large quantities of the protein produced by the concerned gene
 - To integrate the gene in question into the chromosome of a target organism where it expresses itself.
- Even for the latter two objectives, it is essential to first obtain a large number of copies of the concerned genes

RESTRICTION ENZYMES

- A **Restriction Enzyme** (or **restriction endonuclease**) is an enzyme that cuts double-stranded DNA at specific recognition nucleotide sequences known as restriction sites. Restriction enzymes are found in bacteria.
- To cut the DNA, a restriction enzyme makes two incisions, once through each sugar-phosphate backbone (i.e. each strand) of the DNA double helix.
- Recognition sequences vary between 4 and 8 nucleotides, many of them are palindromic
- Recognition sequences in DNA differ for each restriction enzyme, producing differences in the length, sequence and strand orientation
- There are four classes of restriction endonucleases: types I, II, III and IV. All types of enzymes recognize specific short DNA sequences and carry out the endonucleolytic cleavage of DNA to give specific double-stranded fragments with terminal 5'-phosphates.
- Type II are the most commonly available and used restriction enzymes.
- Each enzyme is named after the bacterium from which it was isolated using a naming system based on bacterial genus, species and strain.
- For example, *EcoRI*
 - E – Escherichia –Genus
 - Co – coli –species
 - R-strain
 - I-order of identification

Applications of Restriction enzymes

- Restriction enzymes are used to assist insertion of genes into plasmid vectors during gene cloning and protein expression experiments.
- Restriction enzymes are used to digest genomic DNA for gene analysis by Southern blot
- Restriction enzymes can also be used to distinguish gene alleles by specifically recognizing single base changes in DNA known as single nucleotide polymorphisms (SNPs).

USES OF RESTRICTION ENZYMES

- Restriction enzymes are generally used to manipulate DNA for different scientific applications which includes,
 - To assist insertion of genes / DNA fragments into plasmid vectors in gene cloning and subsequent protein expression experiments
 - To digest genomic DNA for gene analysis by Southern blot to identify number of copies of gene present in an individual
 - To digest genomic DNA for gene analysis experiments such as restriction fragment length polymorphism (RFLP) to identify the polymorphism in the population
 - To distinguish gene alleles by specifically recognizing single base changes in DNA known as single nucleotide polymorphisms (SNPs).

- To digest large DNA molecules into fragments less than 300 base pairs and these fragments can then be used for sequence analysis and arranged to understand the gene structure and its regulation at molecular level.

VECTOR DNA INSERT AND HOST

- Vector" is an agent that can carry a DNA fragment into a host cell. If it is used for reproducing the DNA fragment, it is called a "*cloning vector*". If it is used for expressing certain gene in the DNA fragment, it is called an "*expression vector*".
- The DNA segment to be cloned is called *DNA insert*.
- Recombinant DNAs are introduced into a suitable organism, usually a bacterium; this organism is called *host*, while the process is called transformation. The transformed host cells are selected and cloned.

STEPS IN RECOMBINANT DNA TECHNIQUE / GENE CLONING

- The entire procedure of gene cloning or recombinant DNA technology may be classified into the following five steps
 - Identification and isolation of the desired gene or DNA fragment to be cloned.
 - Insertion of the isolated gene in a suitable vector.
 - Introduction of this vector into a suitable organism/cell called host (transformation).
 - Selection of the transformed host cells.
 - Multiplication /expression/integration followed by expression of the introduced gene in the host.

APPLICATION OF RECOMBINANT DNA TECHNOLOGY

- The first products available through rDNA technology were proteins, such as insulin and human growth hormone.
- These products were relatively simple to produce using genetically engineered *Escherichia coli* bacteria.
- They were followed by more complicated proteins which treated conditions such as blood clots, anemia and hemophilia.
- These more complex proteins required mammalian cell cultures, which are more complicated and costly to produce.
- In addition, rDNA technology is now being used to produce vaccines.

Vaccines

- Biotechnology can be employed to create vaccines that have no pathogenic potential or extraneous material. Vaccines created using rDNA technology introduce plasmid DNA directly into individuals to elicit an immune response.
- It appears that even very small amounts of protein are sufficient to induce immune responses following DNA immunization, and thus, this approach to vaccine development appears to be applicable to a broad number of diseases.
- While the development of biotechnologically derived vaccines is expensive, there appear to be advantages to this approach to immunization.
- Because the immune response is induced by a single gene, rather than the entire organism, infection of the vaccinated individual or infection spreading to others is unlikely.
- Delivery and administration of vaccines in the form of edible transgenic fruits, vegetables or even grains has become a real possibility and an attractive alternative to traditional injectable vaccines.
- Edible vaccines will offer advantages such as ease of production, noninvasive application, and low-budget storage and delivery.
- Potatoes, tomatoes, bananas, soybeans, alfalfa and cereals are the most common foods proposed for edible vaccine delivery.

MODULE-7: GENE CLONING VECTORS

Learning objectives

The learner will learn about

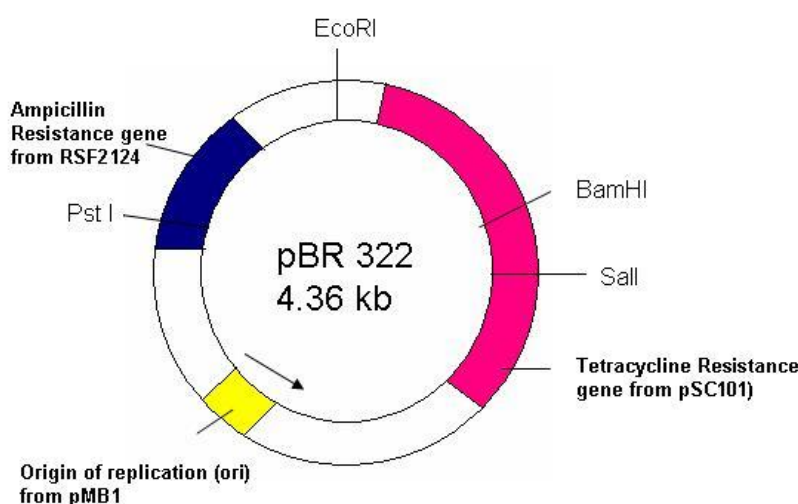
- Cloning vector
- Features of cloning vector
- Types of cloning vector

CLONING VECTOR

- A cloning vector is a DNA molecule that carries foreign DNA into a host cell (usually bacterial or yeast), where it replicates, producing many copies of itself along with the foreign DNA.

FEATURES OF CLONING VECTORS

- Sequences that will permit the propagation of itself in bacteria or in yeast.
- A cloning site to insert the foreign DNA; the most versatile vectors contain a site that can be cut by many restriction enzymes.
- A method of selecting for bacteria or yeast containing a vector with foreign DNA; this is usually accomplished by selectable markers for drug resistance.



GENERAL STEPS IN CLONING

- Prepare the vector and the DNA to be cloned by digestion with restriction enzymes to generate complementary ends.
- Ligate (join) the foreign DNA into the vector with the enzyme DNA ligase.
- Introduce the DNA into bacterial cells or yeast cells by transformation.
- Select cells containing foreign DNA by screening for selectable markers (commonly drug resistance).

Steps in cloning

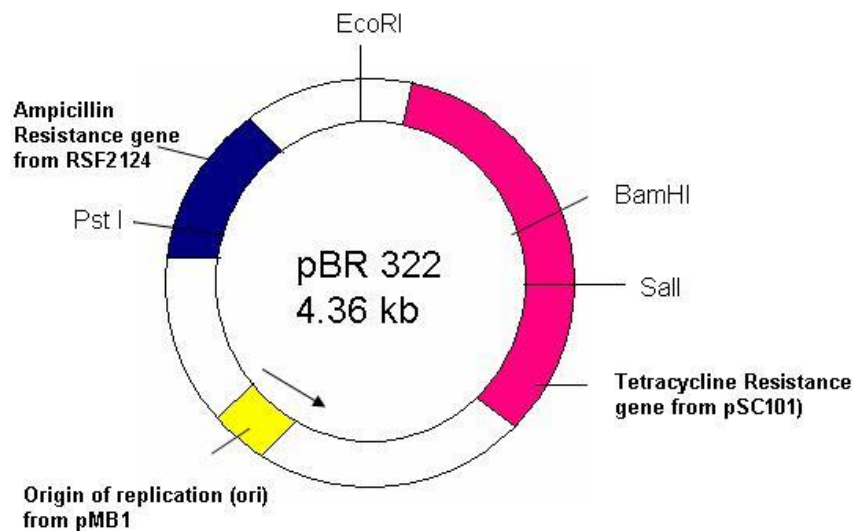
TYPES OF CLONING VECTORS

- Plasmid
- Phage
- Cosmid
- Bacterial Artificial Chromosomes (BAC)
- Yeast Artificial Chromosomes (YAC)

PLASMIDS

- Plasmids are small, circular, extrachromosomal DNA molecules found in bacteria, which can replicate on their own, outside of a host cell.
- They have a cloning limit of 100 to 10,000 base pairs or 0.1-10 kilobases (kb).

- A plasmid vector is made from natural plasmids by removing unnecessary segments and adding essential sequences.
- Plasmids make excellent cloning vectors for various laboratory techniques, including recombinant DNA.

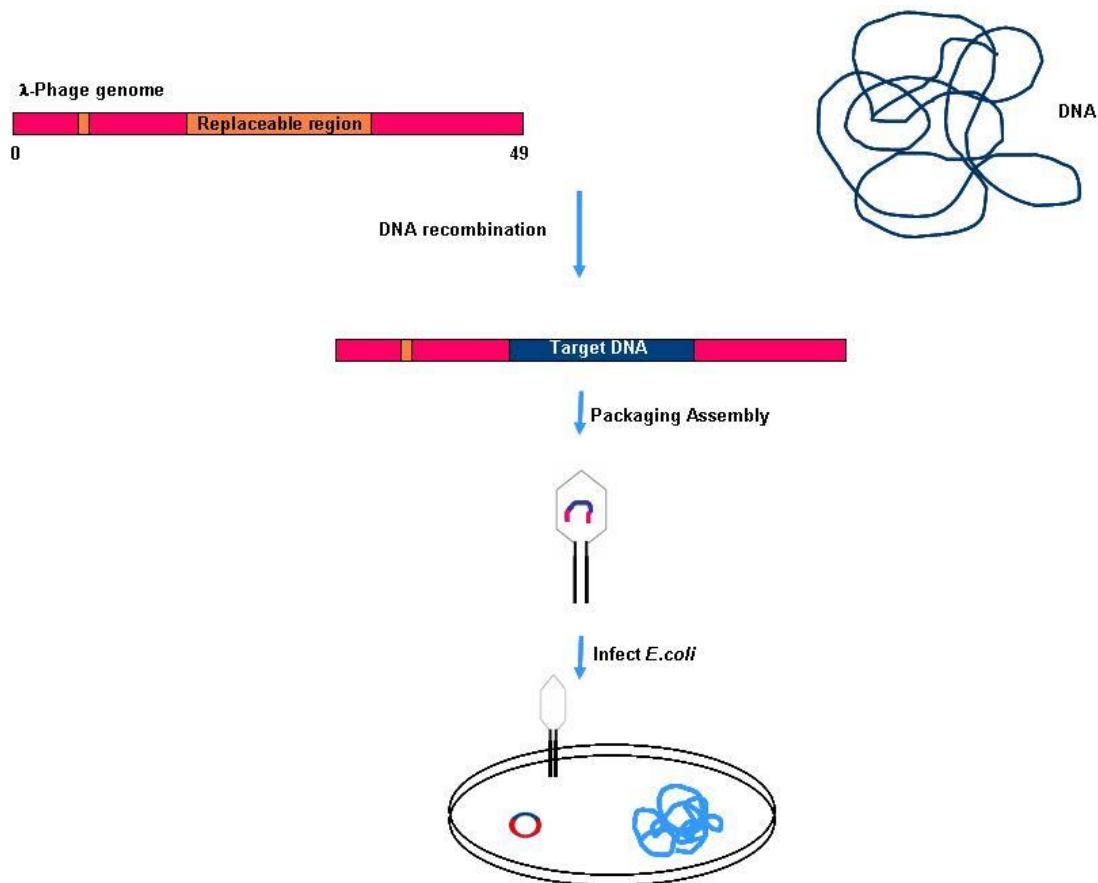


Features of pBR322

- pBR322 is a plasmid and the most commonly used *E. coli* cloning vectors.
- pBR322 was the first artificial plasmid, created in 1977.
- It was named after its Mexican creators, p standing for plasmid, and BR for Bolivar and Rodriguez.
- pBR322 is 4361 base pairs in length and contains a replicon region (source plasmid pMB1), the ampR gene, encoding the ampicillin resistance protein (source plasmid [RSF2124]) and the tetR gene, encoding the tetracycline resistance protein (source plasmid pSC101).
- The plasmid has unique restriction sites for more than forty restriction enzymes.
- 11 of these 40 sites lie within the tetR gene.
- There are 2 sites for restriction enzymes HindIII and ClaI within the promoter of the tetR gene.
- There are 6 key restriction sites inside the ampR gene.
- The origin of replication or ori site in this plasmid is pMB1 (a close relative of ColE1).
- The ori encodes two RNAs (RNAI and RNAII) and one protein (called Rom or Rop).
- The circular sequence is numbered such that 0 is the middle of the unique EcoRI site and the count increases through the tet genes.
- The ampicillin resistance gene is a penicillin beta-lactamase.
- Promoters P1 and P3 are for the beta-lactamase gene.

PHAGES

- Phages are derivatives of bacteriophage lambda (λ phage), a virus which infects *E. coli*.
- They are linear DNA molecules, whose region can be replaced with foreign DNA without disrupting its life cycle.
- The major advantage of the λ phage vector is its high transformation efficiency, which is about 1000 times greater than that of the plasmid vector. They also have a larger cloning limit than plasmids, consisting of 8-25 kb.

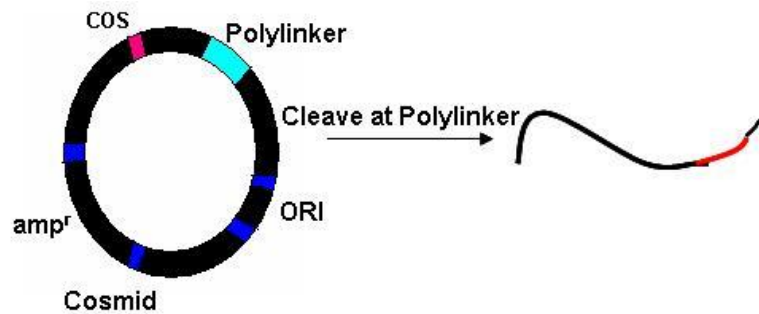


Phage vector

- **Lambda phage vector**
 - Lambda phages are viruses that can infect bacteria. The major advantage of the phage vector is its high transformation efficiency, about 1000 times more efficient than the plasmid vector.
 - The extreme ends of the lambda DNA are known as COS sites, each is single stranded, 12 nucleotides long.
 - Because their sequences are complementary to each other, one end of lambda DNA may base-pair with the other end of a different lambda DNA, forming concatemers.
 - The two ends of a lambda DNA may also bind together, forming a circular DNA.
 - In the host cell, the lambda DNA circularizes because ligase may seal the join of the COS sites.
 - In the assembly process of lambda virions, two proteins Nu1 and A can recognize the COS site, directing the insertion of the lambda DNA between them into an empty head.
 - The filled head is then attached to the tail, forming a complete lambda virion.
 - The whole process normally takes place in the host cell. However, to prepare the lambda virion carrying recombinant lambda DNA, the following in vitro assembly system is commonly used.
 - Proteins Nu1 and A are encoded by the genes in the lambda genome. If the two genes are mutated, lambda DNA cannot be packaged into the pre-assembled head. Because tails attach only to filled heads, the cell will accumulate separate empty heads and tails, which can then be extracted. When the extract is mixed with recombinant lambda DNA and proteins Nu1 and A, the complete lambda virion carrying recombinant lambda DNA will be assembled.

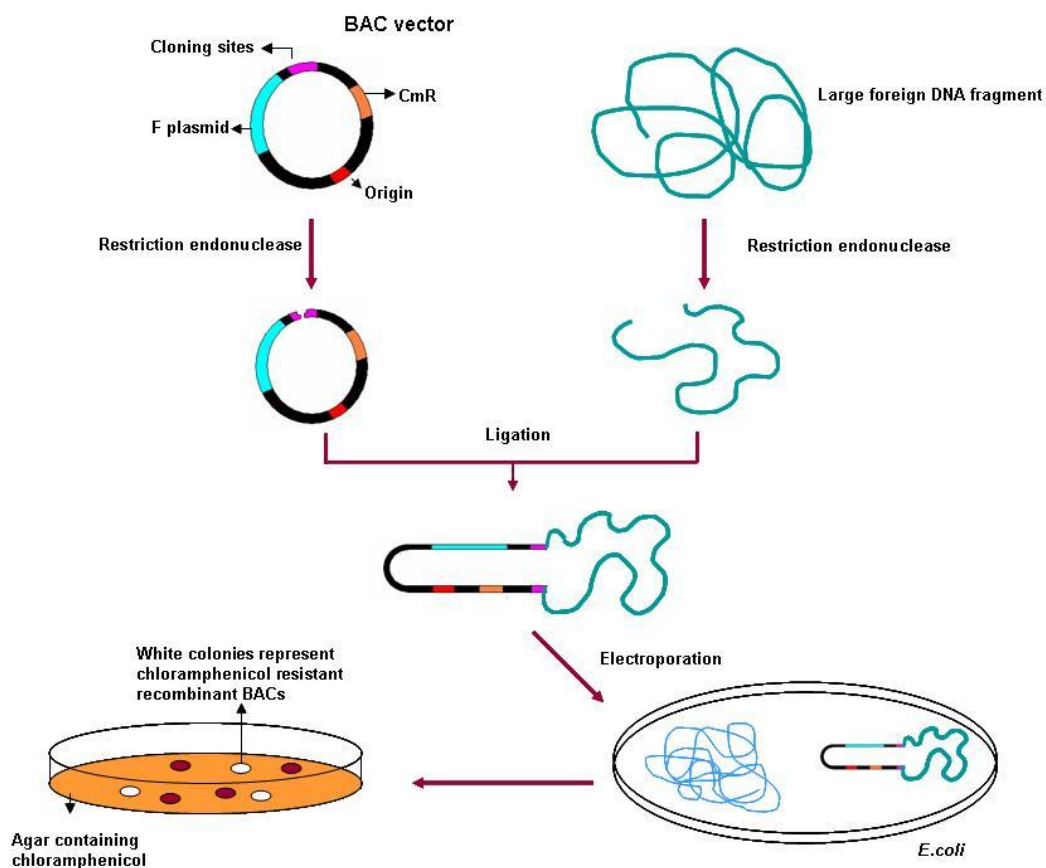
COSMIDS

- Cosmids are extrachromosomal circular DNA molecules that combine features of plasmids and phages. They also have a high transformation efficiency, and their cloning limit of 35-50 kb is larger than that of plasmids or phages.
- The cosmid vector is a combination of the plasmid vector and the COS site of lambda phage which allows the target DNA to be inserted into the lambda head.
- It has the following advantages:
 - High transformation efficiency.
 - The cosmid vector can carry up to 45 kb whereas plasmid and lambda phage vectors are limited to 25 kb.



BACTERIAL ARTIFICIAL CHROMOSOME

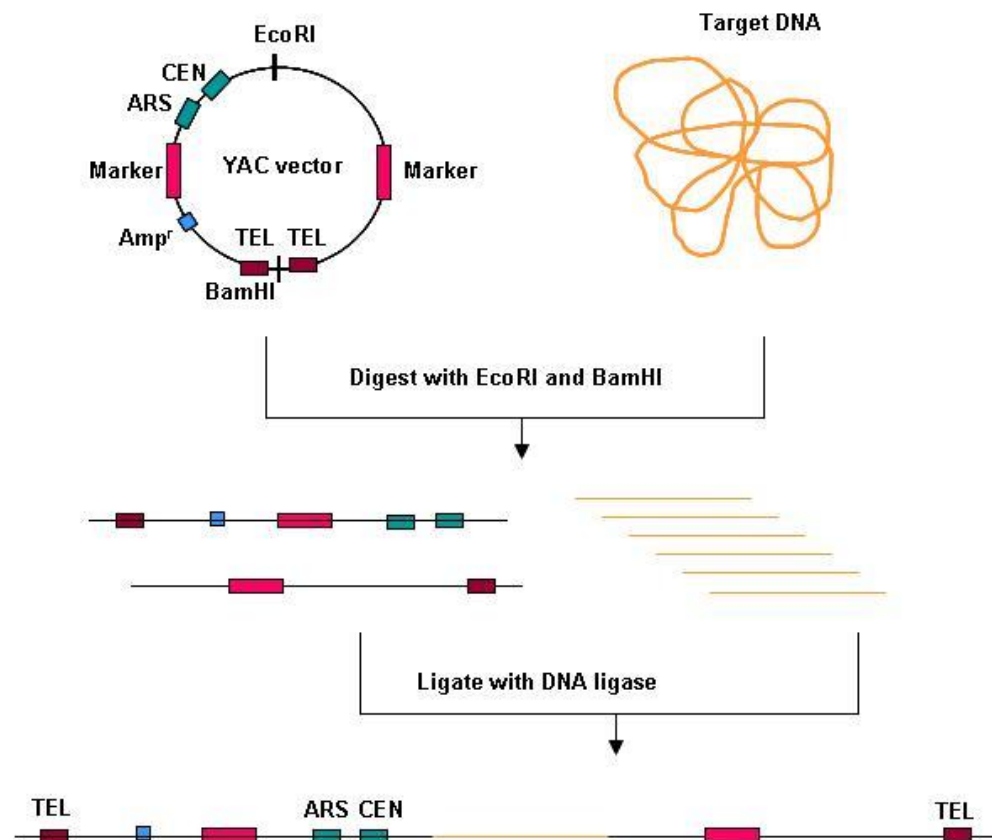
- BACs are based on bacterial mini-F plasmids, which are small pieces of episomal bacterial DNA that give the bacteria the ability to initiate conjugation with adjacent bacteria. They have a cloning limit of 75-300 kb.
- BACs are often used to sequence the genome of organisms in genome projects, for example the Human Genome Project.
- A short piece of the organism's DNA is amplified as an insert in BACs, and then sequenced.
- Finally, the sequenced parts are rearranged in silico, resulting in the genomic sequence of the organism.



YEAST ARTIFICIAL CHROMOSOME (YAC)

- YACs are artificial chromosomes that replicate in yeast cells.
- They consist of
 - Telomeres (TEL), which are ends of chromosomes involved in the replication and stability of linear DNA.
 - Origin of replication sequences necessary for the replication in yeast cells.
 - A yeast centromere (CEN), which is a specialized chromosomal region where spindle fibers attach during mitosis.
 - Autonomous replicating sequence (ARS).
 - A selectable marker for identification in yeast cells.
 - Ampicillin resistance gene for selective amplification.

- Recognition sites for restriction enzymes.
- The yeast artificial chromosome (YAC) vector is capable of carrying a large DNA fragment (up to 2 Mb), but its transformation efficiency is very low.
- Steps in YAC cloning
 - The target DNA is partially digested by EcoRI and the YAC vector is cleaved by EcoRI and BamHI.
 - Ligate the cleaved vector segments with a digested DNA fragment to form an artificial chromosome.
 - Transform yeast cells to make a large number of copies.



MODULE-8: RECOMBINANT GENE EXPRESSION

Learning objectives

The learner will learn about

- Expression vectors
- Expression systems

EXPRESSION VECTORS

- An expression vector, otherwise known as an expression construct, is generally a plasmid that is used to introduce a specific gene into a target cell.
- Once the expression vector is inside the cell, the protein that is encoded by the gene is produced by the cellular-transcription and translation machinery.
- The plasmid is frequently engineered to contain regulatory sequences that act as enhancer and promoter regions and lead to efficient transcription of the gene carried on the expression vector.
- The goal of a well-designed expression vector is the production of large amounts of stable messenger RNA.
- After expression of the gene product, the purification of the protein is required; but since the vector is introduced to a host cell, the protein of interest should be purified from the proteins of the host cell. Therefore, to make the purification process easy, the cloned gene should have a tag. This tag could be histidine (His) tag or any other marker peptide. View image...

Essential features of an expression vector

1. **Selectable marker.** In the absence of selective pressure plasmids are lost from the host. Especially in the case of very high copy number plasmids and when plasmid-borne genes are toxic to the host or otherwise significantly reduce its growth rate. The simplest way to address this problem is to express from the same plasmid an antibiotic-resistance marker and supplement the medium with the appropriate antibiotic to kill plasmid-free cells.
2. **Regulatory gene (repressor).** Many promoters show leakiness in their expression *i.e.* gene products are expressed at low level before the addition of the inducer. This becomes a problem when the gene product is toxic for the host. This can be prevented by the constitutive expression of a repressor protein. The **lac**-derived promoters are especially leaky. These promoters can be controlled by the insertion of a **lac**-operator sequence.
3. **Origin of replication.** The origin of replication controls the plasmid copy number
4. **Promoter.** The promoter initiates transcription and is positioned 10-100 nucleotides upstream of the ribosome binding site. The ideal promoter exhibits several desirable features:
 - It is strong enough to allow product accumulation up to 50% of the total cellular protein.
 - It has a low basal expression level (*i.e.* it is tightly regulated to prevent product toxicity).
 - It is easy to induce.
5. **Transcription terminator.** The transcription terminator reduces unwanted transcription and increases plasmid and mRNA stability
6. **Shine-Dalgarno sequence.** The Shine-Dalgarno (SD) sequence is required for translation initiation and is complementary to the 3'-end of the 16S ribosomal RNA. The efficiency of translation initiation at the start codon depends on the actual sequence. The consensus sequence is: 5'- **TAAGGAGG** -3'. It is positioned 4-14 nucleotides upstream the start codon with the optimal spacing being 8 nucleotides. To avoid formation of secondary structures (which reduces expression levels) this region should be rich in A residues
7. **Start codon.** Initiation point of translation. In *E. coli* the most used start codon is **ATG**. **GTG** is used in 8% of the cases. **TTG** and **TAA** are hardly used.
8. **Tags and fusion proteins.** N- or C-terminal fusions of heterologous proteins to short peptides (tags) or to other proteins (fusion partners) offer several potential advantages:
 - **Improved expression.** Fusion of the N-terminus of a heterologous protein to the C-terminus of a highly-expressed fusion partner often results in high level expression of the fusion protein.
 - **Improved solubility.** Fusion of the N-terminus of a heterologous protein to the C-terminus of a soluble fusion partner often improves the solubility of the fusion protein.
 - **Improved detection.** Fusion of a protein to either terminus of a short peptide (epitope tag) or protein which is recognized by an antibody or a binding protein (Western blot analysis) or by biophysical methods (*e.g.* GFP by fluorescence) allows for detection of a protein during expression and purification.
 - **Improved purification.** Simple purification schemes have been developed for proteins fused at either end to tags or proteins which bind specifically to affinity resins
9. **Stop codon.** Termination of translation. There are 3 possible stop codons but **TAA** is preferred because it is less prone to read-through than **TAG** and **TGA**. The efficiency of termination is increased by using 2 or 3 stop codons in series.

EXPRESSION SYSTEMS

- There are two main systems for the expression of recombinant protein.
- This will be either a prokaryotic (bacterial) or eukaryotic (usually yeast or mammalian cell) system.
- The choice of expression system will decide which vector is needed to clone DNA into as there are different promoters which function in *E. coli* and others that work best with yeast or mammalian systems.

PROKARYOTIC PROTEIN EXPRESSION SYSTEMS

- Prokaryotic recombinant protein expression systems have several advantages.
 - Ease of culture, and very rapid cell growth.
 - Expression can be induced easily in bacterial protein expression systems using IPTG.
 - Purification is quite simple in prokaryotic expression systems and there are several commercial kits available for recombinant protein expression.
- On the other hand, if the expressed proteins have to be used for functional or enzymatic studies, prokaryotic systems are a problem as most proteins become insoluble in inclusion bodies and are very difficult to recover as functional proteins.
- Furthermore, most if not all post-translational modifications are not added by bacteria and therefore the recombinant protein of interest may not be functional. Enzymatic studies thus may be unfruitful.

EUKARYOTIC PROTEIN EXPRESSION SYSTEMS

- Eukaryotic genes are not really “at home” in prokaryotic cells, even when they are expressed under the control of the prokaryotic vectors. One reason is that *E. coli* cells frequently recognize the protein products of cloned eukaryotic genes as outsiders and destroy them.
- Another is that prokaryotes do not carry out the same kinds of posttranslational modification as eukaryotes do. For example, a protein that would ordinarily be coupled to sugars in a eukaryotic cell will be expressed as a bare protein when cloned in bacteria. This can affect a protein’s activity or stability, or at least its response to antibodies.
- Another problem is that the interior of a bacterial cell is not as conducive to proper folding of eukaryotic proteins as the interior of a eukaryotic cell. Frequently, the result is improperly folded, inactive products of cloned genes.
- Eukaryotic systems for the expression of protein include:
 - Yeast
 - Mammalian cells
 - Baculovirus cells (insect)
- All these systems are great eukaryotic systems for the expression of recombinant proteins.
- Advantages of eukaryotic protein expression systems include the fact that very high levels of expression. The proteins are easy to purify using special tags which are included into the vectors including His, and other tags.
- The disadvantages of eukaryotic protein expression systems include the fact that eukaryotic cells do grow slower than prokaryotic cells.

EXPRESSION VECTORS WITH STRONG PROMOTERS

- The main function of an expression vector is to yield the product of a gene- usually, the more product the better.
- Therefore, expression vectors are ordinarily equipped with very strong promoters; the rationale is that the more mRNA that is produced, the more protein product will be made.
- One such strong promoter is the trp (tryptophan operon) promoter. It forms the basis for several expression vectors. It has a trp promoter/operator region, followed by a ribosome binding site, and can be used directly as an expression vector by inserting a foreign gene into the *ClaI* site.
- Alternatively, the trp control region can be made “portable” by cutting it out with *ClaI* and *HindIII* and inserting it in front of a gene to be expressed in another vector

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EXPRESSION VECTORS THAT PRODUCE FUSION PROTEINS

- When most expression vectors operate, they produce fusion proteins. This might at first seem a disadvantage because the natural product of the inserted gene is not made. However, the extra amino acids on the fusion protein can be a great help in purifying the protein product.
- Consider the oligo-histidine expression vectors, one of which has the trade name pTrcHis. These have a short sequence just upstream of the multiple cloning site that encodes a stretch of six histidines.
- Thus, a protein expressed in such a vector will be a fusion protein with six histidines at its amino end.
- The advantage of this, is oligo-histidine regions like this have a high affinity for metals like nickel, so the expressed proteins can be purified by using nickel affinity chromatography.
- After the bacteria have made the fusion protein, simply lyse them, add the crude bacterial extract to a nickel affinity column, wash out all unbound proteins, then release the fusion protein with histidine or a histidine analog called imidazole.
- This procedure allows to harvest essentially pure fusion in only one step.
- This is possible because very few if any natural proteins have oligo-histidine regions, so the fusion protein is essentially the only one that binds to the column.

USES OF RECOMBINANT DNA TECHNOLOGY IN HUMAN HEALTH

Recombinant DNA technology has resulted in a series of medical products. The first two commercially prepared products from recombinant DNA technology were insulin and human growth hormone. Below are the lists of recombinant DNA products which are used successfully for the benefits of human being:

- Recombinant human insulin
 - Recombinant insulin has almost completely replaced insulin obtained from animal sources (e.g. pigs and cattle) for the treatment of insulin-dependent diabetes. A variety of different recombinant insulin preparations are in widespread use.
- Recombinant human growth hormone (HGH, somatotropin)
 - Growth hormone is administered to patients whose pituitary glands generate insufficient quantities to support normal growth and development. Before recombinant HGH became available, HGH for therapeutic use was obtained from pituitary glands of cadavers.
- Recombinant blood clotting factor VIII
 - Recombinant factor VIII is a blood-clotting protein that is administered to patients with forms of the bleeding disorder hemophilia, who are unable to produce factor VIII in quantities sufficient to support normal blood coagulation. Before the development of recombinant factor VIII, the protein was obtained by processing large quantities of human blood from multiple donors, which carried a very high risk of transmission of blood borne infectious diseases, for example HIV and hepatitis B.
- Recombinant hepatitis B vaccine
 - Prevention of hepatitis B infection is controlled through the use of a recombinant hepatitis B vaccine, which contains a form of the hepatitis B virus surface antigen that is produced in yeast cells.
- Diagnosis of infection with HIV
 - Each of the three widely-used methods for diagnosing HIV infection has been developed using recombinant DNA. The antibody test (ELISA or western blot) uses a recombinant HIV protein to test for the presence of antibodies that the body has produced in response to an HIV infection.
- Applications of recombinant DNA technology in Forensic Sciences
 - The applications of molecular biology in forensics center largely on the ability of DNA analysis to identify an individual from hairs, blood stains and other items recovered from the crime scene.

USES OF RECOMBINANT DNA TECHNOLOGY IN ANIMAL SCIENCES

Transgenic animals

- Recombinant DNA technology can be used to introduce foreign genes into organisms for the expression of specific new traits. Animals can also be engineered for a variety of purposes. One such thing is transgenic animals. Transgenic animals not only provide invaluable research tools for studying gene regulation and disease, but they may be genetically modified for the production of pharmaceuticals, vaccines and rare chemicals as well as for food production. Genetically engineered livestock (bioreactors) will yield important products in milk or blood for treating a variety of human diseases and health needs. Among such products might be:
 - Human hemoglobin which could be used during trauma when much blood is lost. Hemoglobin is more desirable than whole blood or red blood cells for transfusions, since it requires no refrigeration and is compatible with all blood types, eliminating the need for blood typing.
 - Human protein C, which helps prevent blood clotting.
 - Human tissue plasminogen activator, which is used to treat patients after a heart attack.
 - Human alpha-1-antitrypsin, which may be useful in treating the people who have α_1 AT-deficiency which predisposes them to a life-threatening type of emphysema.

Vaccines and Diagnostics

- Recombinant DNA technology may be the only way of preventing some of the more widespread and devastating animal diseases found in many developing countries.
- Monoclonal antibodies and recombinant vaccines produced by this technology are effective against deadly bacterial and viral diseases.
- Genetic engineering enables immunogenic proteins produced in bacterial and yeast cells to be used as vaccines that offer several advantages over traditionally produced vaccines and it can dramatically reduce production costs. A pure protein is often produced, eliminating the need for extensive and costly testing since the disease-causing pathogen is not present.

- Recombinant DNA technology also eliminates the need for inactivated vaccine by enabling the antigen to be synthesized separately from the pathogen.
- The genes of different proteins can be fused so that a variety of antigens are present in a single vaccine.
- New disease diagnostic kits based on recombinant DNA technology are available for animal diseases and are more sensitive, rapid and safe.

USES OF RECOMBINANT DNA TECHNOLOGY IN AGRICULTURE

- Crop plants have been and continue to be the focus of biotechnology as efforts are made to improve yield and profitability by improving crop resistance to insects and certain herbicides and delaying ripening (for better transport and spoilage resistance).
- Below are the examples of successful applications of recombinant DNA technology in agriculture:
 - Golden rice
 - It is a recombinant variety of rice that has been engineered to express the enzymes responsible for β -carotene biosynthesis. This variety of rice holds substantial promise for reducing the incidence of vitamin A deficiency in the world's population. Golden rice is not currently in use, pending the resolution of intellectual property, environmental and nutritional issues.
 - Herbicide-resistant crops
 - Commercial varieties of important agricultural crops (including soy, maize/corn, sorghum, canola, alfalfa and cotton) have been developed which incorporate a recombinant gene that results in resistance to the herbicide glyphosate (trade name *Roundup*), and simplifies weed control by glyphosate application. These crops are in common commercial use in several countries.
 - Insect-resistant crops
 - *Bacillus thuringiensis* is a bacterium that naturally produces a protein (Bt toxin) with insecticidal properties. The bacterium has been applied to crops as an insect-control strategy for many years, and this practice has been widely adopted in agriculture and gardening. Recently, plants have been developed which express a recombinant form of the bacterial protein, which may effectively control some insect predators.
 - Producing Genetically Modified Foods
 - A widely debated application of recombinant DNA technology is in the production of genetically modified foods. Genes can be derived from plants or even other organisms to give plants characteristics that are beneficial to both producers and consumers of agricultural products:
 - Delayed fruit ripening for longer shelf life during transportation
 - Enhanced flavor and nutritional content
 - Edible vaccines to prevent widespread diseases in developing countries

MODULE-9: TRANSFORMATION AND TRANSFECTION

Learning objectives

The learner will learn about

- Transformation
- Competent cells
- Steps in transformation
- Transformation methods

TRANSFORMATION

- Transformation is a very basic technique that is used frequently in a molecular biological laboratory.
- The purpose of transformation is to introduce a foreign plasmid into bacteria and to use that bacteria to amplify the introduced plasmid in order to make large quantities of it.

COMPETENT CELLS

- As the DNA is a very hydrophilic molecule, it won't normally pass through a bacterial cell's membrane. In order to make bacteria take in the plasmid, they must first be made "competent" to take up DNA.
- This is done by creating small holes in the bacterial cells by suspending them in a solution with a high concentration of calcium.

- DNA can then be forced into the cells by incubating the cells and the DNA together on ice, placing them briefly at 42°C (heat shock), and then putting them back on ice.
- This causes the bacteria to take up the DNA.
- The cells are then plated out on antibiotic containing media.

PREPARATION OF E.COLI COMPETENT CELLS

- The E.coli cells picked from a single colony is grown freshly until it reaches an OD of 1.6 -1.8 (less than 10⁸ cells / ml).
- The E.coli cells then made competent by CaCl₂ treatment
- The prepared competent cells are aliquoted and stored at -80° C until use

DNA TRANSFORMATION

- The foreign DNA is transformed into the *E.coli* competent cells after heat shock treatment.
- The transformed *E.coli* cells which carry the foreign DNA is propagated to have multiple copies of recombinant DNA.

TRANSFECTION

- Transfection is the process of introducing nucleic acids into eukaryotic cells by using carrier molecules.
- Animal cells are transfected by opening transient pores or holes in the cell membrane to allow the uptake of material.
- Genetic material such as plasmid DNA or siRNA constructs or proteins such as antibodies may be transfected into cells.
- Transfection may cause changes in morphology of target cells.

METHODS

- Various methods are used to introduce foreign DNA into a eukaryotic cell. Several materials are used as carriers for transfection.
 - **Calcium Phosphate method** : One of the cheapest methods of transfection is by calcium phosphate, discovered by F. L Graham and A.J. van der Eb in 1973. HEPES-buffered saline solution containing phosphate ions is combined with a calcium chloride solution containing the DNA to be transfected. Mixing of these two results in precipitate due to binding of positively charged calcium and the negatively charged phosphate. This suspension of precipitate is then added to the cells to be transfected, usually a cell culture grown in a monolayer. The cells take up some of the precipitate and the DNA.
 - **Liposome method** : It is a very efficient method of transfection. Liposomes, a small, membrane-bounded body that are in similar to the structure of a cell and can actually fuse with the cell membrane. The DNA to be transfected is trapped into the liposomes. This DNA loaded liposome is added to the cells to be transfected and release the DNA into the cell.
 - **Cationic polymers** : Cationic polymers such as DEAE-dextran or polyethylenimine. The polycation binds to the negatively charged DNA and the complex is then taken up by the cell via endocytosis.
 - **Gene gun transfection** – It is a method where the DNA is coupled to a nanoparticle such as gold which is then shot directly into the target cell's nucleus.
 - **Optical transfection** - It is a method where a tiny hole is generated in the plasma membrane of the target cell using LASER. In this method, single cell at a time is treated and making it particularly useful for single cell analysis.
 - **Other Substances** - Nucleofectin, lipofectin are also used to transfect nucleic acids into the target cells.

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MODULE-10: POLYMERASE CHAIN REACTION

Learning objectives

The learner will learn about

- PCR
- Components of PCR
- Steps in PCR
- Applications of PCR

POLYMERASE CHAIN REACTION

- PCR was invented by *Kary B. Mullis* in 1983.

PCR

- The PCR is an in-vitro, enzymatic amplification of a desired sequence of DNA using a pair of oligonucleotide primers.

- These primers are complementary to one end of the DNA target sequence.
- These are extended towards each other by a thermostable DNA polymerase in a reaction cycle of three steps; denaturation, primer annealing and polymerization.

COMPONENTS OF PCR

Template

- PCR can amplify as little as one molecule of starting template. Therefore, any source of DNA that provides one or more target molecules can in principle be used as a template for PCR.
- This includes DNA prepared from blood, sperm or any other tissue, from older forensic specimens, from ancient biological samples or in the laboratory from bacterial colonies or plaques as well as purified DNA.

Primers

- Oligonucleotides used for priming, should be at least 16 nts and preferably 20-24 nts in length.
- They should have similar G+C contents so that they anneal to their complementary sequences at similar temperatures.
- They are designed to anneal on opposite strands of the target sequence so that they will be extended towards each other by addition of nucleotides to their 3' ends.
- If the DNA sequence being amplified is known, then primer design is relatively easy.

dNTPs

- The 4 dNTPs, dATP, dGTP, dCTP and dTTP, used at saturating concentration (200 μ M each).

Enzymes

- Thermostable DNA polymerases from a number of thermophilic bacteria are used for PCR.
- The most common is Taq polymerase from *Thermus aquaticus*. It survives the denaturation step of 95°C for 1-2 min, having a half-life of more than 2hr at this temperature.
- It carries a 5'-3' polymerization dependant exonuclease activity, but lack in 3'-5' exonuclease activity (proof reading).
- Hence, it is more prone for introducing errors. There are high-fidelity thermostable enzymes with 3'-5' exonuclease activity. e.g., Vent polymerase, pfu polymerase.

Buffer

- The standard buffer for PCR contains 50 mM KCl, 10 mM Tris.Cl and 1.5 mM MgCl₂. pH is approximately 7.2. The presence of divalent cations is critical (Mg²⁺).

PCR CYCLES

- PCR involves a repetitive series of temperature cycles. Each reaction cycle comprises of *three stages*
 - Denaturation
 - Primer annealing and
 - Extension.
- In the first cycle, the target DNA is separated into two strands by heating to 95°C- denaturation.
- The temperature is reduced to around 55°C to allow the primers to anneal. The actual temperature depends on the primer lengths and sequences- primer annealing.
- After annealing, the temperature is increased to 72°C for optimal polymerization which uses up dNTPs in the reaction mix and requires Mg²⁺.
- If PCR was 100% efficient, one target molecule would become 2ⁿ after 'n' cycles. In practice, 20- 40 cycles are commonly used.

TYPES OF PCR TECHNIQUES

- There are several types of PCR used. The below mentioned are the most commonly used types of PCR.

RT-PCR

- Reverse transcriptase polymerase chain reaction (RT-PCR) is used to convert the RNA template into complementary DNA (cDNA) by using the enzyme reverse transcriptase and then the target is amplified into many copies through PCR steps. RT-PCR is applied to diagnose RNA viruses, mRNA expression etc.

Nested PCR

- Nested PCR is a conventional PCR with a second round of amplification using a different set of primers. This second set of primers is specific to a sequence found within the DNA of the initial conventional PCR amplicon. The use of a second amplification step with the "nested" primer set results in a reduced background from products amplified during the initial PCR due to the nested primers' additional specificity to the region. The amount of amplicon produced is increased as a result of the second round of amplification.

Multiplex PCR

- Multiplex PCR is the term used when more than one pair of primers is used in a PCR. The goal of multiplex PCR is to amplify several segments of target DNA simultaneously and thereby to conserve template DNA, save time, and minimize expense. It is a PCR strategy that enables the amplification of multiple DNA targets in one run.

Real time PCR

- Real-Time PCR, also called quantitative (real-time) PCR or Real-Time Quantitative PCR (RTQ-PCR), is a method of simultaneous DNA quantification and amplification.

APPLICATIONS OF PCR

- Genomic cloning.
- Generating template for sequencing.
- *In-vitro* mutagenesis.
- Analysis of biological materials for forensic applications.
- To study the evolutionary history in the field of molecular palaeontology.
- Medical applications such as pre-natal diagnosis of diseases and sexing of embryos.
- Detection of infectious diseases.

MODULE-11: GENOMIC LIBRARY

Learning objectives

The learner will learn about

- Genomic library
- Steps in genomic library construction

DNA LIBRARIES

- Vectors are used to compile a library of DNA fragments that have been isolated from the genomes of a variety of organisms.
- This collection of fragments can then be used to isolate specific genes and other DNA sequences of interest.
- DNA fragments are generated by cutting the DNA with a specific restriction endonuclease.

- These fragments are ligated into vector molecules, and the collection of recombinant molecules is transferred into host cells, one molecule in each cell.
- The total number of all DNA molecules makes up the library.
- This library is searched, that is screened, with a molecular probe that specifically identifies the target DNA.
- Once prepared the library can be perpetuated indefinitely in the host cells and is readily retrieved whenever a new probe is available to seek out a particular fragment.
- Two main types of libraries can be used to isolate specific DNAs:
 - genomic and
 - cDNA libraries.

GENOMIC LIBRARY

- A genomic library contains DNA fragments that represent the entire genome of an organism.
- The first step in creating a genomic library is to break the DNA into manageable size pieces (e.g. 15–20 kb for phage λ vectors), usually by partial restriction endonuclease digest. This generates a continuum of overlapping fragments.
- The next step is to purify fragments of optimal size by gel electrophoresis or centrifugation techniques.
- The final step is to insert the DNA fragments into a suitable vector. Bacteriophage λ or cosmid vectors are typically used for genomic libraries.
- In humans, the genome size is approximately 3×10^9 bp. With an average insert size of 20 kb, the number of random fragments to ensure with high probability (95–99%) that every sequence is represented is approximately 10^6 clones for humans.

APPLICATIONS OF GENOMIC LIBRARY

- Construction of genomic library is the first step in any DNA sequencing projects.
- Genomic library can be useful in identification of novel pharmaceutically important genes.
- It helps in identifying new genes in the host.
- It helps to understand the complexity of genomes.

MODULE-12: cDNA LIBRARY

Learning objectives

The learner will learn about

- cDNA library
- Steps in cDNA library constructions
- Uses of cDNA library

cDNA LIBRARY

- A *cDNA library* is a collection of cloned cDNA (complementary DNA) fragments inserted into a collection of host cells, which together constitute some portion of the transcripts of the organism.
- cDNA is produced from fully transcribed mRNA found in the nucleus and therefore contains only the expressed genes of an organism.
- Similarly, tissue specific cDNA libraries can be produced.
- In eukaryotic cells the mature mRNA is already spliced, hence the cDNA produced lacks introns and can be readily expressed in a bacterial cell.
- While information in cDNA libraries is a powerful and useful tool since gene products are easily identified, the libraries lack information about enhancers, introns, and other regulatory elements found in a genomic DNA library.

PREPARATION OF cDNA LIBRARY

- cDNA is created from a mature mRNA from a eukaryotic cell with the use of an enzyme known as reverse transcriptase.

- In eukaryotes, a poly-(A) tail (consisting of a long sequence of adenine nucleotides) distinguishes mRNA from tRNA and rRNA and can therefore be used as a primer site for reverse transcription.

mRNA extraction

- The mRNA is obtained and purified from the rest of the RNAs.
- Several methods exist for purifying RNA such as trizol extraction and column purification. Column purification is done by using oligomeric dT nucleotide coated resins where only the mRNA having the poly-A tail will bind. The rest of the RNAs are eluted out. The mRNA is eluted by using eluting buffer and some heat to separate the mRNA strands from oligo-dT.

cDNA construction

- Once mRNA is purified, *oligo-dT* (a short sequence of deoxy-thymine nucleotides) is tagged as a complementary primer which binds to the poly-A tail providing a free 3'-OH end that can be extended by reverse transcriptase to create the complementary DNA strand.
- Now, the mRNA is removed by using a RNase enzyme leaving a single stranded cDNA (sscDNA).
- This sscDNA is converted into a double stranded DNA with the help of DNA polymerase. However, for DNA polymerase to synthesize a complementary strand a free 3'-OH end is needed. This is provided by the sscDNA itself by generating a *hair pin loop* at the 3' end by coiling on itself. The polymerase extends the 3'-OH end and later the loop at 3' end is opened by the scissoring action of *S₁ nuclease*. Restriction endonucleases and DNA ligase are then used to clone the sequences into bacterial plasmids.
- The cloned bacteria are then selected, commonly through the use of antibiotic selection.
- Once selected, stocks of the bacteria are created which can later be grown and sequenced to compile the cDNA library.

USES OF cDNA LIBRARY

- It is commonly used when reproducing eukaryotic genomes, as the amount of information is reduced to remove the large numbers of non-coding regions from the library.
- It is used to express eukaryotic genes in prokaryotes.
- It is most useful in reverse genetics
- It is useful for isolating the gene that codes for that mRNA.

MODULE-13: DNA SEQUENCING

Learning objectives

The learner will learn about

- DNA Sequencing
- Methods of DNA sequencing
- Automated sequencing

STEPS IN AUTOMATED CYCLE SEQUENCING

- There are three major steps in a cycle sequencing reaction (like in PCR), which are repeated for 30 or 40 cycles.
 - *Denaturation at 94°C :*
 - During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop.
 - *Annealing at 50°C :*
 - In sequencing reactions, only one primer is used, so there is only one strand copied in each cycle.
 - *Extension at 60°C :*
 - This is the ideal working temperature for the polymerase (normally it is 72 °C, but because it has to incorporate ddNTP's which are chemically modified with a fluorescent label, the temperature is lowered. When a ddNTP is incorporated, the extension reaction stops because a ddNTP contains a H-atom on the

3rd carbon atom (dNTP's contain a OH-atom on that position). Since the ddNTP's are fluorescently labeled, it is possible to detect the colour of the last base of this fragment on an automated sequencer. Because only one primer is used, only one strand is copied during sequencing, there is a **linear** increase of the number of copies of one strand of the gene.

- Therefore, there has to be a large amount of copies of the gene in the starting mixture for sequencing.

- Suppose there are 1000 copies of the wanted gene before the cycling starts, after one cycle, there will be 2000 copies: the 1000 original templates and 1000 complementary strands with each one fluorescent label on the last base, after two cycles, there will be 2000 complementary strands, three cycles will result in 3000 complementary strands and so on.

-

METHODS OF DNA SEQUENCING

- The two main methods of DNA sequencing are
 - Maxam and Gilbert's chemical method
 - Sanger's dideoxy enzymic method

MAXAM GILBERT METHOD OF SEQUENCING

- The chemical method of Maxam and Gilbert requires that the DNA fragment to be sequenced is labeled at one end, usually by adding either a non radioactive phosphate to the 5' or 3' end or a nucleotide to the 3' end. This method works for both single stranded and double stranded DNA and involves base - specific cleavages which occur in two steps.

- The base is modified by specific chemicals such as Dimethyl sulfate (DMS), Formic acid and Hydrazine. The specific base modifications reactions use DMS to methylate at G base. Formic acid will attack purines A and G. Hydrazine is used to hydrolyse at pyrimidines (C+T) but high salt inhibits the T reaction. Thus four lanes on the sequencing gel (G, A+G, C+T and C) allow the sequences to be determined. This method is useful for sequencing genomic DNA without cloning.

- After the base modification, piperidine is used to cleave the sugar phosphate back bone of the DNA.

SANGER'S DIDEOXY METHOD OF SEQUENCING

- Sanger's dideoxy enzymic method uses specific dideoxy nucleotides (ddNTPs) to terminate enzymically synthesised copies of a template.
- A sequencing primer is annealed to ssDNA template molecule and DNA polymerase extends the primer using ddNTPs.
- This method requires a ssDNA template on which to synthesize complementary copies which means that the DNA has to be cloned into phage vector M13 before sequencing.
- The ssDNA recovered from the phage is annealed to a primer of 15-17 nucleotides which is complementary to the region near the vector- insert junction.
- All sequences cloned into this vector can be sequenced using this universal primer.
- A DNA polymerase enzyme Klenow or T7 DNA polymerase is added to the annealed primer plus template along with a small amount of radioactive labeled ATP.
- It is then divided into 4 tubes each containing a different chain terminator mixed with normal dNTPs (i.e) (tube C would contain ddCTP and dATP, dCTP, dGTP and dTTP) in specific ratios to ensure only a limited amount of chain termination.
- The 4 sets of reaction products are analyzed by Poly Acrylamide Gel Electrophoresis (PAGE).
- Based on the PAGE analysis, the sequence of the DNA is predicted.
- The PAGE results in the sequences copied from of the template. Hence, to know the original sequence of the DNA, the complementary sequence has to be made.

AUTOMATED DNA SEQUENCING

- The complete genome sequencing of important organisms is now possible with the development of automated DNA sequencers.

STEPS IN AUTOMATED CYCLE SEQUENCING

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 - Therefore, there has to be a large amount of copies of the gene in the starting mixture for sequencing.
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SEPARATION OF MOLECULES

- After the sequencing reactions, the mixture of strands, all of different length and all ending on a fluorescently labelled ddNTP have to be separated.
- This is done on an acrylamide gel, which is capable of separating a molecule of 30 bases from one of 31 bases, but also a molecule of 750 bases from one of 751 bases.
- All this is done with gel electrophoresis. DNA has a negative charge and migrates to the positive side.
- Smaller fragments migrate faster, so the DNA molecules are separated on their size.

DETECTION OF FLUORESCENT SIGNALS BY AUTOMATED SEQUENCER

- The fluorescently labelled fragments that migrate through the gel, are passing a laser beam at the bottom of the gel.
- The laser excites the fluorescent molecule, which sends out light of a distinct color.
- That light is collected and focused by lenses into a spectrograph. Based on the wavelength, the spectrograph separates the light across a CCD camera (charge coupled device).
- Each base has its own colour, so the sequencer can detect the order of the bases in the sequenced gene.

ASSEMBLING OF SEQUENCED PART OF A GENE

- For publication purposes, each sequence of a gene has to be confirmed in both directions.
- To accomplish this, the gene has to be sequenced with forward and reverse primers.
- Since it is only possible to sequence a part of 750 till 800 bases in one run, a gene of, for example 1800 bases, has to be sequenced with internal primers.
- When all these fragments are sequenced, a computer program such as DNA star tries to fit the different parts together and assembles the total gene sequence.

RNA SEQUENCING

- Sequencing DNA is much easier than sequencing RNA. A set of 4 RNAses that cleave 3' to specific nucleotides are used to produce a ladder of fragments from end labeled RNA.
- PAGE analysis allows the sequences to be read.

MODULE-14: SOUTHERN BLOTTING

Learning objectives

The learner will learn about

- Southern blotting
- Steps in Southern blotting
- Applications of Southern blotting

SOUTHERN BLOTTING

- Southern blotting is a technique named after its inventor and developer, the British biologist Edwin M. Southern in 1975.
- Southern blotting is a technique which allows the detection of a specific DNA sequence (gene or other) in a large, complex sample of DNA (e.g. cellular DNA).

STEPS IN SOUTHERN BLOTTING

- High molecular weight DNA is digested with restriction enzymes to make it as a small fragments
- The DNA fragments are separated based on their size on an agarose gel
- A nitrocellulose membrane or nylon membrane is placed on the top of the agarose gel in a buffer solution. A pile of tissue papers is then placed on the membrane and weight is placed above it. This makes uniform contact of gel with the membrane and facilitate the transfer of DNA fragments from the gel to membrane (blotting or transfer).
- Nitrocellulose membrane is then baked by exposing it to high temperatures (from 60 to 100 °C). Nylon membrane is exposed to UV radiation. These steps are used to ensure the permanent and covalent crosslink of the DNA present in the bands to the membranes.
- The membrane is exposed to a radiolabeled probe. This probe is a single-stranded DNA fragment which has the sequence of interest that is to be detected. This probe is incubated with the membrane and allowed to hybridize with DNA on the membrane. Probes are usually radiolabeled so that they may be detected on X-ray film, however other probes are also used which are non-radioactive such as fluorescent. After hybridization, excess unbound probe is washed away from the membrane, leaving specifically bound probe.
- The pattern of hybridization is detected by visualization on X-ray film by autoradiography in the case of a radioactive or fluorescent probes, or by development of color on the membrane if a chromogenic detection method is utilized.

APPLICATIONS OF SOUTHERN BLOTTING

- Southern blots are used in several main areas including gene discovery and mapping, evolution and development studies, diagnostics and forensics.
 - Southern blot is used to detect the presence of a particular segment of DNA in a sample
 - To analyze the genetic patterns which appear in a person's DNA.
 - To analyze restriction digestion fragmentation of DNA of a biological sample
 - In the production of genetically modified organisms, Southern blotting is used as a definitive test to ensure that a particular section of DNA of known genetic sequence has been successfully incorporated into the genome of the host organism.

MODULE-15: WESTERN BLOTTING AND NORTHERN BLOTTING

Learning objectives

The learner will learn about

- Western blotting and Northern blotting
- Steps in Western blotting and Northern blotting
- Applications of Western blotting and Northern blotting
- Differences between Southern blotting and Northern blotting
- Western blotting

WESTERN BLOTTING

- Western blotting is an analytical method wherein a protein sample is electrophoresed on an SDS-PAGE and then electrotransferred onto a nitrocellulose membrane.
- The transferred protein on the nitrocellulose membrane is detected using specific primary antibody and secondary enzyme labeled antibody and substrate.
- Western blotting is widely used in the protein detection. Western blotting analysis can detect one protein in a solution that contains any number of proteins and giving the protein information.

STEPS IN WESTERN BLOTTING

- A protein sample is subjected to poly acrylamide gel electrophoresis (PAGE).
- After this the gel is placed over a sheet of nitrocellulose and the protein in the gel is electrophoretically transferred to the nitrocellulose.
- The nitrocellulose is then soaked in blocking buffer (3% skimmed milk solution) to "block" the nonspecific binding of proteins.
- The nitrocellulose is then incubated with the specific antibody for the protein of interest.
- The nitrocellulose is then incubated with a second antibody, which is specific for the first antibody. For example, if the first antibody was raised in mouse, the second antibody might be termed "goat anti-mouse immunoglobulin".
- The second antibody will typically have a covalently attached enzyme which, when provided with a chromogenic substrate, will cause a color reaction. Thus the molecular weight and amount of the desired protein can be characterized from a complex mixture (e.g. crude cell extract) of other proteins by Western blotting.

APPLICATIONS OF WESTERN BLOTTING

- In clinical settings, Western Blotting is routinely used to confirm serious diagnosis suggested by ELISA such as HIV seroconversion.
- In research, Western blotting is extensively used to characterize and quantitate the recombinant expressed proteins
- It is used to characterize the monoclonal antibodies produced against a specific protein of virus etc
- Northern blotting

NORTHERN BLOTTING

- Northern blotting is an RNA blotting technique which was developed in 1977 by Alwine et al.
- In Northern blotting, RNAs are separated by gel electrophoresis, the RNA bands are transferred onto a suitable membrane, e.g., diazobenzylxymethyl (DBM) paper or nylon membranes, and immobilized; the bands are hybridized with radioactive single stranded DNA probes, and the bands showing hybridization are detected by autoradiography.
- Northern blotting is simply an extension of the Southern blotting technique.

STEPS IN NORTHERN BLOTTING

- RNA isolation
 - The total RNA is extracted from homogenized tissue samples. The mRNA is isolated by using an oligo-dT column
- Gel electrophoresis of RNA for separation
 - The mRNA is separated on an agarose gel containing formaldehyde as a denaturing agent for the RNA to limit secondary structure.
- Transfer to membrane (usually positively charged nylon as RNA is negatively charged)
- Cross-linking of RNA to membrane (usually by UV-crosslinking or chemical means)
- Hybridization
 - Probes for northern blotting are composed of nucleic acids with a complementary sequence to all or part of the RNA of interest, they can be DNA, RNA, or oligonucleotides with a minimum of 25 complementary bases to the target sequence.
 - Commonly cDNA is created with labelled primers for the RNA sequence of interest to act as the probe in the northern blot.
- Detection
 - The probes need to be labelled either with radioactive isotopes (^{32}P) or with chemiluminescence. X-ray film can detect both the radioactive and chemiluminescent signals and many researchers prefer the chemiluminescent signals because they are faster, more sensitive, and reduce the health hazards that go along with radioactive labels.

- The same membrane can be probed up to five times without a significant loss of the target RNA.

APPLICATIONS OF NORTHERN BLOTTING

- To study the gene expression at the mRNA level
- To detect the size of the mRNA transcripts
- To study the degradation of RNA and RNA splicing
- To study RNA half-life
- It is often used to confirm and check transgenic / knockout mice

DIFFERENCE BETWEEN NORTHERN BLOTTING AND SOUTHERN BLOTTING

- In Southern hybridization, DNA is separated by gel electrophoresis, while in northern blotting RNAs are separated;
- As a result, in Southern hybridization DNA has to be denatured before blotting, while this step is not needed in northern hybridization;
- Nitrocellulose membrane is generally not used for northern, while it is often used for Southern hybridization; and finally
- Hybridization with the probe produces DNA: DNA hybrid molecules in Southern hybridization but RNA : DNA molecules in northern hybridization.

MODULE-16: NUCLEIC ACID PROBES

Learning objectives

The learner will learn about

- Nucleic acid probes
- Types of nucleic acid probes
- Methods of generation of nucleic acid probes
- Uses of DNA probes

PROBES AND ITS TYPES

- A *probe* is a nucleic acid which has been *labeled* i.e., chemically modified in some way which allows it and hence anything it hybridizes to, to be detected.
- There are three major types of probe:
 - Oligonucleotide probes,
 - DNA probes and
 - cRNA probes (riboprobes)
- *Oligonucleotide probes* are synthesized chemically and end labeled
- *DNA probes* which are cloned DNAs or PCR products and may either be end-labeled or internally labeled during *in vitro* replication
- *cRNA probes (complementary RNA probes) or riboprobes* which are internally labeled during *in vitro* transcription of cloned DNA.
- Riboprobes and oligonucleotide probes are generally labeled as single-stranded molecules. DNA may be labeled as a double-stranded or single-stranded molecule, but it is only useful as a probe when single stranded and must be denatured before use.

PROBE LABELING

- Probes of the highest *specific activity* are generated by *internal labeling*, where many labeled nucleotides are incorporated during DNA or RNA synthesis.
- *End labeling* involves either adding labeled nucleotides to the 3' end of a DNA strand or exchanging the 5' phosphate group for a labeled moiety.

DIFFERENT WAYS OF GENERATING PROBES

Labeling method	Probe	
5' end labeling	Oligo / DNA	Replacement of 5' phosphate group with labeled (gamma) g -phosphate group of free nucleotide catalyzed by T4 polynucleotide kinase
3' end labeling	Oligo / DNA	Tailing with labeled nucleotides using terminal transferase
Nick translation	DNA	Nicks introduced into dsDNA by Dnase I and free 3' ends extended by DNA polymerase I using labeled nucleotides
Random priming	DNA	Short random primers annealed to denatured DNA and extended by DNA polymerase using labeled nucleotides. Higher specific activity probes than nick translation
Primer extension	DNA	As above but using a specific primer. Used to label PCR products during thermal cycling
<i>In vitro</i> transcription	RNA	Single strand RNA produced by <i>in vitro</i> transcription using labeled nucleotide

ISOTOPIC AND NON- ISOTOPIC LABELING SYSTEMS

Isotopic labeling systems

- Traditionally, nucleic acids have been labeled with radioisotopes such as ^{32}P and ^{35}S which are detected by autoradiography. These *radiolabeled probes (hot probes)* are very sensitive but their handling is subject to strict safety precautions and the signal decays relatively quickly.

Non-isotopic probes (cold probes)

- Non-isotopic probes (cold probes)* generate colorimetric or chemiluminescent signals. A widely used label is *digoxigenin*, a plant steroid isolated from *digitalis*.
- This can be conjugated to nucleotides and incorporated into DNA, RNA or oligonucleotide probes and then detected using an antibody.
- Another system uses *biotin*, a vitamin, and the bacterial protein *streptavidin* which binds to biotin. Biotin conjugated nucleotides are incorporated as a label and detected using streptavidin.
- The presence of a target nucleotide sequence in a DNA sample can be determined with a DNA probe. This procedure is called DNA hybridization and depends on the formation of stable base pairs between the probe and the target sequence.
- For a DNA hybridization assay, the target DNA is denatured and the single strands are irreversibly bound to a matrix (e.g. nitrocellulose or nylon), this process is often carried out at a high temperature.
- Then, the DNA probe which is labeled with either a radioisotope (hot probe) or another tagging system (cold probe) is incubated with the bound DNA sample.
- If the sequence of nucleotides in the DNA probe is complementary to a nucleotide sequence in the sample, then base pairing (i.e. hybridization) occurs.
- The hybridization can be detected by autoradiography (hot probe) or other visualization procedures (cold probe), depending on the nature of the probe label.
- If the nucleotide sequence of the probe does not base pair (bind) with a DNA sequence in the sample, then no hybridization occurs and the assay gives a negative results.

ADVANTAGES AND DISADVANTAGES OF HOT AND COLD PROBES

Hot probes

Advantages

- More sensitive in the shortest time.
- More specific in nature.

Disadvantages

- Short half-life.
- Expensive.
- Inconvenience of disposal.
- Hazards of working with the isotopes are more.
- Carcinogenic.
- Requires sophisticated laboratory facilities.

Cold probes

- Though they are not so sensitive as hot probes, the disadvantages of the hot probes can be avoided.
- Generally probes range in length from 100 to more than 1000bp, although both larger and smaller probes can be used.
- Depending on the conditions of the hybridization reaction, stable binding requires a greater than 80% match within a segment of 50 bases.

USES OF DNA PROBES

- For genetic engineering research.
- For identification of specific plasmids and antibiotic resistant genes from clinical isolates.
- For diagnosis of infectious diseases.
- For identification of food contaminants.
- For forensic investigations.
- For epidemiological studies.
- For distinguishing organisms at species and strain level.
- Ante-natal diagnosis of inherited diseases.

MODULE-17: DNA FINGER PRINTING

Learning objectives

The learner will learn about

- DNA finger printing
- Methods of DNA finger printing

DNA FINGER PRINTING

- DNA finger printing (DNA profiling, DNA testing) is a process which uses fragments of DNA to identify the unique genetic makeup of an individual.
- Before the invention of PCR, the only method of establishing and authenticating personal identification was by the DNA fingerprint process.
- Since no two individuals have been found to have identical pattern of ridges on their fingers, this method has been universally accepted as a means of personal identification.
- In 1984, Sir Alec Jeffreys at the University of Leicester in England was able to distinguish differences among individuals based solely on their DNA composition.

METHODS OF DNA FINGER PRINTING

- The main types of DNA fingerprinting methods in use are:
 - Restriction fragment length polymorphism (RFLP)
 - Polymerase chain reaction (PCR)
 - Amplified fragment length polymorphism (AFLP)
 - Short tandem repeats (STR)

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

- Restriction fragment length polymorphism (RFLP) analyzes the length of the strands of the DNA molecules with repeating base pair patterns.
- While about 5% of DNA contains the genes, the other 95% is considered as non-coding genes (junk DNA) but contains identifiable repetitive sequences of base pairs, which are called Variable Number Tandem Repeats (VNTR).
- To extract a DNA fingerprint, a Southern blot is performed and the DNA is analyzed via a radioactive probe.
- The restriction fragment length polymorphism analysis is used to detect the repeated sequences by determining a specific pattern to the VNTR, which becomes the person's DNA fingerprint.
- The drawback with this system is that it requires a considerable amount of DNA in order to be used.

PCR

- The PCR analysis amplifies the DNA molecules using a smaller sample.
- On the forensic front, the PCR found to be useful in identifying DNA fingerprints in criminal matters and in paternity tests because it requires less amounts of DNA as it makes identical copies of the DNA sample.
- The PCR analysis amplified isolated regions on the strands of the DNA under examination.
- The drawback is that it can not as discriminating as the RFLP.

AMPLIFIED FRAGMENT LENGTH POLYMORPHISM

- Amplified fragment length polymorphism (AmpFLP) is a popular DNA finger printing technique
- It remains attractive because of its relatively less complicated operation and the cost-effectiveness of the procedure.
- By using the PCR analysis to amplify the minisatellite loci of the human cell, this method proved quicker in recovery than the RFLP.
- However, due to the use of gel in its analysis phase, there are issues of bunching of the VTRN's, causing misidentifications in the process.

SHORT TANDEM REPEATS

- The short tandem repeat (STR) methodology for extracting DNA is the system most widely used form of DNA fingerprinting.
- This system is based on the features of PCR, as it utilizes specific areas that have short sequential repeat DNA.
- The STR analyzes how many times base pairs repeat themselves on a particular location on a strand of DNA.
- The big advantage in this method is that the DNA comparisons can match the possibilities into an almost endless range.
- DNA fingerprinting has been extremely successful for use in the personal identification of criminal suspects, paternity issues, as well as in identification of the deceased.
- DNA, however, still poses issues because the VNTRs are not evenly distributed in all people because they are inherited.
- However, as forensic science continues its work in DNA testing, there appears to be no limit to the value it can render to society.

MODULE-18: EMBRYO TRANSFER TECHNIQUE

Learning objectives

The learner will learn about

- Embryo transfer in animals
- Steps in embryo transfer
- Applications of embryo transfer

EMBRYO TRANSFER IN CATTLE

- Artificial Insemination has allowed genetic process to be achieved relatively, quickly through the widespread and efficient use of frozen semen for the past several decades. Genetic programmes were limited to male side for genetic contribution because cows could relatively produce only one calf in one year. Today due to the advancement of embryo transfer technique cows can produce many offspring every year.
- Embryo transfer is a method of artificial breeding for the genetic improvement of cattle. Cows have a 21 day oestrus cycle with a day zero characterized by visual display of heat. Buffaloes are silent heaters. Approximately 12 hours after the end of heat a single non-fertilized egg (ova) is released from 1 or 2 ovaries.
- If the cow is bred at the end of heat the egg will be fertilized shortly after ovulation and develop into embryo. The process of embryo transfer is typically the same. But the cow is made to superovulate by hormone treatment so that at one single oestrus period instead of one usual ovum more ova are produced if the cow is bred at this stage more fertilized embryos may result. If these embryos are physically collected from that cow. Each of the fertilized embryo may be transferred to each foster mother so that, more calves can be produced simultaneously from each foster mother. One major advantage of embryo transfer is the cross breeding for genetic improvement can be achieved quickly.
- The first successful transfer of fertilized rabbit eggs was reported in 1891 by *Walter and Heape* at Cambridge. The first successful embryo transfer in farm animals was done in 1934 in Sheep and Goats. The first offspring after transfer of embryos in cattle and swine was reported in 1951.
- Embryo transfer includes a sequence of various steps which are
 - Selection of donors
 - Synchronization of oestrus
 - Induction of superovulation
 - Embryo collection
 - Evaluation of embryos
 - Selection of recipients
 - Transfer of embryos
- Embryo transfer techniques have been extensively used in cattle and more sporadically in Sheep, Goats and Pigs.

SELECTION OF DONORS

- Any regularly cyclic cow or heifer can successfully be termed as donor because technically it will respond to superovulatory treatment followed by embryo transfer.
- Usually there are many reasons that a cow is preferable than a heifer because embryo transfer programme is based on milk yield or genetic superiority. In addition the following are the criteria for selection of donor,
 - Donor should be of age between three years and ten years.
 - Donor should be free from genetic diseases and conformational abnormalities.
 - They should exhibit regular oestrus cycle.
 - Superior production traits of economic importance.
 - Previous record of sound reproductive performance including successive fertility and artificial insemination.

INDUCTION OF SUPEROVULATION

- Superovulation means the induction of multiple ovulations by application of exogenous hormones (PMSG - Pregnant Mare Serum gonadotrophins, FSH - Follicle Stimulating hormone, HMG - Human Menopausal Gonadotrophins) in the early follicular or in the luteal phase of the oestrus cycle in order to collect large number of fertilized eggs. Most frequently PMSG or FSH is used.
- PMSG - one single injection (2000 to 3000 IU) as the substance has a very long half life time.
- FSH - Multiple injections of FSH (35-50 mg) twice daily for four days.
- 48 and 60 hours after beginning the gonadotrophin treatment, Prostaglandins (PG) are administered to induce oestrus. Inseminations are performed at oestrus two days later. Embryos are recovered 8 days after insemination.

SCHEDULE FOR SUPEROVULATION IN CATTLE

Day of cycle	PMSG (2000-3000 IU)	FSH (35-50 mg)
0	OESTRUS (Spontaneous or after synchronization)	
10	PMSG	FSH/FSH
11	---	FSH/FSH
12	PG/PG	PG/PG/FSH/FSH

13		PG/PG/FSH/FSH
14	Anti PMSG Oestrus and inseminations)	
15		
16		
17		
18		
19		
20		
21	Embryo recovery	

EMBRYO COLLECTION

- In the bovine, embryos are recovered by non surgical methods.
- Special catheters(Foley's catheters) are introduced via the cervix into the uterine horn and embryos are flushed with 250 – 300ml flushing medium.
- Recovery rate is influenced by
 - The position of the embryos in the uterus
 - The flushing method
 - The time of recovery
 - Ovarian response
 - Embryo viability
- In other farm animals, Sheep, Pigs and Goats embryo recovery is performed by surgical methods. Disadvantage of the surgical method is the number of repetitions is limited by the occurrence of adhesions.
- After recovering embryos from the flushing medium their further developmental capacity has to be evaluated. Embryo viability can be evaluated by various methods.

Morphological evaluation

- Most frequently the morphological evaluation at various microscopical magnifications is used. Morphological criteria in embryo evaluation are shape, colour, number and compactness of cells, Size of the perivitelline space, number and size of vesicles and status of the zona pellucida. The ideal embryo is even, the perivitelline space is empty and of a regular diameter. According to their morphological appearance, embryos are classified into four groups.
 - *Excellent embryos*: Embryos in the appropriate developmental stage with a perfect morphology.
 - *Good embryos*: Embryos in the appropriate stage of development with slight morphological deviations. Eg. Minor damage of the zona pellucida and excluded blastomeres or vesicles in the perivitelline space.
 - *Degenerated and / or retarded embryos*: Embryos in the appropriate developmental stage with major morphological deviations (degenerated embryos)
 - Unfertilized ova.

Staining methods

- Embryo viability can be evaluated by various vital staining and fluorescence techniques.

SELECTION OF RECIPIENTS

- Recipients are selected based on the
 - Normal physiological and health conditions.
 - The reproductive status.
 - Lack of any reproductive disorders.
 - Compatibility to the donor with respect to size of the foetus.
 - Oestrus synchronization.
- In bovines, heifers and young cows are best suited as recipients.

TRANSFER OF EMBRYOS

- Recently special catheters have been developed to perform non – surgical transfers in cattle.
- It is important to transfer the embryo in to tip of the uterine horn without damaging the endometrium.
- For a successful embryo transfer, the most important is embryo quality. It is very important to transfer the embryo ipsilateral, that means into the horn bearing the corpus luteum.

CRYOPRESERVATION OF EMBRYOS

- Cryopreservation of embryos is an essential part of embryo transfer programme and it allows worldwide shipment of embryos.
- Reliable freezing methods have been developed for bovine and sheep embryos.
- An optimal method for bovine embryos includes a one step addition of 1.4M glycerol as cryoprotectant, a 20 minutes equilibration period and 0.25 ml straws as embryo containers, slow cooling down to -35°C and subsequent plunging to liquid nitrogen (-196°C). Embryos are thawed by placing the straws directly into warm water.

APPLICATIONS OF EMBRYO TRANSFER

- Exploitation of female reproductive capacity (more offspring from valuable donors).
- Significant facilitation of import and export for valuable genetic material.
- Development of new breeding concepts.
- Gene conservation by freezing techniques.
- Twin production (Embryo splitting).
- Introduction of new genes into closed herds.
- Manipulation of embryos.
- Gene transfer.

MODULE-19: IN VITRO FERTILIZATION, SEXING, MICROMANIPULATION AND ANIMAL CLONING

Learning objectives

The learner will learn about

- *In vitro* fertilization
- Steps in *in vitro* fertilization
- Sexing of Embryos
- Micromanipulation
- Animal Cloning

IN VITRO FERTILISATION (IVF)

- Embryo transfer is not used widely because of its cost, technical difficulty and the limited supply of embryos available from super ovulated donors. These limitations would be removed if we could fertilize *in vitro*, the thousands of oocytes that are present in a female's ovaries.
- Breeding programmes would select for female genetics as easily as for male genetics. The genetic influence of a male would be increased further since the amount of semen required for fertilization *in vitro* is fraction of the amount needed for Artificial insemination.
- Fertilization consists essentially the fusion of two cells, the oocytes from the female and spermatozoon from the male to form single cell, the Zygote.
- IVF is generally quite successful, resulting in about 70-80% of fertilized eggs. The practical difficulties arise in sourcing eggs for fertilization and in the development of fertilized zygotes to term.
- IVF involves *three steps* namely
 - Oocyte recovery
 - Oocyte maturation *in vitro*
 - *In vitro* fertilization of matured oocytes

OOCYTE RECOVERY

- In the second (follicular) phase of the oestrus cycle, a number of ovarian follicles (20 or so) grow and become filled with fluid. The fluid-filled space is called the antrum and such follicles are known as 'antral follicles' (or Graafian follicles).
- In the normal course of events just one of these matures and ruptures, releasing the egg for fertilization.
- Super ovulation occurs when many more antral follicles are stimulated to mature by injection of gonadotrophins.
- Pre- ovulatory follicles lie against the surface of the ovary and quite large (about 15 mm in cattle, 8 mm in sheep and pigs).
- Laparoscopy surgery can be used to recover oocytes from these follicles, allowing them to be matured and fertilized *in vitro*.
- A number of eggs can be collected in this way from superovulated donors. The technical demands and cost of surgical procedure of oocyte recovery is high.

IN VITRO CULTURE AND MATURATION OF OOCYTES

- Oocytes for in vitro fertilization are obtained from the oviducts, follicles and surface of ovary after ovulation either from live animals by flushing some medium or from slaughtered animals. The immature oocyte is collected from the ovary and cultured *in vitro* to induce maturation.
- Cow ovaries are collected from the slaughter house and maintained in phosphate buffered saline at 30-32°C for transportation to the laboratory. Following aspiration of follicles, the oocytes are then observed under a dissecting microscope.
- The oocytes are graded according to their morphology, washed twice with HEPES buffer and once with BMOC-3 (Branchat and Oliviant medium for oocyte collection). After washing, 5-10 oocytes are transferred to 0.2 ml of BMOC-3 drops under paraffin oil at 37°C with 5% CO₂ in 95% air for 12-32 hrs.

Oocyte maturation *in vitro*

- Large numbers of oocytes are allowed to mature under *in vitro* conditions.

PREPARATION OF SPERMATOZOA

- The capacitated spermatozoa is essential for *in vitro* fertilization. After capacitation, the acrosome reaction in the head of the spermatozoa allows the release of enzymes for penetration into the zona pellucida of the oocytes.
- The capacitation process is completed during the movement of the spermatozoa from the oviduct to the uterus.
- Capacitation involves the removal, probably by the enzyme action, of macromolecular material located on the surface of the spermatozoa.
- Re-exposure of the spermatozoa to seminal plasma leads to loss of capacitation and the process may well involve the restoration of the surface layer or 'decapacitation factor' which normally coats the spermatozoa as they pass through the male reproductive tract.
- Experimental procedures to induce the capacitation or acrosome reaction of the spermatozoa involve *in vitro* incubation with follicular fluid and serum

IN VITRO FERTILIZATION IN FARM ANIMALS

- The *in vitro* matured oocytes are mixed with the capacitated sperms in a petridish and incubated at 37°C with 5 % CO₂.
- After 24 hours incubation, the cumulus mass is removed manually and examined for fertilization indicated by the presence of 2 pronuclei. Further the fertilized oocytes were incubated for division.
- The morula stage embryos (16 cell stage) are transferred to recipient animals for further development of embryos.
- Among farm animals, *in vitro* fertilization has been achieved in cattle, pig and goat; but the number of new born animals were limited.

SEXING OF EMBRYOS

- Embryo sexing in animals has the advantage to increase the milk yield or to increase meat production. The production of preferred sex is done either by sexing the sperm or by sexing the embryos. In case of sexing sperm, it

could be used both in artificial insemination as well as in the in vitro production of embryos whereas the sexing of embryos could be done either in vivo produced or in vitro produced embryos prior to their transfer to recipient animals.

METHODS

Sexing the sperm

This is done by using flow cytometer attached with a sorter. In this method, the sperm is stained with a stain – Hoechst 33342 and then sex specific sperms are separated by using the flow cytometer's sorter. This method has an accuracy of 90 % sexing sperms. Commercially it is used in cattle.

Embryo sexing

Y-specific DNA probes

- Sexual differentiation of mammalian embryos is determined by the presence or absence of genetic elements located on the Y-chromosome. Molecular genetic techniques allow the detection of specific DNA sequences in interphase cells.
- Sex determination with Y-specific probes involves the identification and cloning of a probe (a DNA fragment with a nucleic acid sequence found only on the Y-chromosome) and the use of this probe to diagnose the presence or absence of Y-chromosomal DNA in a sample of embryonic cells.

PCR

- Using polymerase chain reaction (PCR) to amplify a Y-chromosome-specific repeat sequence, DYZI, that is present 500-8000 times on the Y chromosome. The presence of this amplified target sequence indicates the presence of the Y-specific DNA sequence in the sample DNA and, hence, indicates that the animal is male.
- A disadvantage of this technique is that the absence of the amplified target sequence may be due either to the absence of the sequence in the sample DNA or to a failed reaction,

PCR-RFLP

- Second method involves PCR-based genotyping. In brief, a pseudo-autosomal region (common to both X and Y chromosomes) present in both X and Y chromosomes is amplified. These homologous regions are called ZFY (in the Y chromosome) and ZFX (in the X chromosome).
- The amplified product is then digested with restriction enzymes that take advantage of restriction enzyme fragment length polymorphisms between ZFY and ZFX. The digested DNA is separated via electrophoresis. The ZFX and the ZFY DNA are identified by their different digestion patterns.

MICROMANIPULATION

- In 1966, Lin described the technique of micromanipulating and injecting a mouse egg
- Subsequently, transgenic animals have been produced by introduction of foreign genes at the pronuclear stages of fertilized, one-cell zygotes
- Most of the successes have been with mouse and recently successful production of transgenic rabbit, pig, sheep and goat have been shown
- This technique is a powerful tool for studying gene regulation and physiological functions of gene products in a normal host environment
- Micromanipulation is the technique whereby sperm, eggs and embryos can be handled on an inverted microscope stage, performing minute procedures at the microscopic level via joysticks that hydraulically operate glass microtools.
- The injection of a single sperm into the cytoplasm of the oocyte, or intracytoplasmic sperm injection (ICSI), provided a satisfactory solution to the problems of the assisted fertilization techniques developed earlier.

- In this procedure, a single sperm is first immobilized by touching the sperm tail with an injection pipette (inner diameter 5–7 μm). The injection pipette picks up the immobilized sperm, pierces the ZP and oolemma, and delivers the sperm inside the oocyte cytoplasm.
- In 1976 using hamsters as a model, Uehara and Yanagimachi were probably the first to report the injection of sperm into oocyte cytoplasm (ooplasm).
- It was later attempted on rabbit and human oocytes, although the first successful human pregnancy was not reported until 1992 by the Free University of Brussels' group in Belgium.
- Micromanipulation technology has enabled the reproductive biologist to overcome inefficient steps in mammalian fertilization, the production of chimeric animals through blastocyst injection with embryonic stem (ES) cells and the introduction of specific genes into the genomes of domestic and laboratory animals.
- This technology has also been used for the production of cloned animals and ES cell lineages from cloned embryos, using nuclear transfer. Moreover, micromanipulation is also used for microsurgical embryo biopsy to study the basic developmental biology of embryos during preimplantation development.

ADVANTAGES AND DISADVANTAGES OF MICROINJECTION

Advantages

- The amount of DNA delivered per cell is not limited by the technique and can be optimized. This improves the chance for integrative transformation
- The delivery is precise, again increasing the chance of integrative transformation.
- The small structures can be injected containing only a few cells and with high regeneration potential.
- Since it is a direct physical approach, it is host-range independent.

Disadvantages

- Injection can cause damage that affects embryonic survival and can result in quite high mortalities.
- Only one cell is targeted per injection

ANIMAL CLONING

- Cloning is routine in plants, but in case of animals only a limited success had been achieved so far. Earlier, nuclei from tadpole were transplanted into the cytoplasm of an enucleated fertilized frog egg, and normal frogs were obtained.
- But early in 1997, British scientists announced successful cloning of sheep by transferring the nucleus from the udder cell of an adult sheep into the cytoplasm of an enucleated fertilized egg. The egg was then transplanted into the uterus of a surrogate mother where it developed like a normal zygote into a normal lamb, which grew into a normal adult sheep, called '**Dolly**'.
- Cells from mammary gland of an adult sheep(6yr old ewe) were first cultured *in vitro*. The cultured cells were arrested, induced to enter the G₀ phase (quiescent phase) by reducing the concentration of serum in the medium from 10% to 0.5% for 5 days. The G₀ cells were then fused *in vitro* with the enucleated ova of appropriate stage. Oocyte were recovered between 28 to 33 hr after injection of gonadotropin releasing hormone and enucleated as soon as possible. Cell fusion was induced by electrical pulse, which also activated the oocytes. The fusion product were cultured *in vitro* in ligated oviducts of sheep upto morula or blastula stage before their transfer into the uteri of the surrogate. The rate of success in obtaining normal embryo development was rather low: a total of 277 oocytes were fused with cultured mammary gland cells. Out of these, 29 reached morula/blastula stage, and were transferred into surrogate mothers leading to 13 pregnancies, but only one live birth.
- Cloning in many situations, highly desirable since this allows
 - Indefinite multiplication of an elite desirable genotype without the risk of segregation and recombination during meiosis, which must precede sexual reproduction.
 - The technique holds a great promise in genetic research, and impact of epigenetic changes, such as, imprinting and telomere shortening, etc.
 - This technique should make it feasible to target transgenes in livestock by nuclear transfer from transgenic cell populations developed *in vitro* into enucleated oocytes to recover nonchimaeric transgenic animals.

MODULE-20: TRANSGENIC ANIMALS AND BIOPHARMING

Learning objectives

The learner will learn about

- Transgenic animals
- Steps in the production of transgenic animals
- Applications of transgenic animals
- Biopharming
- Benefits and risks of biopharms
- **Transgenic animals**

TRANSGENIC ANIMALS

- Gordon and Ruddle used the term ‘Transgenic’ for the first time in 1982 to describe genetically modified animals harboring foreign genes within their genome.
- The main objective in the generation of transgenic animals to guarantee that all the somatic cells and germ line cells carry the foreign gene. This necessitates the transfer of foreign genes as early as possible during the development of an animal which means that the foreign genes are introduced into the pronucleus.
- Production of transgenic animals involves a series of steps, starting with cloning of the gene of interest and preparation of DNA samples.
- In principle, both cloned and chemically synthesized DNA fragments can be used for microinjection.
- Microinjected embryos are usually transferred to the recipients after a short period of in vitro culture.
- Several methods are available for introduction of DNA into an animal genome. These include
 - DNA- microinjection into pronuclear stage of embryos.
 - Use of retro viruses.
 - Use of embryonic cells

STEPS IN PRODUCTION OF TRANSGENIC ANIMALS BY DNA MICRO INJECTION

- 4- 6 week old female mice are superovulated by administering 5 IU each of PMSG, HCG and mated to male mice.
- Pronuclear stage – eggs are recovered after 12 hrs.
- Pronuclear transgene – DNA construct is injected into the male pronuclei by using micromanipulator.
- Embryos after microinjection are transferred to recipients and allowed to develop to term.
- Integration of the foreign gene in the new born animals could be confirmed by techniques like
 - Polymerase chain reaction
 - Southern hybridization
 - In situ Hybridization.
 - IIs.

APPLICATIONS OF TRANSGENIC ANIMALS

- To improve economic traits of farm animal like growth promotion, carcass improvement and milk production.
- To develop disease resistant animals.
- To produce a variety of biologicals and pharmaceuticals.
- To understand in vivo gene regulation.

USES OF TRANSGENIC ANIMALS

Basic research

- Knockout mice for determining the function of a gene
 - To study effects of gene products, biochemical pathways, alternative (compensatory) pathways, and developmental pathways
 - To recreate human diseases in animals to establish models to test the beneficial effects of drugs or gene therapy.
- Knockout mice for genetic disease models

Production of useful proteins

- Naked human Hb from pigs
- Human lactoferrin in cows' milk
- Alpha-1-antitrypsin in sheep
- HGH in mouse urine (uroplakin promoters)
- Human antibodies in mice (H and L chain genes to hybridomas)
- Tissue plasminogen activator (TPA) in goats
- Human antithrombin III in goats
- Malaria antigens in goats (vaccine)
- Alpha-glucosidase in rabbits (Pompe's disease)
- Pig organs are rejected acutely due to the presence of human antibodies to pig antigens. Once human antibodies are bound to pig organs, human complement is activated and triggers the complement cascade and organ destruction. Transgenic pigs with complement inhibitors have been produced and are gaining feasibility as a source of xenogeneic organs for transplantation.
- Biopharming

BIOPHARMING

- Biopharming is known as the production of pharmaceutical proteins using genetically engineered plants.
- Bio-pharming has the promise to produce large and low-cost supplies of pharmaceutical drugs which includes vaccines for infectious diseases and therapeutic proteins for treatment of cancer and heart diseases.
- The proteins produced by the genetically engineered plants are called as Plant-made pharmaceuticals (PMPs).
- The most common biopharm crops grown in US field trials are corn, tobacco and rice.
- Other crops being studied include alfalfa, potato, soybean, sugarcane and tomato.

NEED FOR BIOPHARMING

- Many pharmaceutical drugs have been produced in sterile fermenters by mammalian cells or using genetically engineered microorganisms. As the construction of huge fermentation plants incurs huge capital cost, the production costs also very high.
- Another method of production of biopharmaceuticals is to extract it from animal and human tissues such as insulin from pig and cow pancreas or blood proteins from human blood. But these procedures carry the risk of transmitting infectious diseases to humans.
- As there is advance in the techniques of manipulating the plant genome over the past twenty years, plants can be used to produce a wide range of important proteins.
- It is hoped that this will result in therapeutic products at a price significantly cheaper than through current methods of production.

CRITERIA TO SELECT A PLANT FOR BIOPHARM PRODUCTION

- It should be easily engineered
- It should be capable of production of high levels of proteins
- Appropriate technique should be available to extract the proteins from plant tissues
- Ideally the host plant should be non-food crop or the food crop should be completely sterile to avoid cross-pollination with near by field crops

BENEFITS OF BIOPHARMS

- PMPs can be produced at a significantly reduced cost compared to current production methods.
- Plants can be engineered to produce proteins of greater complexity than is possible with microorganisms.
- Plants can be used to produce proteins that cannot be produced in mammalian cell cultures.

RISKS OF BIOPHARMS

- Pollen from plants engineered to produce pharmaceuticals may fertilize nearby food or feed crops of the same species. If this occurs, the pharmaceutical may be produced in seed of the neighboring crop, with potentially negative effects on human or animal consumers of the seed
- The introduced gene or its product may have negative effects on the natural environment.

- Farm workers may be exposed to unhealthy levels of a biopharmaceutical by absorbing products from leaves through their skin, inhaling pollen, or breathing in dust at harvest.
- Unexpected toxins or residues of pesticides used on the crop may contaminate the final drug product

MODULE-21: GENOME MAPPING AND SEQUENCING

Learning objectives

The learner will learn about

- Genome mapping
- Method of genome mapping

GENOME MAPPING

- *Genome mapping* is the creation of a genetic map assigning DNA fragments to chromosomes.
- They also establish the relative distance between genes.
- Once the chromosomal regions have been mapped, it becomes easier to carry out DNA sequencing.
- Genome mapping provides a guide for sequencing experiments by showing the locations of genes as well as other distinguishing genomic features.
- A genome map is the first step toward finding a particular gene of interest, and it shows the arrangement of genes and genetic markers along the chromosomes.
- It offers hints as to where on a chromosome a gene may be located.

METHODS OF GENOME MAPPING

- Two different ways of mapping are distinguished :
 - Genetic mapping uses classical genetic techniques (e.g. pedigree analysis or breeding experiments) to determine sequence features within a genome.
 - Using modern molecular biology techniques for the same purpose is usually

PHYSICAL MAPPING

- In physical mapping, the DNA is cut by a restriction enzyme.
- Once cut, the DNA fragments are separated by electrophoresis.
- The resulting pattern of DNA migration (i.e., its genetic fingerprint) is used to identify what stretch of DNA is in the clone.
- By analysing the fingerprints, contigs are assembled by automated finger printed contigs (FPC) or manual means (Pathfinders) into overlapping DNA stretches.

GENOME SEQUENCING

- Genome sequencing is figuring out the order of DNA nucleotides, or bases, in a genome—the order of As, Cs, Gs, and Ts that make up an organism's DNA. The human genome is made up of over 3 billion of these genetic letters.
- Full genome sequencing (FGS), also known as whole genome sequencing, complete genome sequencing, or entire genome sequencing, is a laboratory process that determines the complete DNA sequence of an organism's genome at a single time.
- This entails sequencing all of an organism's chromosomal DNA as well as DNA contained in the mitochondria and for plants the chloroplast as well.
- Almost any biological sample—even a very small amount of DNA or ancient DNA—can provide the genetic material necessary for full genome sequencing.
- Such samples may include saliva, epithelial cells, bone marrow, hair (as long as the hair contains a hair follicle), seeds, plant leaves, or anything else that has DNA-containing cells.

- Because the sequence data that is produced can be quite large (for example, there are approximately six billion base pairs in each human diploid genome), genomic data is stored electronically and requires a large amount of computing power and storage capacity.
- Macrorestriction is a type of physical mapping wherein the high molecular weight DNA is digested with a restriction enzyme having a low number of restriction sites.
- Once the map is determined, the clones can be used as a resource to efficiently contain large stretches of the genome. This type of mapping is more accurate than genetic maps.
 - referred to as physical mapping.

METHODS OF GENOME SEQUENCING

- **BAC-to-BAC method**, the first to be employed in human genome studies, is slow but sure. The BAC-to-BAC approach, also referred to as the map-based method.
- **Whole genome shotgun sequencing**, brings speed into the picture, enabling researchers to do the job in months to a year. The shotgun method was developed by J. Craig Venter in 1996 when he was at the Institute for Genomic Research (TIGR).

BAC TO BAC SEQUENCING

- These pieces are fingerprinted to give each piece a unique identification tag that determines the order of the fragments.
- Fingerprinting involves cutting each BAC fragment with a single enzyme and finding common sequence landmarks in overlapping fragments that determine the location of each BAC along the chromosome.
- Then overlapping BACs with markers every 100,000 bp form a map of each chromosome.
- Each BAC is then broken randomly into 1,500 bp pieces and placed in another artificial piece of DNA called M13. This collection is known as an M13 library.
- All the M13 libraries are sequenced. 500 bp from one end of the fragment are sequenced generating millions of sequences.
- These sequences are fed into a computer program called PHRAP that looks for common sequences that join two fragments together.

WHOLE GENOME SHOTGUN METHOD

- The shotgun sequencing method goes straight to the job of decoding, bypassing the need for a physical map. Therefore, it is much faster.
- Multiple copies of the genome are randomly shredded into pieces that are 2,000 base pairs (bp) long by squeezing the DNA through a pressurized syringe. This is done a second time to generate pieces that are 10,000 bp long.
- Each 2,000 and 10,000 bp fragment is inserted into a plasmid, which is a piece of DNA that can replicate in bacteria. The two collections of plasmids containing 2,000 and 10,000 bp chunks of human DNA are known as plasmid libraries.
- Both the 2,000 and the 10,000 bp plasmid libraries are sequenced. 500 bp from each end of each fragment are decoded generating millions of sequences. Sequencing both ends of each insert is critical for the assembling the entire chromosome.
- Computer algorithms assemble the millions of sequenced fragments into a continuous stretch resembling each chromosome.
- The small genomes of several viruses and bacteria and the much larger genomes of three higher organisms have been completely sequenced; they are bakers' or brewers' yeast (*Saccharomyces cerevisiae*), the roundworm (*Caenorhabditis elegans*), and the fruit fly (*Drosophila melanogaster*).
- In October 2001, the draft sequence of the pufferfish *Fugu rubripes*, the first vertebrate after the human, was completed; and scientists finished the first genetic sequence of a plant, that of the weed *Arabidopsis thaliana*, in December 2000. Many more genome sequences have been completed since then.

MODULE-22: MARKER ASSISTED SELECTION AND GENE BANKING

Learning objectives

The learner will learn about

MARKER ASSISTED SELECTION

- Marker assisted selection (MAS) is a combined product of traditional genetics and molecular biology.
- MAS allows for the selection of genes that control traits of interest. Combined with traditional selection techniques, MAS has become a valuable tool in selecting organisms for traits of interest, such as color, meat quality, or disease resistance.

- Marker assisted selection
- Marker assisted selection techniques
- Gene banking
- Purposes of gene banking
- **Marker Assisted Selection**

MOLECULAR MARKERS

- Molecular markers are used to find genes of interest that control how plants and animals perform.
- Some molecular markers are pieces of DNA that have no known function or impact on animal and plant performance. Other markers may involve the gene of interest itself.
- One type of molecular marker is called a linked marker. Using well-designed experiments, scientists can find molecular markers that are located very close to major genes of interest.
- The molecular marker is said to be linked to that gene. Linked markers are only near the gene of interest on the chromosome and are not part of the DNA of the gene of interest.
- A second kind of molecular marker is one that is part of the gene of interest. Direct markers are easier to work with after they are found, but they often are more difficult to find than linked markers.

TECHNIQUES USED FOR MARKER ASSISTED SELECTION

- Restriction fragment length polymorphism (RFLP)
- Simple sequence repeats (SSR)
- Single nucleotide polymorphism (SNP)

RESTRICTION FRAGMENT LENGTH POLYMORPHISM

- Restriction fragment length polymorphisms (RFLPs) were the first molecular markers used to diagnose genetic variability in organisms.
- RFLP uses restriction enzymes to digest (cut) the DNA molecule and identify regions linked to a trait.

SIMPLE SEQUENCE REPEATS

- Simple sequence repeats (SSRs), also called microsatellites, are repeated units of two to six nucleotides that occur throughout an organism's genome.
- The sequence ATATATAT is one example of a microsatellite. The sequence GATGATGAT is another example.
- SSRs are useful as molecular markers because they are highly polymorphic (have many forms).
- SSRs have been used successfully as markers in a wide range of analysis, particularly those involving disease diagnosis and forensics.

SINGLE NUCLEOTIDE POLYMORPHISM

- On average, SNPs will occur in an organism's DNA more than 1% of the time. Because only about 3% to 5% of an organism's DNA codes for proteins, most SNPs are found outside the regions of genes of interest.
- SNPs found in a gene of interest are of particular interest to researchers because they are directly associated with a desired trait. Because of the recent advances in technology, SNPs are playing a greater role in selection and diagnosis of genetic traits.

- **Gene Banking**

GENE BANK

- Gene banks mean preservation of genetic materials; it may be a plant or an animal.
- In animals, the sperm and oocytes are frozen in liquid nitrogen containers until further need. By gene banking the most valuable animal's genome is preserved for future use.
- Gene banking is simple and an inexpensive technique which can be used to preserve most valuable germ plasms.
- The bank will keep stock of genes of all rare and precious animals including wild and native animals.

PURPOSE OF GENE BANKING

- The primary purpose for establishing a gene bank is to preserve examples of threatened or endangered species. Each year, untold numbers of plant and animal species become extinct because of natural processes and more commonly, as the result of human activities. Once those species become extinct, their gene pools are lost forever.
- Many livestock breeds are vulnerable and could not be replaced in the case of natural decline or disaster. A typical example is that during the recent outbreak of Foot and Mouth disease in Britain, a large number of minority breeds became highly endangered due to the enforcement of policies to contain the disease.
- Efforts are now underway to establish gene banks for animals. Such banks consist of small colonies of the animals themselves. Animal gene banks are desirable as a way of maintaining species whose natural population is very low.
- Sometimes the purpose of the bank is simply to maintain the species to prevent its becoming extinct. In other cases, species are being preserved because they were once used as farm animals although they have since been replaced by more productive modern hybrid species.
- The Fayoumi chicken native to Egypt, for example, has now been abandoned by farmers in favor of imported species. The Fayoumi, without some form of protection, is likely to become extinct. Nonetheless, it may well have some characteristics (genes) that are worth preserving.

PROGRAMMES OF GENE BANKING

- Several programs are working to preserve the genes of endangered animals. China's Chengdu Research Base of Giant Panda Breeding keeps eggs, sperm and other tissue samples from pandas and other natives species preserved in cold storage.
- The United Kingdom's Frozen Ark Project has taken on the mission of creating a network of similar gene banks around the world devoted to endangered animals.
- Scientists in India have taken the idea a step farther by working to eventually reintroduce the Indian cheetah back in to the wild, more than half a century after it was declared extinct. While the researchers lack cryogenically preserved tissue, they have been able to collect skin and bone tissues from museums and zoos. They hope to fill in the genetic blanks by studying the genes of similar cheetah populations in modern-day Iran.
- Some gene banks have set out to document and store DNA from major livestock breeds. The United Kingdom's Department of Agriculture and Rural Development founded a semen archive to safeguard the genetic profiles of rams in Great Britain and Northern Ireland.
- To preserve genetic information for animals, scientists must cryogenically freeze diverse specimens of sperm, eggs, hair, skin and blood from target species to provide the best chance for future cloning. To achieve this, scientists would use a female from a related species as a surrogate mother.
- They'd take one of the surrogate's eggs and fuse it with a cell from the animal to be cloned. The mother would, in effect, give birth to another species -- making her a biologic mother, but not a genetic one.

MODULE-23: NUTRITIONAL BIOTECHNOLOGY

Learning objectives

The learner will learn about

- Biotechnology in animal nutrition
- Applications of DNA technologies for animal nutritional physiology and rumen biology

ANIMAL NUTRITION BIOTECHNOLOGY

- Biotechnology research in the third field of animal nutrition concentrates on improvements in the enzymatic treatment of feed; the addition of inoculant to ensilaged fodder for better and faster conversion and
- Decreasing the anti-nutritional factors in certain plants, such as legumes, which are used as feed.
- In developing countries, such techniques might eventually increase the potential range of crops used to feed larger herds of livestock.

DNA TECHNOLOGIES IN NUTRITIONAL PHYSIOLOGY

- Applications are being developed for improving the performance of animals through better nutrition.
- Enzymes can improve the nutrient availability from feedstuffs, lower feed costs and reduce output of waste into the environment.
- Prebiotics and probiotics or immune supplements can inhibit pathogenic gut microorganisms or make the animal more resistant to them.
- Administration of recombinant somatotropin (ST) results in accelerated growth and leaner carcasses in meat animals and increased milk production in dairy cows.
- Immunomodulation can be used for enhancing the activity of endogenous anabolic hormones.
- In poultry nutrition, possibilities include the use of feed enzymes, probiotics, single cell protein, and antibiotic feed additives.
- The production of tailor-made plant products for use as feeds and free from antinutritional factors through recombinant DNA technology is also a possibility.
- Plant biotechnology may produce forages with improved nutritional value or incorporate vaccines or antibodies into feeds that may protect the animals against diseases.

DNA TECHNOLOGIES IN RUMEN BIOLOGY

- Rumen biotechnology has the potential to improve the nutritive value of ruminant feedstuffs that are fibrous, low in nitrogen and of limited nutritional value for other animal species.
- Biotechnology can alter the amount and availability of carbohydrate and protein in plants as well as the rate and extent of fermentation and metabolism of these nutrients in the rumen.
- The potential applications of biotechnology to rumen microorganisms are many but technical difficulties limit its progress. Current limitations include: isolation and taxonomic identification of strains for inoculation and DNA recombination; isolation and characterisation of candidate enzymes; level of production, localisation and efficiency of secretion of the recombinant enzyme; stability of the introduced gene; fitness, survival and functional contribution of introduced new strains.
- Methods for improving rumen digestion in ruminants include the use of probiotics, supplementation with chelated minerals, and the transfer of rumen microorganisms from other species.

MODULE-24: GENETIC MANIPULATION OF MICROBES FOR IMPROVED FEED UTILIZATION AND HEALTH

Learning objectives

The learner will learn about

- Rumen manipulation
- Steps to optimize rumen digestion
- Applications of recombinant technologies to manipulate rumen micro organisms

NEED FOR RUMEN MANIPULATION

- Fibrous feeds and cereal crop residues of low digestibility comprise the major proportion of feed available to most ruminants.
- Because of feeding of ruminants with the feed of low digestible fibrous feeds, productivity of these livestock has been extremely low evidenced by low growth rates, late maturity, low reproduction rates, late maturity, low reproduction rates, low milk yield and stunted mature body size.
- Rumen fermentation has to be optimized to improve feed utilization in ruminants.

- This is done to maximize the digestion of lignocellulosic compounds and the use of non-protein nitrogen, while allowing other nutrients (i.e., proteins, starch and lipids) to escape rumen fermentation.

STEPS TO OPTIMIZE RUMEN DIGESTION

- There are three steps to optimize digestion,
 - Supplying the nutritional requirements of rumen microflora to ensure maximum microbial activity and microbial growth yield.
 - Modifying microbial population (bacteria, protozoa and fungi) to select the species that are most suitable to supply biomass and metabolites that the host animal requires.
 - Altering the physiology of the digestive tract (i.e., salivary secretion, rumen motility, rates of liquid-phase and particle-phase outflow).
- The first step requires that the known quantitative and qualitative needs of the ruminal microbial populations be met. This can be done by controlling the diet (nature, amount, physical form of feedstuff, balancing nutrients). The other two steps requires the manipulation of the rumen microbes.
 - The primary objective of the manipulation of rumen organisms is to increase the rate and extent of digestibility of these fibrous feeds as this is a primary limiting factor.
 - The fastest way to improve rumen function in an animal is to introduce digestion-enhancing bacterial species from other animals or to selectively increase populations of species that inhabit the rumen only at low levels.
- Rumen ecology may also be modified by altering the function of bacteria using genetic engineering techniques. Many approaches have been taken in attempts to transform rume

ORGANISMS

- Fermentative digestion of forages occurs in ruminants in the well developed fore stomach (the reticulo-rumen). This organ is maintained in a relatively stable continuous anaerobic culture of mixed population of protozoa, bacteria, fungi and bacteriophage.
- The rumen fluid pH is maintained at close to neutrality by continuous saliva which balances the production of acidic end products of fermentation.
- The short chain fatty acids are either absorbed directly or pass out of the rumen in the digesta to the intestines where they are absorbed.
- The anaerobic bacteria are the major organisms in the rumen which help in fibre digestion by attached to and attack damaged parts of plants and digestion occurs from outside towards the centre.
- The fungi are mostly fibrolytic with the major substrates being cellulose and hemicellulose. Their major role may be weakening of feed particle structure as most species have radii which penetrate plant tissues and therefore tend to digest plant materials from inside.
- *Prevotella ruminicola* (formerly *Bacteroides ruminicola*) is one of the most numerous groups of rumen bacteria and is found in ruminants fed on many different diets. These are strictly anaerobic and involved in the degradation and utilization of starch and of plant cell polysaccharides such as xylans and pectins. These bacteria are also believed to play an important role in the degradation of proteins and in the uptake and fermentation of peptides.
- *Ruminobacter amylophilus* accounts for about 15% of isolates from rumen and involved in starch degradation. They also possess significant proteolytic activity.
- *Fibrobacter succinogenes* is one of the most widespread cellulolytic bacteria of the rumen. This accounted for around 20% of the isolates recovered from a cow fed wheat straw; in cows fed other diets, *F. succinogenes* accounted for about 5% of the isolates. *Succinivibrio dextrinosolvens* ferments dextrins in animals fed starch. In addition to their capacity to digest starch, some strains also possess enzyme which could enable products of plant cell wall breakdown to be used.
- *Anaerovibrio lipolytica* has two key properties that probably reflect its ecological roles, the hydrolysis of lipids and utilization of lactate. *Ruminococcus flavefaciens* and *R. albus* are involved in plant fibre degradation in the rumen.
- n bacteria by introducing a foreign gene or genes into the bacteria.

APPLICATION OF RECOMBINANT DNA TECHNOLOGY TO RUMEN ORGANISM

- The type of alteration that is most desirable is
 - an increased ability by rumen organisms to digest coarse plant fibre,
 - by the increased production of cellulolytic and xylanolytic enzymes.
 - *Succinogenes* are cellulolytic and are unable to digest xylan, while *B. rnmnicola* has only xylanolytic activity. By giving the dominant populations of bacteria with digestive capability for multiple cell wall constituents, it may be possible to enhance fibre digestion by a sort of mixed populations that occur naturally.

- Many bacterial genes have inducible promoters and such switches may be useful for the timed induction of nutrient synthesis, antibiotic production, anti-helminthic synthesis, etc. The initial step in this process is to identify inducible genes in the ruminal anaerobes and isolate promoter sequences responsible for control functions.
- Gene libraries have been constructed and used to isolate the genes for cellulose enzymes which can be expressed as active molecules in the *E. coli* host cells.
- The genetic manipulation of rumen microorganisms by altering the inherited characteristic is the most powerful potential tool for enhancing the rate and extent of digestion of fibrous feeds within the rumen.
- Its success depends on the identification of the specific genetic information, the development of appropriate genetic techniques and creation of new organisms with high probability of survival in the harsh environment of the rumen of animals fed on highly fibrous feeds.

MODULE-25: CELL CULTURE AND CELL LINES

Learning objectives

The learner will learn about

- Cell culture and cell lines
- Primary cell culture

CELL CULTURE AND CELL LINES

- **Tissue culture:** The term tissue culture is used as the generic term to include organ culture and cell culture
- Cell culture is the *in vitro* propagation of cells harvested from animal organs or tissues.
- Organ culture involves the maintenance of whole organ or parts of an organ in a way that may allow differentiation and preservation of the architecture and/or function. In primary explant culture, fragments of tissues are placed at a glass-liquid interface, where following attachment, migration is promoted in the plane of the solid surface.
- Cell culture involves the growth and maintenance of individual cell types *in vitro*.
- *In vivo*, the vascular system provides cells with nutrients and growth factors and also with a means of removing metabolic byproducts. This maintains the viability of the cells. The extracellular environment is also necessary for *in vitro* culture systems.
- Nutrients are provided by the culture medium which may also provide a stable pH and a defined salt concentration. Chemically defined media were developed to replace the biological fluids and it has the advantages of consistency between batches of media, ease of sterilization and reduced chance of microbial contamination. It is usually necessary to add serum to provide growth factors.
- Media formulations used include a complex mixture of carbohydrates, amino acids, salts, vitamins, hormones and growth factors. The salt concentration is isotonic to prevent osmotic imbalances. Bicarbonate is often included to act as a buffer system in conjunction with the carbon-di-oxide enriched environment (5-10% CO₂/ 95% air) in which the cells are cultured. This allows the cultures to be maintained around the optimal pH for growth of about 7.4.
- For most cultures, the addition of serum to the medium (5- 10% V/V) is required to maintain cell growth. The sera used mostly in tissue culture are calf, fetal, bovine, horse and human. Calf serum is the most widely used. Fetal bovine second. Antibiotics such as penicillin and streptomycin are added to reduce the bacterial or fungal contamination of the cell culture.

PRIMARY CELL CULTURE

- A primary cell culture is established by inoculating cells taken directly from animal or human tissue into growth medium. The tissue is generally fragmented into small pieces and the excised fragments are treated with a proteolytic enzyme, Trypsin, which disaggregates the tissue into individual cells.
- Primary cell cultures retain most of the characteristics of the cells from which they originated and generally they are anchorage dependant. These cells exhibit a phenomenon called “contact inhibition” which results in cells lining up in strongly oriented parallel strands. In any vessel these cells multiply until they reach a maximum density after which they stop growing further. With regard to chromosomal number, these type of cells usually retain their diploid karyotype.

Primary culture

- Freshly isolated cultures from mammalian systems are known as primary cultures until it is subcultured. At this stage they are usually heterogenous but still closely representative of the parent cell types and in the expression of tissue specific properties.
- After the first sub culture- or passage the primary culture becomes cell lines and may be propagated or subcultured several times. After several subcultures onto fresh media, the cell line will either die out or transform to become a continuous cell line.
- Such cell lines show many alterations from the primary cultures, including changes in
 - Cytomorphology
 - Increased growth rate
 - Increase in chromosome variation
 - Tumorigenicity
- In vitro* transformation is primarily the acquisition of an infinite life span. Animal cells can be grown either in an unattached suspension culture or attached to a solid surface. They do not exhibit the property of contact inhibition and some are anchorage independent and could be established in suspension cultures. Cell lines show a great variation in karyotype. Continuous cell lines are usually aneuploid and often have a chromosome number between the diploid and tetraploid value.
- There are two types of cells
 - Anchorage dependant cells*: Cells attach to a solid surface Eg. Primary or normal diploid cells.
 - Anchorage independent cells*: Cells do not require any solid surface for their attachment. Suspension cultures: Eg. Hela, BHK 21 (Razi).
- Roller tubes and bottles are used for anchorage dependent cells.

CELL LINES

- Cell lines can be divided into two types: adherent (monolayer cells) and non-adherent (suspension cells).
 - Adherent cells attach to the plastic surface of a flask or plate and therefore need to be detached from this surface before they can be used.
 - Suspended cells do not normally attach to the surface of the culture vessel.
- The cell lines may be propagated in an unaltered form for a limited number of cell generations beyond which they may either die out or give rise to continuous cell lines.
- The continuous cell lines are often aneuploid and have larger variation in chromosome number. The alteration in culture, giving rise to a continuous cell line is commonly called *in vitro* transformation.

REGULARLY USED CELL LINES

Name		
Finite cell lines		
MRC 5	Fibroblast	Human embryo lung
W 138	Fibroblast	Human embryo lung
IMR 90	Fibroblast	Human embryo lung
Continuous cell lines		
BHK 21	Fibroblast	Newborn Syria hamster kidney cells
CHOK 1	Fibroblast	Adult Chinese hamster ovary
HeLa	Fibroblast	Adult human
Vero	Fibroblast	Adult monkey kidney

PRODUCTS OBTAINED FROM CELL LINES

Cell line	Product
Human leucocytes	Interferons
Mouse fibroblasts	Interferons
Human kidney	Urokinase
Dog kidney	Canine distemper vaccine
Chick embryo fluid	Foot –and-mouth disease vaccine

Chick embryo fluid	Vaccines for influenza, measles, and mumps
Duck embryo fluid	Vaccines for rabies and rubella
Cell lines of mouse, rat or human origin	Monoclonal antibodies
Chinese hamster ovary cells (CHO)	Tissue-type plasminogen activator (tPA), beta-gamma interferons, factor VIII

MODULE-26: TUMOR MARKERS AND ACUTE PHASE PROTEINS

Learning objectives

The learner will learn about

- Tumor markers
- Importance and uses of tumor markers
- Acute phase proteins
- Applications of acute phase proteins
- **Tumor Markers**

TUMOR MARKERS

- Tumor markers are substances, usually proteins, that are produced by the body in response to cancer growth or by the cancer tissue itself.
- It may be detected in blood, urine, or tissue samples.

IMPORTANCE OF TUMOR MARKERS

- Some tumor markers are specific for a particular type of cancer, while others are seen in several cancer types. Most of the well-known markers may also be elevated in non-cancerous conditions. Consequently, tumor markers alone are not diagnostic for cancer.
- The importance of the tumor marker is to be able to screen for and diagnose cancer early, when it is the most treatable and before it has had a chance to grow and spread.

USEFUL TUMOR MARKERS

- So far, the only tumor marker to gain wide acceptance as a screening test is Prostate Specific Antigen (PSA) for prostate cancer in men. Some people are at a higher risk for particular cancers because they have inherited a genetic mutation.
- *Alpha fetoprotein (AFP)*: is a plasma protein that is normally produced by the fetus. The level of AFP goes down in baby's blood after the birth. AFP production is essentially nil after a year of age. However, it starts up again in some diseases of liver such as, viral hepatitis, cirrhosis of liver, hepatomas, teratocarcinoma and embryonal cell carcinomas. Hence, a person's serum AFP level can therefore be used to help detect these conditions and monitor their treatment.
- BRCA1 and BRCA2 are examples of gene mutations related to an inherited risk of breast cancer and ovarian cancer.
- Carcinoembryonic antigen:
 - Carcinoembryonic antigen (CEA) is a protein found in many types of cells but associated with tumors and the developing fetus. CEA is tested in blood.
 - The normal range is <2.5 ng/ml in an adult non-smoker and <5.0 ng/ml in a smoker. The most common cancers that elevate CEA are in the colon and rectum.
- *Others*: cancer of the pancreas, stomach, breast, lung, and certain types of thyroid and ovarian cancer. Levels over 20 ng/ml before therapy are associated with cancer which has already metastasized (spread). CEA is useful in monitoring the treatment of CEA-rich tumors. If the CEA is high before treatment, it should fall to normal after successful therapy.
- *Neuron-specific enolase (NSE)*: Neuron-specific enolase (NSE) is a substance that has been detected in patients with certain tumors, namely: neuroblastoma, small cell lung cancer, medullary thyroid cancer, carcinoid tumors, pancreatic endocrine tumors, and melanoma.

- **Acute Phase Proteins**

ACUTE PHASE PROTEINS (APP)

- Acute phase proteins (APPs) are a large and varied group of glycoproteins in the serum which are unrelated to immunoglobulin.
- They are synthesized by liver parenchymal cells and released into the bloodstream in response to a variety of stressors such as local inflammation, bacterial infection, endotoxin injection, neoplasia and thermal or mechanical injury as part of the acute phase of the inflammatory reaction.

SYNTHESIS AND BIOLOGICAL FUNCTIONS OF APPs

- The synthesis of APPs is thought to be stimulated by monokines such as IL-1, IL-6 and tumor necrosis factor (TNF).
- Although the response pattern of individual APPs to various stressors or disease may differ, changes in their plasma concentrations are generally regarded as being sensitive, although non-specific, indicators of inflammation.
- The biological functions of APPs are also highly variable with some functioning as proteinase inhibitors, enzymes, transport proteins, coagulation proteins, and modulators of the immune response.
- However, all APPs appear to play a role in the restoration of homeostasis after injury, tissue necrosis, or infection.

APPLICATIONS OF ACUTE PHASE PROTEINS

- Some APPs such as C-reactive protein (CRP) and serum amyloid A (SAA) in humans and haptoglobin in ruminants, are considered "major" APPs and have been used to detect and monitor infection, inflammatory disease, and cancer.
- Acute phase proteins are also being evaluated for their utility in antemortem and postmortem meat and poultry inspection as part of the Hazard Analysis Critical Control Point (HACCP) program.
- The concentration of alpha-acid glycoprotein has been measured in dogs with malignant neoplasia. Continued high levels of this acute phase protein for treatment for conditions where the protein level is high initially suggests that treatment is not working or not appropriate.
- Levels above 1000 ug/ml of serum indicate a poor prognosis especially if subsequent measurements show increasing levels.

MODULE-27: APPLICATION OF PCR AND DNA PROBES IN DISEASE DIAGNOSIS

Learning objectives

The learner will learn about

- Applications of PCR in disease diagnosis
- Applications of nucleic acid probes in disease diagnosis

APPLICATIONS OF PCR IN DISEASE DIAGNOSIS

- PCR has many exciting varied applications:
 - PCR can be used to amplify a specific gene present in different individuals of a species and even in different somatic cells or gametes say humans sperms. These copies can be used for cloning.
 - Alternatively they can be sequenced to obtain information on the mutational changes in gametes in genes of different individuals, cells or gametes such data can be used in disease diagnosis, population genetics, estimation of recombination frequencies.
 - PCR permits early diagnosis of malignant diseases such as leukaemia and lymphomas. Which is currently the highest development in cancer research.
 - PCR assays can be performed directly on genomic DNA samples to detect translocation specific malignant cells at a sensitivity which is at least 10,000 fold higher than other methods.
 - PCR also permits identification of non-cultivable or slow growing micro organisms such as Mycobacteria, Anaerobic bacteria or viruses from tissue culture assays and animal models.
 - The basis of PCR diagnostic application is the detection of infectious agents and the discrimination of non-pathogenic from pathogenic strains by virtue of specific genes.

- Viral DNA can like wise be detected by PCR. The primers used need to be specific to the targeted sequences in the DNA of a virus and the PCR can be used for diagnostic analysis or DNA sequencing of the viral genome. The high sensitivity of PCR permits viral detection soon after infection and even before the onset of disease. Such early detection may give physicians a significant lead in the treatment . The amount of virus (viral load) in a patient can be quantified by PCR based DNA quantification techniques.
- Tumor cells shed from solid primary tumors can be detected by the PCR based on the selective amplification of mutated tumor genes expressed in tissue specific manner when tumor specific alterations are amplified, few tumor cells can be detected in excess of normal cells derived from the same tissue. Thus malignant cells can be detected specifically in pancreatic juice, urine, sputum.
- Viruses:
 - Human retroviruses eg: Human Immuno deficiency virus types 1 and 2 and Human T- cell Lymphotropic virus types 1 and 2 replicate through an RNA intermediate.
 - The RNA intermediate can be detected after performing a reverse transcriptase step to generate a DNA template for PCR.
- As a consequence of replicative cycle and the reduced fidelity of the viral-replicative polymerase and the reverse transcriptase enzymes, considerable heterogeneity between viral isolates is seen. So it is necessary to target highly conserved regions of the viral genome for a PCR assay.
- Other clinically important viruses:
 - Hepatitis B virus (HBV)
 - Human papilloma virus (HPV).
 - *Bacteria*: Diseases caused by *salmonella*, *shigella* and *vibrio* mainly by faecal contamination of water and the presence of the bacterial pathogens in the conditioning tanks such as *legionella pneumophila*, causing legionnaires disease are of concern to be monitored by the way of PCR amplification of a target gene sequence followed by hybridization with specific gene probes provides both specificity and sensitivity for the monitoring.
 - Yet another is the use in the detection of bacterial antibiotic resistance such as bacterial antibiotic resistance such as methicillin resistance in *staphylococcus aureus* using primers specific for these genes.

APPLICATIONS OF DNA PROBE/DNA HYBRIDIZATION IN DISEASE DIAGNOSIS

- The application of Insitu PCR has been mainly confined to two areas foreign gene detection and identification if gene alterations, although many other areas remain to be fully explored because of its extremely high sensitivity. Insitu PCR for DNA is limited to the detection of gene sequence that are not normally present in the tissue samples of interest.
- Insitu PCR is a new molecular technique that combines the extreme sensitivity of PCR with the cellular localization provided by In situ hybridization (ISH). It is based on the principles of amplification of gene sequences within intact cells or tissue sections in order to increase copy number to levels detectable by ISH.
- Insitu PCR can be used in detecting viral infection. In case of HIV one can detect the extent of infection and the effects of treatment. One can utilise in gene therapy for verification of integration and expression of a desired gene in vivo.
- Insitu can also be used to determine tumour burden before and after chemotherapy where specific genetic aberrations are associated with certain types of malignancy or to determine pre neoplastic by examining P⁵³ mutations.
- They can be used to identify infectious agents, tumours, marker genes, cytokines growth factors and their receptors as well as in gene therapy. It can be used to do immuno - histo chemistry together with RNA and DNA amplification at a single cell level.
- The successfully amplified and detected are
 - Human immuno deficiency virus (HIV-1)
 - Simian immunodeficiency virus (SIV)
 - Human papilloma virus (HPV)
 - Hepatitis B virus (HBV)
 - Cytomegalovirus (CMV)
 - Epstein- Barr virus (EBV)
 - Human-herpes virus-6 (HHV-6)
 - Herpes simplex virus (HSV)
 - Lymphogranuloma venereum (LGV)
- P⁵³ and its mutations, mRNA for surfactant protein A, estrogen receptors, inducible nitric oxide synthetase (iNOS) gene sequences associated with multiple sclerosis in various tissues including peripheral mononuclear cells, lymph nodes, spleen, brain, skin, breast, lungs, cytological specimens, tumours, cultured cells, tissues.
- It is a good tool to assist gene therapy in tracing the whereabouts of foreign genes and pinpointing the exact locations of introduced gene sequences after gene therapy delivery.

Applications of PCR and DNA probes for animal disease diagnosis

S.No.	Species	Disease	Target Gene
1.	Cattle	a. Mycobacterium bovis b. Haemorrhagic septicaemia c. Clostridium chauvoei d. Foot and Mouth disease	a. IS1081 b. 16S and 23S rRNA c. 16S rRNA d. C-coding sequence
2.	Sheep & Goats	a. Mycobacterium avium subspecies paratuberculosis b. Staphylococcus aureus c. Brucella d. Sheep pox virus e. Pestivirus f. Babesia ovis g. Theileria h. Toxoplasma gondii	a. IS900 b. se a, b, se c & d gene sequences c. IS711 d. Inverted Terminal Repeats e. 5'-UTR f. Small subunit ribosomal RNA g. Small subunit ribosomal RNA h. B1 gene
3.	Dog	a. Canine distemper b. Canina parvo c. Rabies	a. NP gene b. VP1/VP2 c. Nucleoprotein gene
4.	Poultry	a. Newcastle disease b. Infectious bronchitis c. Infectious bursal d. Marek's disease	a. Fusion gene b. Nucleo protein gene c. VP gene d. Meq gene

MODULE-28: MONOCLONAL ANTIBODY PRODUCTION**Learning objectives**

The learner will learn about

- Monoclonal antibody production
- Steps in monoclonal antibody production
- Applications of monoclonal antibodies

MONOCLONAL ANTIBODIES

- Each B lymphocyte can produce only one type of antibody. Monoclonal antibodies are antibodies produced by a group of antibody secreting B cells grown from a single B cell that is directed against a single epitope of an antigen.
- In 1975 the scientists, *Kholer* and *Milstein* first showed the ways to produce monoclonal antibodies from hybrids between myeloma tumor cells and antigen stimulated B lymphocytes.
- Monoclonal antibody production involves the creation of hybrid cells derived from two parental cell types: normal antibody producing cells and cancerous myeloma cells. The hybrid cells are formed by fusing the myeloma cells and the antibody secreting B lymphocytes using a chemical, polyethylene glycol (PEG). Hybrid cells have characters of both parents, that is the infinite growth of the cancerous myeloma cells and antibody secreting property of B lymphocytes.

STEPS IN PRODUCTION OF MONOCLONAL ANTIBODIES

- Immunization
 - The animal used for immunization is BALB/c mice. The purpose of immunization is to sensitize the B lymphocytes (in the spleen) against the antigen for which monoclonal antibodies required. Immunization is done by repeated injection of antigen into the mice.
- Fusion
 - The purpose of the fusion is to produce hybrids cells between the B lymphocytes and the cancerous myeloma cells (Sp2/o). The fusion is done by mixing the immunized B lymphocytes with the myeloma cells in the presence of polyethylene glycol (PEG). The PEG helps to fuse the cells.
- Selection of hybrid cells
 - The fused cells are grown in HAT (Hypoxanthine, Aminopterin and Thymidine) medium. The selection of hybrid cells formed between only B lymphocyte and the myeloma cell is achieved by growing the fused cells in the HAT medium.
- Cloning of hybrid cells
 - The hybrid cells growing in HAT medium are then screened for their ability to produce and secrete the desired antibody. This is done by enzyme linked immunosorbent assay (ELISA). Then cloning under limiting dilution is done to ensure the monoclonality of the hybrid cells. Limiting dilution is a dilution technique by which hybrid cells are distributed at a concentration of 1 cell per well. Within 2 weeks, the 'one' cell in the well form a group of cells and secrete monoclonal antibodies against single epitope of the antigen used in the immunization.
- Large scale production of monoclonal antibodies
 - For large scale production, hybridomas can be grown either in tissue culture, where they secrete up to 100 ug / ml (usually 10 – 50 ug / ml) or *in vivo* as tumors in the peritoneal cavity of BALB/c mice (hybridoma) where they produce up to 40 mg / ml (usually 2 – 20 mg / ml)

APPLICATIONS OF MONOCLONAL ANTIBODIES

- Antigen detection / disease diagnosis.
- Leucocyte identification.
- Parasitic identification.
- Hormones detection.
- Detection of carcinoembryonic protein.
- Detection of tumor related antigens.

MODULE-29: VACCINES

Learning objectives

The learner will learn about

- Types of vaccines
- Steps in preparation of subunit vaccines
- Advantages and disadvantages of subunit vaccines

VACCINES

- Vaccination protects a recipient from pathogenic agents by establishing an immunological resistance to infection. An injected or oral vaccine induces the host to generate antibodies against the disease-causing organisms.
- *Dr. Edward Jenner* discovered the principle of vaccination. Cowpox is a mild cattle disease cause human infection. Where as small pox is an extremely virulent disease with high death rate in human beings.
- Jenner inoculated James Phipps, an 8-year old, with exudates from a cowpox pustule. The boy was found to be fully protected against human small pox.
- Modern vaccines typically consist of either a killed (inactivated) or a live, non-virulent (attenuated) form of an infectious agent. The infectious agent is grown in culture, purified and either inactivated or attenuated without losing its ability to evoke an immune response.
- The introduction of recombinant DNA technology has provided a means for creating a new generation vaccines that overcome the drawbacks of traditional vaccines.

SUBUNIT VACCINES

- Vaccines generally consist of either killed or attenuated forms of the whole pathogenic agent.
- The antibodies elicited by these vaccines initiate an immune response to inactivate (neutralize) pathogenic organisms, by binding to proteins on the outer surface of the agents.
- In order to avoid the whole organisms for vaccines, for their disadvantages, purified outer-surface viral proteins, either capsid or envelope proteins were tried.

- Recombinant DNA technology, in which a section of DNA from one species is inserted into the DNA of another, is proving to be useful in the manufacture of vaccines that contain only the desired antigen - usually one or several proteins - without the need for attenuated toxins or modified versions of the disease causing virus or bacterium. This results in the production of an immune response without the risk of actual contraction of the original disease, and is potentially more cost effective and commercially viable.
- An example of how the recombinant vaccine process works can be seen in the production of Tickgard vaccine. Here, genes responsible for the manufacture of the gut protein Bm86 in the cattle tick, *Rhipicephalus microplus*, are inserted into the plasmid DNA and then transformed into the *E.coli*. These microbes are cultured, and express the Bm86 protein on a large scale. The protein can then be purified and injected into cattle, with the result that their immune system will protect them against the Bm86 antigen introduced in a real tick bite.
- Current research has found that, although the Bm86 gene can be adequately expressed in recombinant *E. coli* bacteria and strains of the fungus *Aspergillus*, the yeast, *Pichia pastoris* has proved to be the most successful at secreting the protein. One reason for this is its rapid growth rate and its ability to grow on inexpensive media.

- They are sufficient for eliciting neutralizing antibodies in the host organism. Vaccines that use components of a pathogenic organism rather than the whole organism are called subunit vaccines.

CREATING A SUBUNIT VACCINE

- E.g. Foot and Mouth disease vaccine.
- The primary requirement for creating any subunit vaccine is identifying the component(s) of the infectious agent that elicits antibodies that react against the intact form of the infectious agent.
- The major antigenic determinant inducing neutralizing antibody is the capsid viral protein 1 (VP1). Thus the gene for VP1 became the target for cloning.
- The genome of FMDV is composed of ssRNA. First double stranded cDNA was synthesized for the entire genome. This cDNA was then digested with restriction enzymes and the fragments cloned in an *E. coli* expression vector.
- The product of the VP1 coding sequence was identified immunologically as part of a fusion protein. The fusion protein was 396 amino acids long and consisted of a portion of the carrier protein as well as the entire sequence of FMDV VP1 protein. The fusion protein containing the VP1 protein fragment was able to generate neutralizing antibodies to FMDV

ADVANTAGES AND DISADVANTAGES OF SUBUNIT VACCINE

Advantages

- Using purified protein (s) as an immunogen ensures that the preparation is stable and safe.
- They are precisely defined chemically.
- Free of extraneous proteins and nucleic acids that can initiate undesirable side effects.

Disadvantages

- Purification of a specific protein can be costly.

RECOMBINANT DNA VACCINE

- The isolated protein may not have the same conformation as it does *in situ* (i.e. within viral capsid or envelope), with the result it is antigenically altered.

RECOMBINANT VACCINE FOR ANIMAL USE

- Several commercially available recombinant vaccines used on animals employ a vector based delivery system. These include the VRG vaccine, which protects animals against rabies, and the Purevax recombinant feline leukaemia vaccine.
- As mentioned above, the VRG vaccine consists of a recombinant *Vaccinia* virus that carries the gene for a rabies glycoprotein. The virus has been modified in several ways, one of which involves the removal of the thymidine kinase gene, making it safer to administer than in its original form. Studies have shown that it has not caused any side effects in over 10 avian species and 35 mammalian species.
- The Purevax leukaemia vaccine contains a harmless recombinant canarypox virus that incorporates the FeLV gene. This gene produces a protein identical to that produced by the FeLV (feline leukaemia) virus, with the result that the cat's immune response is triggered without the danger of the actual virus being introduced. The canarypox virus is also used as a vector in dog and ferret vaccines.

RECOMBINANT VECTORED VACCINES

- It is possible to introduce genes that encode major antigens of especially virulent pathogens into a non-pathogenic or avirulent viruses or bacteria. These organism serves as a vector, replacing within the host and expressing the gene product of the pathogen. A number of organisms have been used for vector vaccines, including vaccinia virus, the canary poxvirus, attenuated poliovirus, adenoviruses, attenuated strains of *Salmonella*, and the BCG strain of *Mycobacterium bovis*.
- *Vaccinia* vector has widely been employed as a vector vaccine. This large, complex virus, with a genome of 200 genes, can be engineered to carry several dozen foreign genes without impairing its capacity to infect host cell and replicate. The genetically engineered vaccinia expresses high levels of the inserted gene product, which can then serve as a potent immunogen in an inoculated host.
- The canary poxvirus has recently been tried as a vector vaccine. Like its relative vaccinia, the canary poxvirus is a large virus that can be easily engineered to carry multiple genes. Unlike vaccinia, the canary poxvirus does not appear to be virulent even in individuals with severe immune suppression. Another possible vector is an attenuated strain of *Salmonella typhimurium*, which has been engineered with genes from the bacterium that causes cholera. The advantage of this vector vaccine is that *Salmonella* infects cells of the mucosal lining of the gut and therefore will induce secretory IgA production.

MODULE-30: FERMENTATION TECHNIQUES

Learning objectives

The learner will learn about

- Fermentation technology
- Upstream and down stream processes of fermentation technology
- Types of fermenters
- Applications of fermentation technology

FERMENTATION TECHNOLOGY

- Fermentation forms the core of the old biotechnology and continues to constitute the most fundamental and the most mature area of contemporary biotechnology.
- Man has been using fermentation technology for the processing of food and beverages since time immemorial. One of the earliest applications of fermentation technology was in the brewing industry. Later, it was used for the production of chemicals, and still later, of antibiotics.
- "*Fermentation*" may be defined as the biochemical activity of a microorganism in its growth, development, reproduction and possibly even senescence and death. A fermenter may likewise be defined as the "container, whether conceptual or physical, which contains the fermentation".
- A fermentor is the person who operates or controls the fermentation process in a fermenter.

FERMENTATION PROCESS

- At the heart of any microbial fermentation process is the fermenter or the vessel or container in which fermentation occurs.
- All the operations before starting the fermenter are collectively called '*upstream*' processes and those after the fermentation has occurred are called '*downstream*' processes.

- Examples of upstream operations include the preparation and sterilization of the culture medium, the sterilization of the fermenter itself, and the preparation and growth of a suitable inoculum culture of the microbial strain.
- Downstream operations involve the collection or harvesting of the product, its concentration, purification and further processing such as separation from contaminants or unwanted components and also a proper treatment of the spent culture medium or effluent.
- The fermenter is also known as *bioreactor*. It provides an optimum environment in which organisms or their enzymes can interact with a substrate, forming the desired product. Several kinds of fermenters are being used for various preparation.

TYPES OF FERMENTERS

- Some commonly used fermenters are
 - Stirred tank fermenter (usually used for making antibiotics).
 - Tubular tower fermenter (for making beer, wine, vinegar, cider and so on).
 - Internal recycle airlift fermenter (for producing oil from yeast).
 - External recycle airlift fermenter (producing methanol from bacterial biomass).
 - Nathan fermenter (currently popular in brewing industry).

APPLICATIONS OF FERMENTATION TECHNOLOGY

- Production of biomass. E.g. of spirulina and yeast.
- Production of enzymes and nucleic acids.
- Production of metabolites, both primary (e.g. ethanol, lactic acid) and secondary (e.g. antibiotics, antimetabolites).
- Enzymatic conversion of specific substrates. E.g. glucose to fructose.
- Enzymatic conversion of multiple substrates. E.g. biological wastes.

MODULE-31: FERMENTATION PROCESS FOR MILK, MEAT AND LEATHER

Learning objectives

The learner will learn about

- Fermentation process of milk
- Fermentation process of meat
- Fermentation process of leather
- **Fermentation Process for Milk**

MILK FERMENTATION PROCESS

- Fermentation of milk has been practised all over the world for very long period. This process was used to preserve a highly perishable product such as milk. In the early periods, the fermentation of milk was done by natural process i.e., fermentation of milk by its normal microflora. Now, lactic acid producing microorganisms are added to milk to decrease the pH of the milk and produce different fermented milk products.
- The fermentation of milk involves the production of lactic acid from lactose in the milk. This process lowers the pH of the milk which results in coagulation of the milk protein casein – curdling.

FERMENTED MILK PRODUCTS

- Yogurt
- Sour cream
- Kefir
- Cheese

YOGURT

- Yogurt is produced by using whole milk or skim milk.

- The milk is inoculated with a starter culture which contains *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. These bacteria convert the lactose in the milk into lactic acid, causing the milk into curdle to form yogurt.

SOUR CREAM

- Sour cream is produced like yogurt but light cream is used instead of whole milk or skim milk.
- *Streptococcus* spp. and *Lactobacillus* spp. are used as starter cultures to produce lactic acid from lactose in the milk.
- The lowered pH results in coagulation of cream which results in sour cream.

KEFIR

- Kefir is a fermented milk product which utilizes a wide variety of microorganisms to produce different products such as ethanol, free fatty acids and acetaldehyde in addition to lactic acid.
- These products are produced by addition of *Lactobacillus* spp and non-pathogenic *Streptococcus* spp., yeasts such as *Saccharomyces delbrueckii* and *Saccharomyces cerevisiae*.
- These bacteria and yeasts are added to milk and allowed to ferment for 18 to 24 hours to produce various products mentioned above.

CHEESE

- Cheese is another fermented milk product produced by addition of bacteria to produce lactic acid to coagulated casein and then formation of cheese by addition of enzyme, rennet.
- The cheese can also be produced without addition of rennet such as cottage cheese or cream cheese.

MEAT FERMENTATION

- A food is termed 'fermented' if it 'has been subjected to the action of microorganisms or enzymes so that desirable biochemical changes cause significant modification of the food'.
- Dry cured, unground raw meats are mainly preserved by salting and drying, and excellent raw hams can be prepared without significant microbial activity. Rather, the activity of meat enzymes is important for the development of the aroma and tenderness of these products. Bacteria are needed mainly for the reduction of nitrate which is still frequently used as a curing agent, and bacteria have also been reported to improve the flavour. In addition, injection of lactic acid bacteria along with sugar has been suggested in order to lower the pH of hams and facilitate water removal. Some perishable meat products may also be preserved by addition of selected strains of lactic acid bacteria antagonistic to pathogens and spoilage flora.
- Fermented meats are generally ground, spiced and made into sausage. These meats also can be smoked, cooked, cured or uncooked. After the meat is prepared, it is generally hung and left to ferment for varying amounts of time depending on the type. Common types of fermented meats are salami, pepperoni and summer sausage.

FERMENTED SAUSAGES

- Fermented sausages are defined as ground meat mixed with salt and curing agents, stuffed into casings and subjected to a fermentation process in which microorganisms play a crucial role.
- Most fermented sausages are dried and can be stored with little or no refrigeration.

BIOTECHNOLOGY PROCESSES IN LEATHER MAKING

Soaking

- Soaking enzymes are used to shorten production time by attacking solidified fats and non-collagenous proteins that interpose themselves between the fibers. These proteins sometimes cover the external surfaces of the hide, making contact between collagen fibers and water difficult.
- Rather than targeting a specific reaction, the enzymes used in soaking target a broad-spectrum of reactions to obtain both solubilization and removal of the interfibrillar proteins, enabling easy rehydration of the skin.
- The types of enzyme used in the soaking of hides are carbohydrases and proteases.

- The advantages of an enzyme soak include shorter wetting times, loosening of the SCUD, initiation of FIBER OPENING, and production of a product with a less wrinkled grain. However, a major disadvantage of their use is the added costs involved.

Liming

- The conventional process of liming uses high proportions of lime and sulfide. These materials are a source of pollution in the spent lime liquors. An advantage of using lime is that it is a poorly soluble alkali thus providing limited availability of dissolved alkalis. However, a disadvantage is that it generates large quantities of solid waste. Although sulfide is toxic, it is the prime depilant in the de-hairing process.
- It is now possible to reduce sulfide at its source using enzyme-assisted processes. Enzymes used in de-hairing are generally proteolytic, catalyzing the breakdown of proteins. Their origin can be animal (e.g. from bovine or porcine pancreas), bacterial, fungal or plant. Enzymatic de-hairing generally uses proteolytic enzymes along with small amounts of sulfide and lime.
- The mixture is painted onto the fleshside, causing the hair to be loosened by selective breakdown of the cementing substances in the hair follicle, thereby keeping the hairs intact.

Bathing

- Bating is the process of removing proteins other than collagen using proteolytic enzymes. The most important function of the enzymatic bate is removal of the coagulable protein of skin.

Degreasing

- The degreasing process is another stage of the tanning process where the use of enzymatic products is feasible. The degreasing process can be broken down into three successive stages:
 - breakdown of the proteic membrane of the fat-containing sac,
 - removal of the fat, and
 - emulsification of the fat in water or solubilization in solvent. An enzymatic preparation, therefore, needs a triple-action (proteolysis, lipolysis and emulsification) to be an effective degreasant.

MODULE-32: ETHICS AND REGULATORY ISSUES IN BIOTECHNOLOGY

Learning objectives

The learner will learn about

- Ethical issues in biotechnology
- Regulations for animal biotechnology

ETHICAL ISSUES IN BIOTECHNOLOGY

- Ethics are the rules or standards that govern the way people behave and their decisions on the 'right' thing to do. It asks basic questions about what is right and wrong, how we should act towards others and what we should do in specific situations.
- It is important to note that ethics relating to biotechnology and its applications are not fundamentally different from other situations. Ethics are practiced by everyone, every day.
- One common feature of ethics is that different people with different values often disagree on the 'right thing' for individuals and society. One reason for this disagreement is that one thing may benefit some people but not others.
- An example is embryonic stem cell research. Some people see this as having great potential to develop cures for diseases. But, others object because it involves the destruction of human embryos that have the potential to become a human being.
- For instance, someone who has a strong environmental outlook might see the use of genetically modified (GM) crops as unnatural. But, someone who has a strong scientific-based view of the world might see the use of GM crops as a natural extension of traditional crop breeding technologies.

Commercial outcome and ethics

- In some areas of biotechnology development, the money needed to fund research projects is out of the range of individuals or small groups; it can only be undertaken by multinational or overseas companies. Some perceive this as acceptable, because it helps local researchers form links with wealthy larger companies. But others do not think it is not acceptable, because local research and development leave the community and are then controlled by international corporations.
- There are particular ethical positions that are commonly shared. One of these is the view that all

FOOD SAFETY

- The main question posed about the safety of food produced through animal biotechnology for human consumption is, “Is it safe to eat?” National Academies of Science (NAS) report listed three specific food concerns: allergens, bioactivity and the toxicity of unintended expression products.
- The potential for new allergens to be expressed in the process of creating foods from genetically modified animals is a real and valid concern, because the process introduces new proteins. While food allergens are not a new issue, the difficulty comes in how to anticipate these adequately, because they only can be detected once a person is exposed and experiences a reaction.
- Another food safety issue, bioactivity, asks, “Will putting a functional protein like a growth hormone in an animal affect the person who consumes food from that animal?”
- Finally, concern exists about the toxicity of unintended expression products in the animal biotechnology process. While the risk is considered low, there is no data available. The NAS report stated it still needs be proven that the nutritional profile does not change in these foods and that no unintended and potentially harmful expression products appear.
- biotechnology products must be safe for humans and the environment.

ENVIRONMENTAL CONSIDERATIONS

- Another major concern surrounding the use of animal biotechnology is the potential for negative impact to the environment.
- These potential harms include
 - the alteration of the ecologic balance regarding feed sources and predators,
 - the introduction of transgenic animals that alter the health of existing animal populations and
 - the disruption of reproduction patterns and their success.
- To assess the risk of these environmental harms, many more questions must be answered, such as: What is the possibility the altered animal will enter the environment? Will the animal’s introduction change the ecological system? Will the animal become established in the environment? and Will it interact with and affect the success of other animals in the new community? Because of the many uncertainties involved, it is challenging to make an assessment.

REGULATIONS FOR ANIMAL BIOTECHNOLOGY

- The regulation of animal biotechnology currently is performed under existing government agencies. To date, no new regulations or laws have been enacted to deal with animal biotechnology and related issues. The main governing body in USA for animal biotechnology and their products is the FDA. Specifically, these products fall under the new animal drug provisions of the Food, Drug, and Cosmetic Act (FDCA). In this use, the introduced genetic construct is considered the “drug.” This lack of concrete regulatory guidance has produced many questions, especially because the process for bringing genetically engineered animals to market remains unknown.
- Currently, the only genetically engineered animal on the market is the GloFish, a transgenic aquarium fish engineered to glow in the dark. It has not been subject to regulation by the FDA, however, because it is not believed to be a threat to the environment.
- The European Medicines Agency is responsible for the approval of pharmaceuticals derived through animal biotechnology, and the EFSA is responsible for approval of food derived from animal biotechnology. Regarding regulation of food biotechnology, the EFSA is responsible for scientific risk assessment. Directive 2001/18/EC regulates the distribution of GMOs and GMO use in food products, but there are no specific European regulations for food products derived from biotechnology, such as cloning that does not involve genetic modification. No distinction is made between animal or plant products .
- China currently is the sixth largest producer of GM crops, and its government has made a strong commitment to both plant and animal biotechnology. Animal biotechnology in China is governed primarily by three agencies: the Ministry of Health, the Ministry of Science and Technology, and the Ministry of Agriculture.

- There are two main international protocols that affect animal biotechnology. The Codex Alimentarius Commission (Codex) and the Cartagena Protocol on Biosafety to the Convention on Biological Diversity.
- The Codex, jointly administered by two United Nations agencies—the World Health Organization and the Food and Agriculture Organization—sets international safety standards for foods. Before a food produced by biotechnology can be marketed, it is subjected to a pre-market assessment that evaluates both the direct and unintended effects on food safety and nutritional aspects that might arise because of the use of technology. Although it is a thorough risk assessment of the food safety issues, the Codex does not address the environmental, ethical, moral, or socioeconomic impacts of the technology.
- The Cartagena Protocol on Biosafety to the Convention on Biological Diversity primarily is an environmental treaty. Its main purpose is to protect biological diversity from risks posed by “living modified organisms” (LMOs), taking into account potential risks to human health. Although the Cartagena Protocol thus far primarily has focused on plant biotechnology, its definition of LMOs equally encompasses animals. The protocol adopts a precautionary approach; if a potential but not yet scientifically proven risk might exist, that potential risk may be used as a reason to limit the importation or use of an LMO. There are 157 parties to the Cartagena Protocol, including India, most European countries and China. The United States and Australia are not parties.

Biotechnology authorities which controls biotechnology activities in India

1. Genetic Engineering Approval Committee (GEAC) - functions under Ministry of Environment and Forests (MOEF).
2. Review Committee on Genetic Manipulation (RCGM - function under Department of Biotechnology (DBT).
3. Review Committee on Genetic Manipulation (RCGM) - function under Department of Biotechnology (DBT).
4. Monitoring Cum Evaluation Committee (MEC)
5. Institutional Biosafety Committee (IBC) - functions at research institution/ Organization level
6. State Biotechnology Coordination Committee (SBCC) - functions under the state government where biotech research occurs.
7. District-Level Committee (DLC) - functions under the district administration where biotech research occurs.

MODULE-33: INTELLECTUAL PROPERTY RIGHTS

Learning objectives

The learner will learn about

8. Intellectual property rights (IPR)
9. Categories of IPR
10. Where research occurs

INTELLECTUAL PROPERTIES

- The term intellectual property refers broadly to the creations of the human mind.
- Intellectual property rights protect the interests of creators by giving them property rights over their creations.

CATEGORIES OF INTELLECTUAL PROPERTY

- Intellectual property is divided into two categories:
 - Industrial property, which includes inventions (patents), trademarks, industrial designs, and geographic indications of source
 - Copyright, which includes literary and artistic works such as novels, poems and plays, films, musical works, artistic works such as drawings, paintings, photographs and sculptures, and architectural designs. Rights related to copyright include those of performing artists in their performances, producers of phonograms in their recordings, and those of broadcasters in their radio and television programs.

INDUSTRIAL PROPERTY

- Industrial property takes a range of forms, these include patents to protect inventions and industrial designs, which are aesthetic creations determining the appearance of industrial products.

- Industrial property also covers trademarks, service marks, layout-designs of integrated circuits, commercial names and designations, as well as geographical indications, and protection against unfair competition.

COPYRIGHT

- Copyright relates to artistic creations, such as poems, novels, music, paintings, and cinematographic works.
- In most European languages other than English, copyright is known as author's rights.
- The expression copyright refers to the main act which, in respect of literary and artistic creations, may be made only by the author or with his authorization. That act is the making of copies of the literary or artistic work, such as a book, a painting, a sculpture, a photograph, or a motion picture.
- The second expression, author's rights refers to the person who is the creator of the artistic work, its author, thus underlining the fact, recognized in most laws, that the author has certain specific rights in his creation, such as the right to prevent a distorted reproduction, which only he can exercise, whereas other rights, such as the right to make copies, can be exercised by other persons, for example, a publisher who has obtained a license to this effect from the author.

PATENTS

- Patents, also referred to as patents for invention, are the most widespread means of protecting the rights of inventors. Simply put, a patent is the right granted to an inventor by a State, or by a regional office acting for several States, which allows the inventor to exclude anyone else from commercially exploiting his invention for a limited period, generally 20 years.
- By granting an exclusive right, patents provide incentives to individuals, offering them recognition for their creativity and material reward for their marketable inventions. These incentives encourage innovation, which in turn contributes to the continuing enhancement of the quality of human life.
- In return for the exclusive right, the inventor must adequately disclose the patented invention to the public, so that others can gain the new knowledge and can further develop the technology.
- The disclosure of the invention is thus an essential consideration in any patent granting procedure. The patent system is so designed as to balance the interests of inventors and the interests of the general public.
- The word patent, or letters patent, also denotes the document issued by the relevant government authority. In order to obtain a patent for an invention, the inventor, or the entity he works for, submits an application to the national or regional patent office.
- In the application the inventor must describe the invention in detail and compare it with previous existing technologies in the same field in order to demonstrate its newness.
- In order to obtain a license to this effect from the author.

CONDITIONS OF PATENTABILITY

- Not all inventions are patentable. Laws generally require that an invention fulfill the following conditions, known as the requirements or conditions of patentability:
 - *Industrial Applicability (utility)*: The invention must be of practical use, or capable of some kind of industrial application.
 - *Novelty*: It must show some new characteristic that is not known in the body of existing knowledge (referred to as prior art) in its technical field.
 - *Inventive step (non-obviousness)*: It must show an inventive step that could not be deduced by a person with average knowledge of the technical field.
 - *Patentable subject matter*: The invention must fall within the scope of patentable subject matter as defined by national law. This varies from one country to another. Many countries exclude from patentability such subject matter as scientific theories, mathematical methods, plant or animal varieties, discoveries of natural substances, methods for medical treatment (as opposed to medical products), and any invention where prevention of its commercial exploitation is necessary to protect public order, good morals or public health.

PRODUCT PATENT AND PROCESS PATENT

- The creation of a new alloy is an example of a product invention.
- The invention of a new method or process of making a known or new alloy is a process invention.
- The corresponding patents are usually referred to respectively as a product patent and a process patent.

PATENTEE AND EXCLUSIVE RIGHTS

- The person to whom a patent is granted, is known as the patentee, the owner of the patent or the patent holder.
- Once a patent has been granted with respect to a particular country, anyone who wishes to exploit the invention commercially in that country must obtain the authorization of the patentee.
- In principle, anyone who exploits a patented invention without the patentee's authorization commits an illegal act. The protection is granted for a limited period, generally 20 years.
- Once a patent expires, the protection ends, and the invention enters the public domain. The patentee no longer holds exclusive rights to the invention, which then becomes available for commercial exploitation by others.
- The rights conferred by a patent are not described in the patent itself. Those rights are described in the patent law of the country in which the patent is granted.
- The patent owner's exclusive rights generally consist of the following in the case of a product patent, the right to prevent third parties without the owner's consent from making, using, offering for sale, selling or importing for these purposes the product; in the case of a process patent, the right to prevent third parties without the owner's consent from using the process; and to prevent third parties from using, offering for sale, selling or importing for these purposes the products which were obtained directly by that process.

TRADE MARK

- A trademark is a sign, or a combination of signs, which distinguishes the goods or services of one enterprise from those of another.
- A trademark is a sign used on goods or in connection with the marketing of goods.
- The trademark may appear not only on the goods themselves but also on the container or wrapper in which the goods are sold.
- When used in connection with the marketing of the goods the sign may appear in advertisements, for example in newspapers or on television, or in the windows of the shops in which the goods are sold.

COLLECTIVE MARKS

- Collective marks are owned by an association, such as an association representing accountants or engineers, whose members use the mark to identify themselves with a level of quality and other requirements set by the association.

CERTIFICATION MARKS

- Certification marks, such as the Woolmark, are given for compliance with defined standards, but are not confined to any membership.

TRADE NAMES

- Another category of industrial property covers commercial names and designations.
- A commercial or trade name is the name or designation that identifies an enterprise. In most countries, trade names may be registered with a government authority.

GEOGRAPHICAL INDICATIONS

- A geographical indication is a sign used on goods that have a specific geographical origin and possess qualities or a reputation that are due to that place of origin.
- Agricultural products typically have qualities that derive from their place of production and are influenced by specific local factors, such as climate and soil.

WORLD INTELLECTUAL PROPERTY ORGANIZATION

- The World Intellectual Property Organization (WIPO) is an international organization (a specialized agency of the United Nations) dedicated to ensuring that the rights of creators and owners of intellectual property are protected worldwide and that inventors and authors are thus recognized and rewarded for their ingenuity

MODULE-34: BIOINFORMATICS

Learning objectives

The learner will learn about

- Bioinformatics
- Components of bioinformatics
- Fields of bioinformatics
- 1. **Structural bioinformatics**
- 2. **Genome bioinformatics**

BIOINFORMATICS

- Bioinformatics is conceptualizing biology in terms of molecules and applying “informatics techniques” derived from disciplines such as applied maths, computer science and statistics to understand and organise the information associated with these molecules, on a large scale.

COMPONENTS OF BIOINFORMATICS

- Bioinformatics has three components,
 - The creation of databases allowing the storage and management of large biological data sets.
 - The development of algorithms and statistics to determine relationships among members of large data sets.
 - The use of these tools for the analysis and interpretation of various types of biological data, including DNA, RNA and protein sequences, protein structures, gene expression profiles, and biochemical pathways.

IMPORTANCE OF COMPUTERS IN BIOINFORMATICS

- First, many bioinformatics problems require the same task to be repeated millions of times. For example, comparing a new sequence to every other sequence stored in a database or comparing a group of sequences systematically to determine evolutionary relationships. In such cases, the ability of computers to process information and test alternative solutions rapidly is indispensable.
- Computers are required for their problem-solving power. Typical problems that might be addressed using bioinformatics could include solving the folding pathways of protein given its amino acid sequence, or deducing a biochemical pathway given a collection of RNA expression profiles. Computers can help with such problems, but it is important to note that expert input and robust original data are also required.

IMPORTANT FIELDS OF BIOINFORMATICS

- Structural bioinformatics
- Drug designing
- Phylogenetics
- Computational biology
- Population genetics
- Splicing site prediction
- miRNA prediction
- RNA structure prediction
- Gene prediction
- Transcription factor binding site prediction
- Genome annotation
- Ancestry prediction

STRUCTURAL BIOINFORMATICS

- It involves, predicting the 3D structure of a protein from its protein sequence.
- Homology modelling is the best method for predicting the protein structures by using already structured or crystallized protein as a template.
- MODELLER is one of the best software for Homology modelling. Protein Data Bank is the data base for 3D co-ordinates of a protein.

DRUG DESIGNING

- Drug design is the approach of finding drugs by design, based on their biological targets.
- Typically a drug target is a key molecule involved in a particular metabolic or signalling pathway that is specific to a disease condition or pathology, or to the infectivity or survival of a microbial pathogen.

PHYLOGENETICS

- Predicting the genetic or evolutionary relation of set of organisms. Mitochondrial SNPs and Microsatellites (DNA repeats) are mostly used in Phylogenetics.
- MEGA, PAUP are some of the important softwares. Maximum Parsimony and Maximum Likelihood are mostly used methods.

COMPUTATIONAL BIOLOGY

- Computational biology is an interdisciplinary field that applies the techniques of computer science, applied mathematics, and statistics to address problems inspired by biology.

POPULATION GENETICS

- Population genetics is a study of genotype frequency distribution and the change in the genotype frequencies under the influence of natural selection, genetics drift, mutation and gene flow.
- Coalescent theory is one of the most used theory to predict the most recent ancestor.
- Arlequin is one of the best and most used software in population genetics.

SPLICING SITE PREDICTION

- Splicing prediction is a very important application of bioinformatics which is very important in Gene expression studies.

miRNA PREDICTION

- miRNA (micro RNA) emerged as a new gene regulatory element and gained more space in research. 20 -23 base pair RNA which regulates a gene or genes. So many methods and softwares have been developed to predicting this tiny RNAs. But still they are not precise in predicting. It means that we need some more information from experimental labs to predict.
- miRNA binds to the gene and regulates the gene. Most of the time it down regulate the gene expression. Predicting the miRNA target is also a very important problem in Bioinformatics.

RNA STRUCTURE PREDICTION

- The functional form of single stranded RNA molecules frequently requires a specific tertiary structure.
- The scaffold for this structure is provided by secondary structural elements which are hydrogen bonds within the molecule. This leads to several recognizable "domains" of secondary structure like hairpin loops, bulges and internal loops.
- There has been a significant amount of bioinformatics research directed at the RNA structure prediction problem.

GENE PREDICTION

- Predicting the Gene by the predefined conditions.
- Comparative genomics is the best method for predicting the gene.
- Some of the softwares include GeneMark, Genscan

TRANSCRIPTION FACTOR BINDING SITE PREDICTION

- Predicting the transcription factor. Most common method is to use "Comparative genomics".
- And finding clusters of motifs in the noncoding part of gene.

GENOME ANNOTATION

- Predicting the genes, coding and noncoding sequences are called genome annotation.
- Most of the people follow comparative genomics to annotate the newly sequenced genomes.
- GOLD is the database for

ANCESTRY PREDICTION

- Predicting the Ancestry of an individual based on his/her genetic signatures or SNPs. Mitochondrial SNPs are used in predicting maternal ancestry because mitochondria is passed ONLY through mother to the child.
- Y chromosome SNPs are used in predicting paternal ancestry because Y chromosome is passed from father to the child.
- Ancestry is one of the successful fields in bioinformatics.
- ongoing genome projects.

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