DEPARTMENT OF FOOD & INDUSTRIAL MICROBIOLOGY

Study material

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INDEX

Lecture 1

Prospectus of Biotechnology-new development in the science of gene manipulation – a rash of new companies - to commercialize the new technology - Human genome sequencing project - potential for human therapy - Regulation of transcription of particular genes -making easy to use cloning kits - recombinant DNA technology in all biological disciplines

Biotechnology (sometimes shortened to "**biotech**") is a field of applied biology that involves the use of living organisms and bioprocesses in engineering, technology, medicine and other fields requiring bio products. The United Nations Convention on Biological Diversity defines biotechnology as

"Any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use."

In other terms: "Application of scientific and technical advances in life science to develop commercial products" is biotechnology.

Biotechnology draws on the pure biological sciences (genetics, microbiology, animal cell culture, molecular biology, biochemistry, embryology, cell biology) and in many instances is also dependent on knowledge and methods from outside the sphere of biology (chemical engineering, bioprocess engineering, information technology, biorobotics).

Several branches of biotechnology :

- **Bioinformatics** is an interdisciplinary field which addresses biological problems using computational techniques, and makes the rapid organization and analysis of biological data possible. Bioinformatics plays a key role in various areas, such as functional genomics, structural genomics, and proteomics, and forms a key component in the biotechnology and pharmaceutical sector.
- **Blue biotechnology** is a term that has been used to describe the marine and aquatic applications of biotechnology, but its use is relatively rare.
- **Green biotechnology** is biotechnology applied to agricultural processes. An example would be the selection and domestication of plants via micropropagation. Another example is the designing of transgenic plants to grow under specific environments in the presence (or absence) of chemicals. An example of this is the engineering of a plant to express a pesticide, thereby

ending the need of external application of pesticides. An example of this would be Bt corn. Whether or not green biotechnology products such as this are ultimately more environmentally friendly is a topic of considerable debate.

- **Red biotechnology** is applied to medical processes. Some examples are the designing of organisms to produce antibiotics, and the engineering of genetic cures through genetic manipulation.
- **White biotechnology**, also known as industrial biotechnology, is biotechnology applied to industrial processes. An example is the designing of an organism to produce a useful chemical. Another example is the using of enzymes as industrial catalysts to either produce valuable chemicals or destroy hazardous/polluting chemicals. White biotechnology tends to consume less in resources than traditional processes used to produce industrial goods the investment and economic output of all of these types of applied biotechnologies is termed as **bioeconomy.**

The living beings have a basic characteristic of producing young ones. The off springs of all the organisms resemble their parents in many respects. This resemblance is due to inheritance or heredity. For example, an E.coli can divide to another E.coli only, and a human can produce only human. Although the off springs are similar to their parents, they differ not only from one another but also from their parents.

Every activity of living organism is under the control of genes. Genes are the expressible units of genetic material. The total genetic material of a cell is called genome. The genome of a cell organized as a single chromosome in viruses and bacteria. But in higher organisms the genome is organized in more than one chromosome, as an example a human cell consists of 23 pairs of chromosomes. Nucleic acids were first isolated by Friedrich Miescher in 1869. They were so named as they were found by him in the nuclei of pus cells.

Biochemical nature of genes is nucleic acids. Among the two types of nucleic acids(DNA and RNA) – except in some viruses in all the living organisms DNA is the genetic material. DNA, as the genetic material is confirmed by using transformation, life cycle of bacteriophages etc., Nucleotides are the building blocks of nucleic acids. In this unit the structure of genetic material and its expression in prokaryotes is discussed.

The genetic material is copied with high exactness in the replication. Protein expressing unit of DNA is first transcripted to RNA. Then the nucleotide language is translated to protein language, which carries out cellular functions.

DNA is the material of heredity, with minor exceptions as in some RNA viruses. Early geneticists like Mendel demonstrated that genes are the elements carrying and transferri8ng inherited characteristics from parent to offspring

Lecture 2

Molecular genetics - chromosomes - genome - genes - Chromonemata - nucleosomes -Heterochromatin - Organization of chromosomes - nature of gene - definitions of gene -classical - modern - gene as unit of - physiological function - transmission - mutation -Cistron - Recon - Muton - Number of genes - functions of genes

CHROMOSOME

The most important structures within the nucleus are the chromosomes which are the carriers of the hereditary units or genes. Their presence was demonstrated first by WALDEYER in 1888. Forty years prior to this HOFMEISTER while studying the pollen mother cells of Tradescantia portrayed the chromosomes.

The chromosomes are visible only one the onset of cell division. When they appear as little rods which stain deeply. They are always present in a fixed number characteristic of an organism. The chromosomes are capable of self-reproduction and of maintaining their structural and functional properties through successive cell divisions. They play an important role in heredity, mutation and evolution and control the various vital processes of the cell.

The nuclei of the reproductive cells contain only one set of chromosomes (haploid). In the zygote nucleus there are two sets of chromosomes, one derived from the maternal and another from the paternal germ cells. Such nuclei are described as diploid. All the nuclei formed by mitosis retain the diploid condition.

Number of chromosomes :

Mostly the number of chromosomes for any species (animals or plants)is fixed and constant. Amongst the diploid organisms, the lowest possible number of two is found in a strain of an animal *Ascairs megalocephala* (horse nematode). On the other extreme is a fern plant, *Ophiolossum* which has 1020 chromosomes. The chromosome numbers of some of the common plants and animals are given below.

Name of the Organism	Chromosome Number Hapoid no.	 Dipoid no.
Aspergillus nidulans	4 or 8	
Penicillium sp.	2	
Chlamydomonas reinhardi	8	
*Allium cepa (*Onion*)*		16
*Triticum aestivum (*Wheat*)*		42
*Oryza sative (*Rice*)*		24
Lycopersicum esculentum		24
*Nicotiana tabaccum (*tobacco*)*		48
*Pisum sativum (*pea*)*		14
Solanum tuberosum (Potato*)*		48
Animals : *Canis familiaris (*Dog)		78
*Felis catus (*Cat*)*		38
*Mus musculus (*Horse*)*		40
*Rana pipiens (*Frog*)*		26
*Musca domestica (*Housefly		12
*Homo sapiens (*Man)		46

Structure : The chromosome is made of two different substances, namely a strongly chromatic axial body or filament and an achromatic substance which surrounds it. The latter is the matrix. The axial body is made up of a pair of spirally twisted chromonema threads. The number of chromonema threads of a chromosome varies in different stages of cell division. Thus the prophase chromosome has a pair of chromonemata and metaphase chromosome has a pair of chromonemata and metaphase chromosome consists of two pairs of chromonemata.

Centromere : Every chromosome shows a special achromatic region called the centromere or kinetochore or primary constriction. Centromere has less DNA than else where. The two portions of the chromosome on either side of the kinetochore are known as arms. The arms may be of equal length (metacentric) or unequal length (sub-metacentric), or one of the arms is very small and the other long (acrocentric).

Chromosomes lacking a centromere fail to arrange themselves in the metaphase plate, lag behind in anaphase, and are finally eliminated. The centromere is the region to which the spindle fiber is attached in the metaphase

Types of Chromosomes :

The position of centromeres in chromosomes varies. Accordingly various types of chromosomes are as follows:

1. **Metacentric** : If the centromere is present near about in the middle of the chromosome, then it is called as metacentric

Eg: Amphibians.

2. **Sub-metacentric** : These chromosomes possess the centromeres at one end forming a long arm and a very short arm. Sub-metacentric chromosomes are L or J-shaped.
3. **Telocentric** : When centromere is at the terminal or proximal position, then the chromosome is called telocentric.
4. **Acentric :** If centromere is lacking the chromosome is termed acentric

a) **Secondary constriction** : Besides the primary constriction, secondary constriction, is also present. The small segment of the chromosome distal to this constriction is known as the satellite. All the chromosomes of a nucleus do not possess the satellites. Only one or two chromosomes have them and they are called Sat-chromosomes. The secondary constrictions of such chromosomes are concerned with the formatin of nucleoli at the end of mitosis. The number of the nucleoli in a nucleus corresponds to the number of Sat-chromosomes.

b) **Telomere** : The extremities of the chromosome are described as telomeres. When chromosomes are fractured by X-rays, the resulting segments do not fuse at the telomere region although they unite at the broken ends. This shows that the telomeres exhibit a marked polarity.

c) **Chromomeres** : The chromonema threads of the chromosome bear a number of small beads in a linear fashion. These are called chromomeres. It has been experimentally demonstrated that the hereditary characteristics depend on the arrangement of the chromomeres. Chromomeres are also regarded as specialized areas where DNA is synthesized during the interphase stage.

In the matrix are embedded one or more chromonemata (singular, chromonema). Chromonemata are considered to be permanent and essential part of the chromosome. Chromomnema is helically (spirally) coiled in the matrix. The number of chromonemata per chromosome is disputed. But there is enough proof to show that there are at least two (may be four) chromonemata per chromosome (in anaphase)

Chromomeres are composed largely of nucleoprotiens. Very rich in nucleic acids. Chromosomes probably contain the “genes” which are the units of heredity. Each chromonema is made up of many microfibrils per chromonema is not certain, but may be above 64 in number.

Ultra structure of Chromosome:

By electron microscope the smallest visible unit of the chromosome is the fibril which is 100 A^0 in thickness. This fibril contains 2 DNA double helix molecules separated by a space abour 25 A^0 across and associated protein.

Next largest unit of chromosome is the half chromatid. The half chromatid consists of four 100 A fibrils, so that it is 400 A in thickness and contains eight double helixes of DNA and the associated protein. Two half-chromatids form a complete chromatid consisting of 16 double DNA helix molecules. As the chromosome consists of two chromatids, thus total number of helixes will be 32 and diameter 1600 A^0 thick before duplication or synthesis. After duplication chromosome has 64 double helixes of DNA with corresponding diameter of 3200 A^0. The number of DNA helixes in each unit above fibril level varies according to species. Thus each chromosome is composed of numerous microfibrils, the smallest of which is a single nucleoprotein molecule.

Chromosomes consist largely of nucleo-proteins formed by the nucieic acids and proteins. There are two basic kinds of nucleic acids; RNA and DNA. DNA is found inside the chromosomes and RNA in the cytoplasm and the nucleus (nucleolus). A model of DNA was proposed by Watson & Crick in the year 1953. The DNA molecule is aspiral, ladder-like structure which is twisted so that the two vertical strands form a double helix. The two strands are connected by rungs made of purines and pyrimidines.

Chromonemata and Nucleosomes : Eukaryotic chromosomes are composed of ochromatin, which consiosts chemically of about 60 percent protein, about 35 percent DNA and perhaps 5 percent RNA. The DNA of each chromosome consist of a single, very large, linear molecule. The protein component can be broadlydivided into two classes: histone and non-histone. The non-histone represents different proteins including DNA polymerase and RNA polymerase. The histones designated H2A,H2B,H3 and H4 are components of nucleosomes.

Heterochromatin an euchromatin : The dark staining portions of the chromosome are called “heterochromatin and light staining protions are called “euchromatin”.

Metabolically euchromatin appears to be more active than heterochromatin as functional genes are present in euchromatin.

ORGANISATINO OF CHROMOSOMES :

(a) A chromosome consists of a string of nucleic acid units, to which the protein is attached.
(b) A chromosome consists of a continuous molecule of DNA. The multiple loopings of the molecule, perhaps represent the enlargement of the molecule.
(c) A chromosome consist of a series of molecules of DNA connected to one another in a llinear fashion. It has been estimated that about 95% of DNA of chromosomes is present in chromomers and 5% in the interconnecting regions.

Models of Chromosomal Organisation : Several models were proposed to explain the organization of chromosomes. These models tend to explain in the manner in which the DNA is packed in the chromosomes.

1. **Solenoid model** : Finch and Klug's (1976) model called the solenoid model explains the formation of nucleosomes. They postulated that the double helix DNA molecule associates itself with the various types of histones to form beaded structures called the nucleosomes that have a diameter of 100-125 A. The portion of strand between the nucleosomes is called a string, which is again coiled to form a solenoid which is 300 A. This coiling or folding is 6.28 times as compared with the degree of coiling during the formation of beads or nucleosomes (7 fold). The solenoid is further coiled to form a supersolenoid or the chromonea which is 2000-4000 A thick. It has a folding ratio of 17.8 – 35.6 times.
2. **Dangler – String Model** : Another recently proposed model is called the Dangler – String Model. It was proposed by J.R.Paulson and U.K. Laemmli (1977). This model visualizes that a coromosome has structure in which there is central chromatin string like structure giving of a number of lateral loops in all directions. There loops are variously folded to give a compact appearance. The loops are about 300 A thick and are made up of chromatin. The chromatin fibres are formed by the coiling of DNA – nulceosome beaded string in the manner discribed under the solenoid model.

The solenoid model discussed above was also supported by F.Thoma and his co-workers in 1979. The two models are now supported by most of the recent workers.

GENE STRUCTURE AND FUNCTION

The hereditary units that are transmitted from one generation to the next generation are called genes. Gene is the fundamental biological unit. There are various

hypotheses to explain the nature of genes. De Vries postulated one gene-one character hypothesis. Bateson and Punnet proposed the presence and absence theory. But all these theories were discarded by Morgan, who proposed the particulate gene theory in 1926. He considered genes as corpuscles which are arranged in a linear order on the chromosomes and appear like beads on a string.

There are two types of definitions about gene. They are

(i) Classical (ii) Modern

1. **Classical definition of a gene** : Classically a gene was assumed to be a genetic unit by the following criteria.

(a) **Gene is a unit of physiological function** : According to this. The gene, that occupies a definite locus in the chromosome is responsible for a specific phenotypic character.

(b) **Gene is a unit of transmission or segregation** : It means, gene can be segregated and exchanged at meiosis by way of crossing over.

(c) **Gene is unit of mutation** : By the spontaneous or induced mutational changes, gene can give rise to a new phenotypic expression.

Nature of Gene : In almost all organisms except plant viruses genes are made up of DNA. This was shown experimentally by Shapiro. Experiments conducted by Avery. Mc.Leod and Mc.Carty shown that DNA is the genetic material. Chromosomes contain DNA, proteins and small amounts of RNA, out of which only DNA acts as the genetic material. The total hereditary or genetic material can be divided into genome and Plasmon.

Genome is the hereditary material of the chromosomal complex. Plasmon is the total hereditary material present outside the chromosomes. On the basis of work done on *Drosophila maelanogaster* the gene was considered to be the shortest segment of a chromosome.

The gene was th0ough to the indivisible unit of chromosomes controlling some phenotypic character. However, recombination show that crossing over not only takes place between the genes but also with in the gene. Beadle and Tatum considered the gene as a unit which controls the synthesis of a single enzyme. Stahl has defined the gene as a polynucleotide chain consisting of segments each controlling the expression of a particular trait.

2. **Modern definition of a gene** : After the discovery of DNA, the gene has been defined as cistron, recon and muton. The classical gene is the smallest unit that could undergo

a mutational change. A gene further divided into smaller units of function, mutation and recombination.

Symour Benzer (1955-USA) coined the terms cistron, recon, and muton to explain the relationship between DNA and genetic phenomena.

(a) **Cistron** : It is the unit of function. Cistron represents a segment of the DNA molecule and consists of a linear sequence of nucleotides, which controls some cellular function.

In E. Coli cistron may contain about 1500 base pairs. Some cistrons may contain as many as 30,000 base pairs. The cistron begin with initiation codon and ends with a terminating codon. Each cistron is responsible for coding one m-RNA molecule which in turn controls the formation of one polypeptide chain. Each cistron consists of hundreds of mutons and recons.

(b) **Recon** : It is a unit of recombination. It is the smallest unit capable of recombining genetically. Recombination studies on microbes indicate that structurally the recon consists of one or two pairs of nucleotides, possibly only one pair.

(c) **Muton** : It is a unit of mutation. The shortest chromosomal unit capable of undergoing mutation has been called the muton. The muton consists of one or many pairs of nucleotides within the DNA molecule.

Number of genes : The no. of genes that an organism contain has some relations with its complexity. Viruses are parasitic on host cells. They require very few enzymes hence viruses consist very few number of genes. Bacteriophage R-17 has single stranded RNA and contains only three genes.

Lecture 3

Fundamentals of molecular biology - DNA as genetic material - Hershey and chase experiment - RNA as genetic material - Viruses and bacteriophages - Genetic material organization - Transcription – Translation

DNA AS GENETIC MATERIAL :

The discovery of the nature and features of genetic material started from 1928 with the work of Fred Griffith. Griffith found that when Streptococcus pneumoniate heat killed virulent forms are injected into mice, the mice were not affected and no Pneumococci recovered. But when a combination of heat killed virulent bacteria recovered. But when a combination of heat killed virulent bacteria and a living non virulent strain were given the mice died. Griffith called this change of non virulent bacteria into virulent pathogens as transformation. The Griffiths experiment is not describing about the cellular molecule and its nature, participating in transformation.

In 1944 Oswald T. Avery, Colin M. Macleod and Maclyn Macarty made the discovery that the substance active in transforming the non virulent to virulent strain was DNA. They confirmed this by using enzymes. Transformation was unaffected, When transforming substance was treated with proteases or RNAase. But with the treatment of DN as the transformation substance was readily inactivated. This experiment by Avery, Macleod and Macarty indicated DNA as the genetic material.

Hershey and chase experiment :

Roger Herriott suggested that virus injects its genetic material, like a needle, into the bacterial host. Alfred Hershey and Martha Chase confirmed Herriott suggested in 1952. In this experiment they also confirmed that DNA as genetic material using radioactive elements ^{32}P and ^{35}S. As except the central nuclear material all the remaining structural components are of proteinaceous.

(a) Bacteriophage T_2 was grown in a medium containing radioactive elements ^{32}P and ^{35}S. Then in the newly produced phages the phosphate of DNA were labeled with ^{32}P and sulfur of capsid protein was with ^{35}S. The reason for selecting these two radioactive elements was phosphate is present in DNA and absent in protein, whereas sulphate is present in protein and absent in DNA.

(b) The labeled phages were added to E.coli culture grown on normal medium. The T_2 phages bind to E.coli cells. Then only genetic material of phage enters

into host and the remaining structural components will remain on host cell, which are called as ghost.

(c) To confirm whether DNA or protein entered into the cell shear the phage ghosts from host cells using a blender. The sheared phages are removed from the bacteria suing centrifugation. The removed ghosts were found to contain most of ^{35}S and the bacteria contain most of ^{32}P.

With this Hershey and Chase found that only the DNA of phage was essential for the production of progeny. They confirmed DNA is the hereditary material by confirming that only the DNA of phage was required for the production of new phage progeny.

RNA AS GENETIC MATERIAL :

Generally RNA serves as a carrier of genetic information from DNA to ribosomes. Most of genetic systems studied to date contain genetic material made of DNA. But some viruses, including several bacteriophages, plant and animal viruses, have RNA as their genetic material. For example HIV (Human immune deficiency virus has RNA as its genetic material.

OVERVIEW OF GENETIC MATERIAL ORGANIZATION

In case of prokaryotes genetic material is located as nucleoid in cytoplasm, without having a specific membrane. But in case of eukaryotes the DNA is located in the nucleus of cells.

The organization of cell is simple in prokaryotes. The total genetic material will be as a single chromosome without involvement of histone proteins. In case of E.coli the number of base pairs in DNA are 4.65×10^6 with a length of 1.6 mm.

In case of eukaryotes three specific important features are

(i) Presence of centromeres, which are attachment points for the mitotic spindle and telomeres which occur at the ends of chromosomes.

(ii) The fundamental unit of organization of DNA is nucleosome with 200 base pairs and histone proteins.

(iii) Many genes of eukaryotic cells and very occasionally in bacteria, are interrupted by introns are called exons.

TRANSCRIPTION

Enzymatic process whereby an RNA strand is synthesized complementary to the genetic information in DNA is called transcription. The strand of duplex DNA copied as RNA is called template strand. Differences between DNA and RNA are (a) 2' deoxyribose is present in nucleotides of DNA whereas ribose in RNA nucleotidies; and (b) RNA contain uracil in place of thymine in DNA; and usually the DNA is double stranded but RNA is single stranded.

During replication the whole genetic material is copied. But transcription is at selective sites of genetic material. Particularly genes are only transcripted at a time. Depending on the cellular need some genes may be expressed at high rate and some at low rate, some may be expressed continuously and some are restricted . So transcription must be regulated.

The RNA synthesis is catalyzed by the enzyme RNA polymerase. Many number of transcriptional units are present in DNA. Before the transcriptional start point a special region called promoter should be recognized and bound by RNA polymerase. The polymerase moves along DNA from this point, synthesizing RNA, until it reaches termination site. Sequences before start point of transcription are called up stream sequences and the sequences after transcription start point are called down stream sequences. The upstream nucleotides are indicated with numbers -1, -2, -3, etc and down stream nucleotides with numbers as +1, +2, +3, etc.

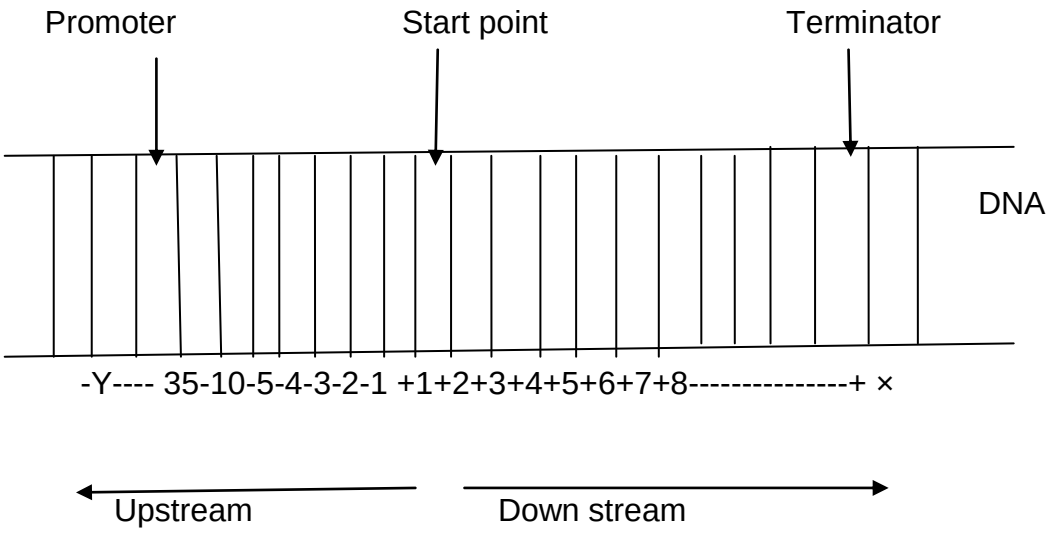


Fig. 3.24. Transcription unit

PROKARYOTIC TRANSCRIPTION :

In prokaryotes transcription requires RNA polymerase, promoter sequences and topoisomerases. RNA polymerase is to polymerize nucleotides to form RNA strand. Promoter sequences are required to bind RNA polymerase exactly at transcriptional unit. Topoisomerases are to remove the top0logical stress developed in DNA during translation.

A. **RNA polymerase** : E.coli RNA polymerase holoenzyme is a complex multi9-subunit protein. Its molecular weight is 450 KD. The core enzyme of α_2ßß^1 joins with σ subunit to form the holoenzyme. The subunit function in accurate recognition of promoter sites to be transcribed. ß and ß^1 subunits form the catalytic site of RNA polymerase and α subunits are essential for assembly of subunits and regulation by other proteins.

B. **Promoter** : The DNA sequences near the transcriptional unit, which promotes the binding of RNA polymers and initiates its activity are called promoters. Prokaryotic promoters are 20 to 200 bp length. The promoter region generally extends between – 70 and +30. All the promoters for all prokaryotes are not identical. But certain nucleotides are particularly common forming consensus sequences. At -10 region a 6 base pairs AT-rich consensus sequence 5'TATAAT3' called pribnow box or TATA box occurs. Another consensus sequence at -35 region is 5'TTGACA3'. The RNA polymerase binds from around -35 region to a few bases downstream to the start site.

MECHANISM OF TRANSCRIPTIN IN PROCARYOTES :

The transcription mechanism in prokaryotes involves three steps.

(A) Initiation (B) Elongation (C) Termination.

(A) **Initiation** : Promoter of transcription unit on double stranded DNA is recognized by the core enzyme (α_2ßß^1) of RNA polymerase. This association forms closed complex. The σ factor associates with core enzyme of RNA polymerase. Then the double stranded DNA melts in the promoter region forming open complex. After formation of open complex and polymerization of RNA to 8 – 9 bases length the σ factor dissociates and core enzyme continue the transcription process in 5'→3' direction.

The factor plays a major role in initiation of transcription. E.coli has several types of σ factor, each of which causes RNA polymerase to initiate, at a set of promoters, with a specific -35 and -10 consensus sequences. The σ 70 is used for general transcription. When the template is negati9vely super coiled the efficiency of promoters will be more. Rifamycin B and rifampicin are inhibitors of transcription.

(B) **Elongation** : Once a short oligonucleotide of 8-9 bases length RNA is synthesized σ factor dissociates. Elongation of the RNA transcript is catalyzed by the core enzyme of polymerase only. At a rate of 20-50 nucleotides polymerization per second the RNA chain is elongated. As the RNA polymerase moves along the template strand of DNA, the DNA double helix is unwound and recloses after the polymerase passed. Cordycepin is the nucleoside, which inhibits chain elongation.

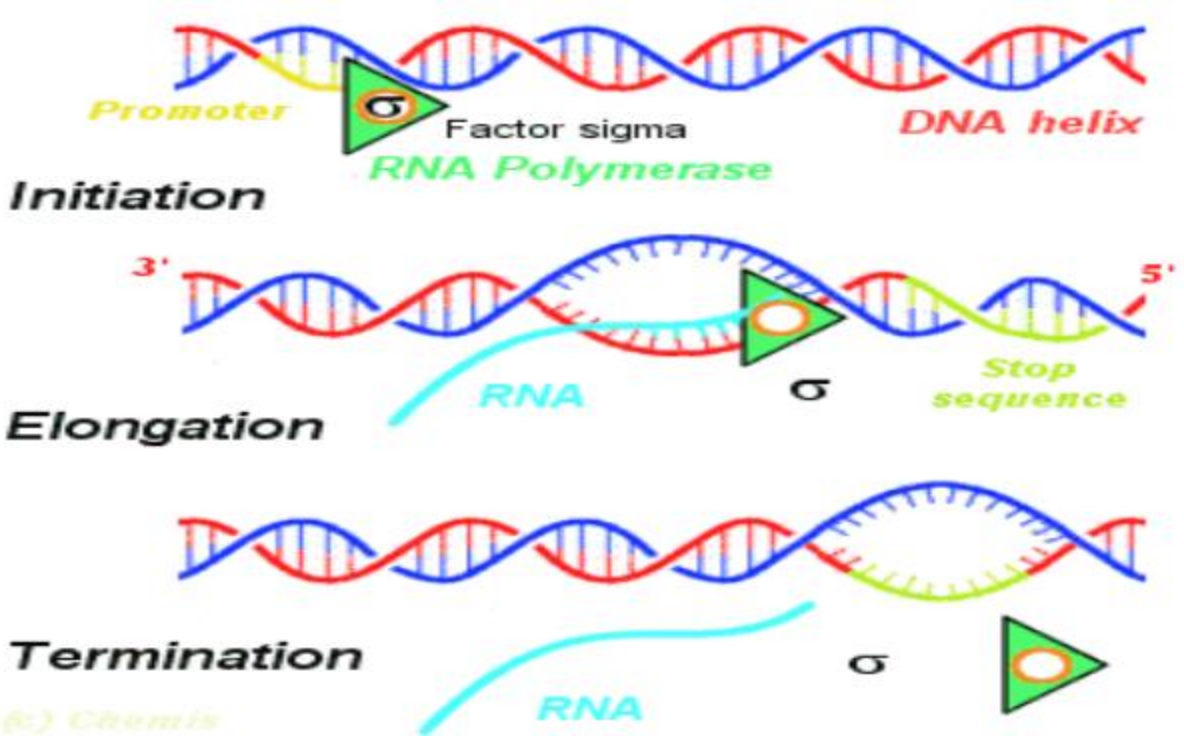


Fig. Initiation ,elongation and termination in prokaryotes

C. **Termination** : Depending on the nature of nucleotide sequences in the template DNA strand the elongation of RNA is terminated. The termination of transcription is of two types.

(i) ρ (rho) independent

(ii) ρ(rho) dependent

(i) **ρ independent** : This type of transcription termination is not dependent on any termination factors. This is determined by specific sequences in DNA called termination sites. The features of termination site are presence of G-C rich inverted repeats. This feature leads to the formation of stem-loop structure in RNA, with hydrogen bonding between inverted repeats. The inverted repeat is followed by 6 to 8 adenines in the DNA template. These adenines code for uridines in RNA. As A:U base pair between the transcript and the DNA template stand are unstable than the A:T base pairs between the template and other DNA strand. So the RNA spontaneously dissociates from DNA.

(ii) **P dependent** : In transcription units not ending with sequence of A's the termination requires termination factor called (rho). It is an ATP dependent RNA:DNA hybrid duplex. It binds to RNA transcript and catalyzes the releasing of the transcript from template DNA.

EUKARYOTIC TRANSCRIPTION :

Most of the general aspects of mechanism of eukaryotic transcription is similar to prokaryotic transcription. Three types of RNA polymerases are present in eukaryotes. In addition to promoters other control units called enhancers are located for eukaryotic genes. The primary transcripts of mRNA are called hnRNA (heterogeneous nuclear RNA). Maturation of eukaryotic mRNA from the primary transcript is through a complex process called posttranscriptional modifications.

A. **RNA polymerases** : Three types of RNA polymerases are present in eukaryotes. They are RNA PI, RNA P II and RNA PIII. The RNA PI is present in nucleolus, where as the remaining two enzymes in nucleoplasm. They can be differentiated bases on their sensitivity to α- amanitin

Polymerase	Location	RNA	Sensitivity to Synthesized α-amanitin
RNA PI	Nucleolus	r RNA	Not sensitive
RNA PII	Nucleoplasm	hn RNA	Sensitive
RNA PIII	Nucleoplasm	tRNA	Moderately Sensitive

B. **Transcription factors** : In order to form active transcription complex RNA polymerase requires other proteins called as transcription factors (TF). TATA-binding protein (TBP) binds to TATA box along with TFIIB. The TBP-TFIIB complex on TATA box is stabilized by TFIIA. TFIIF binds to RNA polymerase and prevents binding of nonspecific DNA sequences. TFIIH as DNA helicase unwinds DNA at transcription site.

C. **Promoters and enhancers** : Different promoter sequences have been identified for different RNA polymerases.

(i) **RNA Polymerase I** : The RNA polymerase I promoter has two consensus sequences –one at -107 to -180, rich in GC and other at -45 to +20 overlapping transcription start site.

(ii) **For RNA polymerase II** : The promoter sequences for RNA polymerase II are similar to prokaryotic promoter sequences. The consensus sequences are TATA box at around -25 position with the sequence TATA AT or TATA TA; and a CAAT box at around -75; and enhancer elements.

The enhancers may be located upstream; downstream or with in the transcriptional region. They may cause up to 200 fold increase in transcription rate.

MECHANISM OF TRANSCRIPTION IN EUKARYOTES:

As in prokaryotes the eukaryotic transcription of mRNA involves three stages.

(1) Initiation (2) Elongation (3) Termination

(1) **Initiation** : RNA polymerase II binds to the promoter along with six transcription factors-TFIIA, TFIIB,TFIID,TFIIE,TFIIF and TFIIH. Different models are proposed to explain the mode of binding of all these factors. The Complex of all them binding on transcriptional unit is called preinitiation complex or open complex. TFIIA stimulates transcription by stabilizing interaction of TFIID with TATA box.

(2) **Elongation** : With the involvement of two transcription factors TFIIF and TFIIS, the RNA polymerase II elongate the RNA transcript. TFIIF accelerates uniform RNA chain elongation. TFIIS relieves obstructions in the path of elongation.

(3) **Termination** : The termination sequences of transcription in eukaryotes are present away from 3' end of mRNA. These are rich in A's with sequence AAUAAA. These can be removed during posttranscriptional modifications to generate the 3' end of mRNA. The protein called small nuclear RNA-proteins is supposed to be playing a role in cleaning the extra 3' sequences.

The transcripted primary RNA's called hn RNA are processed to form mRNA. The processing s are like capping at 5' end, methylation of Guanine or adenines, addition of adenine at 3' end to form poly (A) tail, removal of introns (non coding regions) by splicing mechanism.

POST TRANSCRIPTIONAL MODIFICATIONS:

Prokaryotes will have no nuclei. So translation of an mRNA into protein can begin from the 5^1 end of the mRNA even while the 3^1 end is still being synthesized by RNA polymerase. But in eukaryotes nucleus has a membrane separating from cytoplasm. Transcription takes place in nucleus and translation in cytoplasm. The transcripts of

eukaryotic genes are called pre-mRNA, as they have to undergo several modifications called RNA processing or post transcriptional modifications. After modifications functional mRNA enters into cytoplasm to be translated into protein. Thus transcription and translation can not occur concurrently in eukaryotic cells.

Almost all eukaryotic pre-mRNA are modified at the ends and also internally.

(a) **Capping** : As the 5^1 end of a nascent RNA chain emerges from the surface of RNA polymerase II, it is immediately acted on by several enzymes that together synthesize the 5^1 cap. The capping is connection of 7-methyl guanylate to the terminal nucleotide of the RNA by an unusual 5^1-5^1 triphosphate linkage. The cap protects an mRNA from enzymatic degradation and also supports the export of mRNA to cytoplas

(b) **Tailing** : At about 100 – 250 adenylic acid residues are added at the 3' hydroxyl group of pre-mRNA. The addition is catalyzed by the enzyme poly7 (A) polymerase. The poly (A) tail length is shorter in yeasts and invertebrates than in vertebrates. The poly (A) tail protect the mRNA from nucleases.

(c) Splicing : The final step in the processing of many different eukaryotic mRNA molecules is RNA splicing. The mechanism of internal cleavage of a transcript to excise the introns, followed by ligation of the only coding exons.

TRANSLATION

Synthesis of protein from mRNA is translation. It involves translating the language coded in mRNA into language of amino acids in proteins. Proteins are the end products of most gene expressions. A single cell requires thousands of different proteins at a given moment. Depending upon the cellular need proteins are synthesized and transported to its functional site with great regulation mechanisms.

Protein synthesis is a complex process. About 300 different macromolecules cooperate to synthesize polypeptides. Many of these macromolecules are organized into the complex three-dimensional structure of the ribosome. In a single bacterial cell 20,000 ribosomes, 1,00,000 relate protein factors and 2,00,000 tRNAs are continuously involved in protein synthesis. The protein synthetic rate in a E.coli is 20 amino acids per second.

GENETIC CODE :

The set of three-nucleotide code (triplet-code) in mRNAs which stand for the 20 amino acids in proteins is called genetic code. It possesses 64 codons. Out of 64 codons 61 codes for all different 20 types of amino acids. The remaining three codons,

which are not specifying for any amino acid are called nonsense or stop codons. During protein synthesis on to the each codon in mRNA its corresponding amino acyl tRNA binds, with its anticodon. The anticodon's 5' base interacts with 3' base of codon and vice versa.

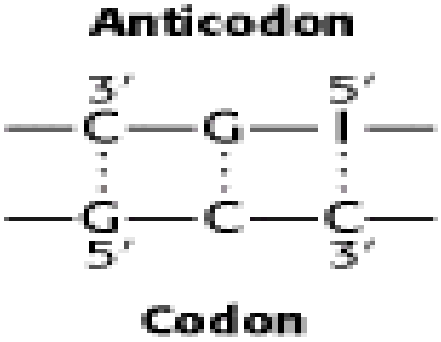


Fig. Interaction of codon and anticodon

Codon is universal : The genetic code is almost common in several organisms. A codon can represent for same amino acid in all the types of cells. For example animal insulin gene when introduced into a E.coli cell produce same protein.

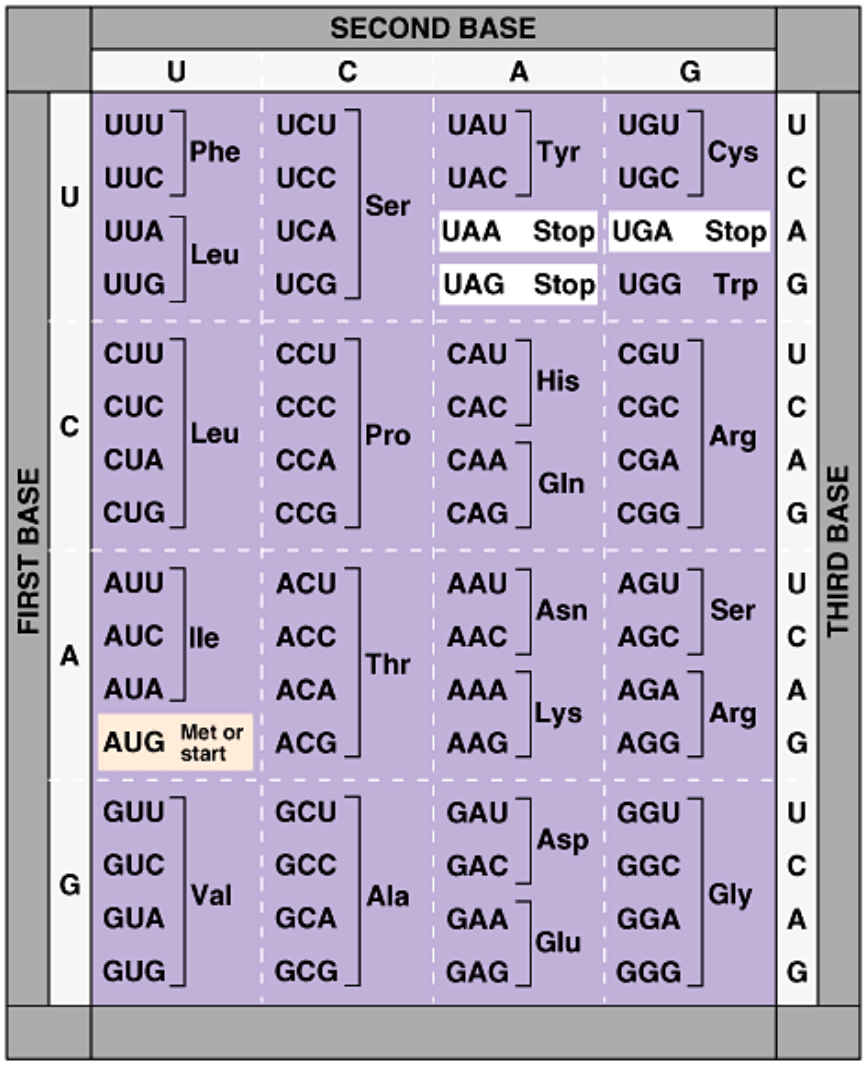


Fig. The genetic code

Triplet code : Fancis cris and Leslie Bernelt by using frame shift mutants discovered that a codon consists of three bases. As the natural amino acids are of twenty different types and the mRNA has only four types of bases, single bases can not specify amino acids. If two bases as a codon are supposed 4^2 (16) non identical doublets are only possible. So three base sequences are initially proposed to be a code fro a amino acid and later confirmed with different experiments like frame shift mutations, triplet binding assay, etc.

Codon synonyms : A single amino acid may be represented by many codons degeneracy or codon redundancy. This is possible as the interaction of 3' base of codon with 5' base of anticodon is not highly specific. For example CUU,CUC, CUA,CUG code for same amino acid leucine. The significance of codon degeneracy is that it can minimize the consequences of mutations.

Codons are nonoverlapping : The reading of genetic information in mRNA during protein synthesis involving each base as a part of one condon. For example in the sequence GCAUGC the codons are GCA and UGC only as the codons are nonoverlapping.

Code is commaless : During translation process the genetic code in mRNA in the form of nucleotides is read continuously without having any gaps. So it is said to be commaless. This feature of genetic code was confirmed using frame shift mutation.

Wobble hypothesis : Crick pin 1966 proposed some special features in the binding of tRNA anticodon to mRNA codon, called wobble hypothesis. This explains that between third base of codon and first base of anticodon a nonstandard base pairing (pairing other than AT and GC) is happening. This feature of codon pairing is supporting codon degeneracy.

Table: 3.7.Wobble pairing

First base of tRNA anticodon	Third base of mRNA codon
A	U only
C	G, C only
G	C or U
U	A or G
I (Inosine)	A,U or C

Start and stop codons : The initiating or start codon in prokaryotes is N-formyl methionine and in eukaryotes is methionine. After initiation polypeptide chain is elongated by addition of successive amino acids. This will be terminated reaching the stop codons UGA, UAG, UAA. The genetic code is not strictly universal. In certain eukaryotic nuclei, prokaryotes and mitochondria, codons representation for amino acids will have some exceptions. For example UGA codes for tryptophan in protozoa, Neewiospora crassa.

MECHANISM OF TRANSLATION :

In prokaryotes transcription and translation are coupled. Ribosomes begin associating with an mRNA and start synthesizing proteins even before fully finishing mRNA synthesis.

One more significant feature in prokaryotic translation is polycistronic mRNA, coding for more than one protein. Selection of right initiation codon, AUG is made by ribosome in prokaryotes.

In contrast, in eukaryotes after finishing the complete synthesis of mRNA, it moves into the cytoplasm and is translated. Eukaryotic mRNA is monocistronic and initiation occurs at the first AUG near the 5'- terminal.

The translation of mRNA to proteins if always in 5'→ 3' direction of mRNA. The protein synthesis starts with its amino terminus amino acid and ends with carboxyterninus amino acid.

Protein synthesis occurs on the subcellular organelles, ribosomes. In prokaryotes 70S ribosomes and in eukaryotes 80S ribosomes are the site of translation. The ribosomes will provide three main sites of it for protein synthesis. A (amino acyl) site is for binding of incoming new amino acyl- tRNA, which is going to be added to growing eptide chain. P(peptidyl) site is the site for nascent peptidyt- tRNA, to which the amino acid in A site is added. From E-exit site the finished polypeptide is released out from ribosomes and mRNA complex.

Each amino acid participating in translation has to be first activated with ATP and then attached to tRNAs by the amino acyl tRNA synthetases. The tRNA has an anticodon for delivering amino acid to an mRNA positioned on ribosomes.

Proteins synthesis involves four stages:

(1) Activation of amino acids
(2) Initiation
(3) Elongation
(4) Termination

1. **Activation of amino acids** : During this stage of translation, each of the 20 amino acids are covalently attached to specific tRNA utilizing ATP energy. The reactions being catalysed by a group of Mg^{2+} dependent activating enzymes called amino acyl tRNA synthetases. These enzymes are very specific for amino acids and its corresponding tRNAs 20 different amino acid are esterified to their corresponding tRNAs by 20 types of amino acyl tRNA synthetases. However, for amino acids that have two or more corresponding tRNAs also the same amino acyl tRNA synthetase usually activates them. Because of this high specificity interaction between the enzyme and tRNA is called second genetic code. In E.coli the only exception to this rule is lysine, for which there are two amino acyl tRNA synthetases.

Amino acyl tRNA synthetases have been divided into two classes, based on differences in their structure and reaction mechanisms. The class I enzymes load the amino acyl group initially to the 2' – hydroxyl group of the 3' – hydroxyl group of adenylate by transesterification reaction. The class II enzymes load the amino acyl group directly to the 3'-hydroxyl group of terminal adentlate. Example of class I amino acyl tRNA synthetases are arginine, cysteine, etc and for class II are alanine, asparagines, lysine, serine, etc. The reaction catalyzed by amino acyl tRNA synthetases.

2. **Initiation** : During Initiation of protein synthesis in all cells a ribosome assembles with a mRNA and an activated amino acyl initiator tRNA. The iniatiator amino acyl tRNA should be correctly positioned at the start codon. Initiation in prokaryotes and eukaryotes is through different factors and mechanisms.

   (A) **Initiation in prokaryotes** : The initiation of prokaryotic translation begins with assembly of the small ribosomal unit (30S) with the initiation factors IF-1, IF-2, and IF-3. The resulting complex is called preinitiation complex. This complex binds with the mRNA along with $^{f}met^{\text{-RNA met}}$ to a specific 30S subunit forming 30S $^{f}met^{\text{-tRNA met}}$ initiation complex. Association of large (50S) subunit of ribosome to the 30S initiation complex forms 70S initiation complex. The starting point of translation on mRNA is identified by 16S rRNA of 30S ribosomal subunit. This is with the eight nucleotide sequences in mRNA called, Shine-Dalgarno sequence : This sequence is located before start codon. 16S rRNA has the complementary sequences to Shine-Dalgarno sequence and binds to it.

   (B) **Initiation in eukaryotes** : Initiation of translation in eukaryotes also starts with assembly of eukaryotic initiation factors eIF, eIF-IA, eIF-2, GTP with small subunit (40S) forming preinitiation complex. The recognition of start codon in eukaryotes is different from that of prokaryotes. The first AUG at 5' cap site of eukaryotic mRNA is the start codon of eukaryotes. mRNA's 5' end alongwith eIF-4 bind to pre-initiatin complex using ATP energy. After recognition of cap at 5' end of mRNA by eIF-4 the proteins on mRNA moves along it and stops at the first AUG. However the selection of AUG start codon is facilitated by kozak sequences. The 40S initiatin complex is formed from pre initiation complex with the correct positioning of met- tRNA ^{met} at start site. Finally large (60S) subunit joins forming 60S initiation complex.

3. **Elongation** : The initiation complex is elongated with successive linkage of amino acids by peptide bond. In this process the next amino acyl-tRNA arrive at the A site and be available for linkage of this amino acid with the amino acids in

P site. Chain elongation continues by a repetitive mechanism adding one amino acid at a time in every repetition. Except during addition of first amino acid in initiation all the amino acyl tRNA,s of elongation are loaded on to the a site of ribosome.

Lipman showed that incoming aminoacyl- tRNA is accessed to the ribosomal A site by its interaction with elongation factors (EFs). The elongation factors in prokaryotes are EF-T_u (temperature unstable) – EF-T_s (temperature stable) and EF-G. (GTP binding).

EF-T_u complexes with GTP to form EF-T_uGTP. This interacts with the amino acyl tRNA (AA n+1) going to be loaded on to A site forming (AA n+1) A^an+1 $tRNA^{n+1}$,EF T_u –GT complex. This complex binds to second codon (n+1) of the mRNA in A site of ribosome. GTP is hydrolyzed during binding process to GDP and Pi. After binding of complex to mRNA the EF-T_u. GDP complex required for next elongation process is regenerated from EF-T_u. GTP complex required for next elongatin process is regenerated from EF-T_u. GDP by the aid of EF-T_s. After loading new amino acyl tRNA in A site the next step in elongation is formation f peptide bond between amino acids in Psite and new amino acid in A site. The process is called transpeptidation. The peptide in P site is transferred on to the A site amino acid. This forms peptide bond between carboxyl group of P site amino acid and free amino group of amino acid in A site. The last step in elongation is translocation of ribosome. In this step ribosome moves on mRNA towards 3' end by the distance of one codon. As the peptidyl- tRNA is still on n+1 codon of the mRNA, the movement of ribosome shifts the peptide from the A site to the P site. The deacylated tRNA in the P site is removed moves to the E site and released from there. This moment of ribosome again exposes the next codon to code amino acid of protein to the A site. The translocation process is mediated by elongation factor EF-G and GTP complex.

Mechanism of eukaryotic elongation is similar to prokaryotes. The eukaryotic elongation factors are eEF-1 and eEF-2. eEF-1 has two subunits α and ß. The α subunits is similar to EF-T_u and ß subunit to EF-T_s. The eEF-2 is same as EF-G.

Termination : The process of elongatin continues till the noncoding codon(stop or nonsense condon) in mRNA reaches the A site of ribosome. The stop codons are UAA, UAG, and UGA. Normally tRNAs with anticodons for these three amino acids will not exists. So no amino acid is coded by these three condons. Stop codons are recognized by the release factors-RF1,RF2 and RF_3. Among these RF_1 recognized the stop codons UAA and UAG; and RF_2 recognizes UGA and UAA. RF_3 acts to promotethe cleavage of the peptidyl tRNA releasing the completed protein chain.However, in eukaryotes a single release factor eRF recognizes all three termination codons.

Lecture 4

Chemistry and biology of DNA - Structural elements of nucleic acids - sugar - Anionic group- Nitrogenous bases - Purines - pyrimidine's - Nucleosides - Nucleotides - Phosphoric acid- ATP - GTP - CTP - UTP - TTP - Functions of nucleotides - chemical energy carriers -building blocks of nucleic acids - Physiological messengers - Components of coenzymes- enzymes effectors- Active substrates

STRUCTURAL ELEMENTS OF NUCLEIC ACIDS

The chemical structures of nucleic acids were elucidated largely through the efforts of Phoebus Levine and Alexander Todd in the early 1950's. Nucleic acids are polymers of nucleotides and hence may be called polynucleotide sequences. There are two types of nucleic acids – deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Both types of nucleic acids are present in all organisms except viruses. In case of the viruses either DNA or RNA is present, but not both.

Each nucleotide consists of a pentose sugar, a nitrogenous base and phosphate group(s). DNA have 2' – deoxyribose sugar and four types of nitrogenous bases – Adenine (A), Guanine (G), Cytosine (C) and Thymine (T) where as RNA have ribose sugar and contains Uracil (U) in place of thymine besides other bases – Adenine, guanine and cytosine.

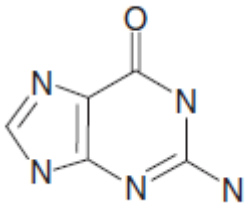
Table: 3.1. Structural elements of nucleic acids

Elements	DNA	RNA
Sugar	D-ribose	D-2' – deoxyribose
Anionic group	Phosphoric acid	Phosphoric acid
Nitrogenous bases		
a) Purines	Adenine, Guanine	Adenine, Guanine
b) Pyrimidines	Cytosine, Thymine	Cytosine, Uracil

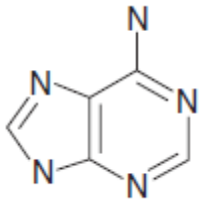
NITROGENOUS BASES :

The nitrogenous bases of nucleic acids are of two structural types – purines and pyrimidines.

(A) **Purines** : The nitrogenous bases derived from parent purine ring are called purines. The purine ring contains a six-membered pyrimidine ring fused to the five membered imidazole ring. Major types of purines present in DNA and RNA are adenine and guanine.

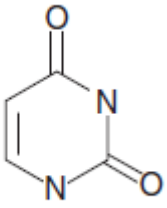


Guanine

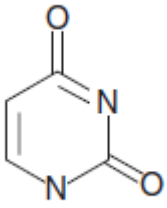


Adenine

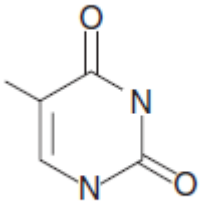
(B) **Pyrimidines** : The nitrogenous bases derived from parent pyrimidine ring are called Pyrimidines. The Pyrimidine ring is heterocyclic compound ring. Major types of Pyrimidines present in nucleic acids are Cytosine, Uracil and Thymine.



Uracil



Cytosine



Thymine

(C) **Modified nitrogenous bases** : Along with the five major nitrogenous bases some other minor bases called Modified nitrogenous bases also occur in polynucleotide structures. Methylation is the most common form of modification. Some naturally occurring modified nitrogenous bases are 5-methyl cytosine, 6-methyl adenine, 5hydroxymethyl cytosine, pseudouracil, etc. Some other nitrogenous bases are formed as products of nucleotide metabolism like Xanthine, hypoxanthine, uric acid etc,.

SUGAR :

A five carbon pentose sugar in a ring form is present in nucleic acids. DNA and RNA contain different sugar moieties. They are distinguished mainly on the basis of the type of sugar. DNA has the deoxyribose sugar and RNA has the ribose sugar. Both the sugars are present in ß- furanose form.

Sugars in nucleic acids

NUCLEOSIDES

Conjugating the nitrogenous bases to the pentose sugar, ribose or deoxyribose, by a ß-glycosidic linkage forms the compounds called nucleosides. In DNA purines or pyrimidines are linked to deoxyribose. The nucleosides containing deoxyribose are called deoxyribonucleosides. In RNA purines or pyrimidines are linked to ribose. The nucleosides containing ribose are called ribonucleosides. The ß-glycosidic linkage is formed between N-9 of purine or N-1 of pyrimidine and C-1 of ribose or deoxyribose. Because the glycosidic linkage is to a nitrogen of purine or Pyrimidine the nucleosides are called as N-glycosides. The N-9 of purine is involved, so they are said to be N-9 glycosides and the N-1 of Pyrimidine is involved, so they are said to be N-1 glycosides.

Purine nucleosides are immediately hydrolysed by acids, but the pyrimidines are hydrolysed only after prolonged treatment with acid.

In addition to the common nucleosides of adenine, guanine, cytosine, thymine and uracil some other nucleosides are also present in cells. Pseudouridine is an unusual nucleoside present in the tRNA. 3' – azidodeoxythymidine (AZT) and 2' – 3' – dideoxycytidine (DDC) are therapeutically used for the treatment of acquired immunodeficiency (AIDS) caused by HIV (Human immune deficiency virus). Their triphosphated incorporated in growing DNA stands inhabit further polymerization of DNA strand.

PHOSPHORIC ACID :Phosphoric acid is a strong acid containing three monovalent hydroxyl groups and a divalent oxygen atom linked to a pentavalent phosphorus.

A nucleotide contains phosphpate group. The phosphate can be attached through the oxygen of a C-5' of sugar. This phosphate group of nucleotides links one nucleotide to other with phosphodiester linkage to form nucleic acids.

NUCLEOTIDES :

The phosphoric esters of nucleosides are called nucleotides. In the nucleotides the phosphoric acid group has been attached to the sugar mmolecule of nucleosides by esterification.

In the ribonucleosides phosphorylatinis possible at positionos C-2', C-3' and C-5'. But as C-2' position of deoxynucleosides is deoxygenated, phosphorylation only. The C-1' and C-4'd of sugar are involved in furanose ring formation.

The nucleosides may have one, two or three phosphate groups. These are correspondingly called nucleoside monophosphates (NMP) nucleoside diphosphpates (NDP) or nucleoside triphosphated (NTP). Nucleotides are named depending on type of nitrogenous base in them.

Along with the above mentioned types of nucleotides some unusual nucleotides also occur in physiological systems. Pseudouridine monophosphate is present in tRNA. Inosine monophosphate of purine nucleotide de nove biosynthetic pathway.

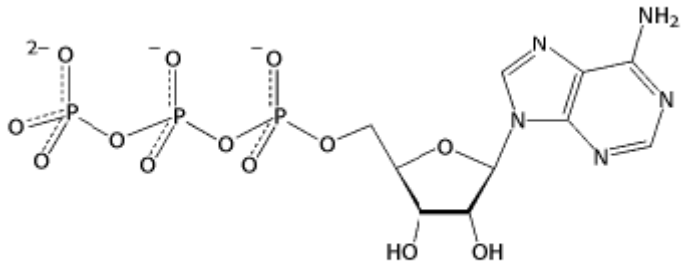


Fig. 3.9. Structure of Adenosine triphosphate (ATP)

Functions of nucleotides:

(a) Chemical energy carriers : Nucleoside triphosphates are the common energy carriers in any living system. With in them Adenosine triphosphate (ATP) is most predominant carrier of energy. Guanine triphosphate (GTP), Uridine triphosphate (UTP) and Cytidine triphosphate (CTP) are the carriers of energy for particular reactions only.

(b) Building blocks of nucleic acids: Nucleoside triphosphates are the substrates for nucleic acid polymerases. DNA is the polymer of deoxyribonucleotides and RNA is the polymer of ribonucleotides.

(c) Physiological messengers: The signals of hormones are carried by the secondary messengers like cyclic Adenosine monophosphate (cAMP) and cyclic Guanosine monophosphate (cGMP).

(d) Components of coenzymes : Many coenzymes like NAD+NADP+, FAD, coenzyme A contain AMP as part of their structure.

(e) Enzyme effectors : Nucleotides regulates some enzymes activity also by acting as allosteric effectors. For example in case of Aspartate transcarbamonlase CTP and ATP are the effectors.

(f) Activate substrates: Some biochemical reactions require activated substrates. For example amino acids need to be activated before for binding to tRNA in protein synthesis. UDP glucose is a key intermediate in glycogen and glycoprotein synthesis.

Lecture 5

Primary conformation of DNA - Secondary conformation of DNA - Watson and Crick model-Types of DNA- A, B, Z - Tertiary conformation of DNA - Higher level of chromatin structure- Denaturation and renaturation -Types of RNA - mRNA- rRNA- tRNA - sRNA - hn RNA -functions-ScRNAs - Sn RNAs

STRUCTURE OF DNA

The polymer form of deoxyribonucleotides is called deoxyribonucleic acid (DNA). It is present in the chromosomes of all cells as genetic composition except in some viruses. DNA is also present in the cellular organelles mitochondria and chloroplasts. In case of bacterial and some yeast, DNA is present as extrachromosomal genetic material called plasmids.

Structure of DNA is organized in three conformations. This perfect compact packing of that large DNA make it suitable to fit in a verylittle space. In humans the length of DNA in a cell is about 1.74 meters. It has to be accomidated in the cell's nucleus. The three conformations of DNA are

1. Primary conformation of DNA.
2. Secondary conformation of DNA.
3. Tertiary conformation of DNA.

PRIMARY CONFORMATION OF DNA

The sequence of the nucleotides in the DNA is primary confirmation. Normally the four different nucleotides Adenosine deoxyribonucleotide, Guanosine deoxyribonucleotide, Cytosine deoxyribonucleotide and Thymidine deoxyribonucleotide are found as monomers of DNA. These nucleotides are linked to one another with the phosphodiester linkage, between 3'-hydroxyl group of a cucleotide's sugar and the adjacent nucleotide's 5' hydroxyl group by phosphodiester linkage.

The purine and pyrimidine bases of DNA carry genetic information, where as the sugar and phosphate groups plays a structural role. The end of the DNA strand, which bears a free 5' phosphate group without involbing in phosphodiester linkage is called the 5' – end. The other end with a free 3' – hydroxyl is called the 3' end. The backbone of the primary structure is the linear strand of interconnected sugar phosphate residues.

The purine and pyrimidines connected with the sugar residues projects laterally from the backbone.

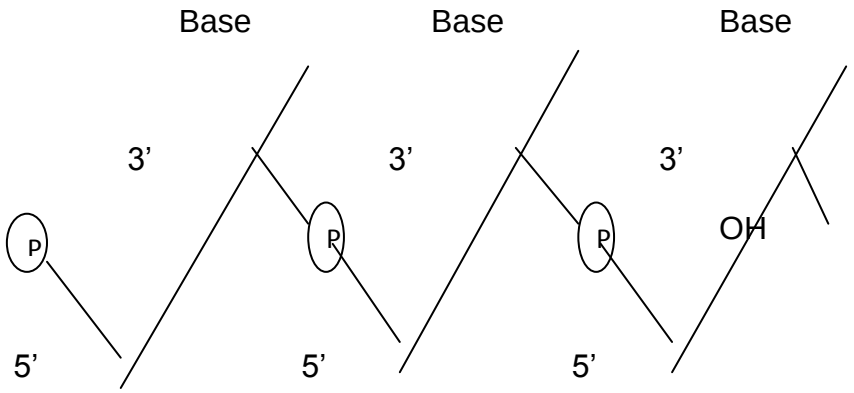


Fig. 3.11. Representation of nucleotide components in organization of

DNA stand

SECONDARY CONFORMATION OF DNA:

The two helical polynucleotide chains are coiled around a common central axis. The two chains are held together by hydrogen bonds between bases. The structures of double helical DNA was determined by James Watson and Francis Crick in 1953. Most important clue for this double helical structure of DNA was given by Erwin chargaff and his colleagues in 1940s.

Chargaff’s clues :

Chargaff has given many clues regarding the base composition of DNA:

1. From species to species the base composition varies. For example in E.coli K_{12} puring : Pyrimidine ratio is 1.08, where as in *Saccharomyces cerevisiae* the ratio is 1.00.
2. The base composition of a particular species is same in all its different cells and it will not change naturally.
3. The number of purines in a double stranded DNA are equal to the number of pyrimidines. A+G = T+C.
4. The ratio of Adenine to thymine or Guanine to cytosine is always one i.e., A/T or G/C = 1.

Watson-Crick model of double helical DNA :

Rosalind Franklin and Maurice Wilkins used X-ray diffraction to study DNA structure. They showed the characteristic X-ray diffraction pattern of DNA in 1950. In 1953 James D. Watson and Fancis Harry Compton Crick using all the available information they proposed a three-dimensional model of DNA. Watson and Crick along with Wilkins got the Nobel prize for their discovery in medicine and physiology in 1962. Till today many types of conformations of DNA were found to be adopted by DNA. However, Watson

and Crick's double helix is the predominant conformation and is now referred to as B-form of DNA.

Features of Watson-Crick model of DNA:

1. The DNA molecule consists of two polynucleotide strands.
2. They coil around a common axis to form double helical structure.
3. The helix is turning towards right so the B-form of DNA is a right handed double helix structure.
4. The two strands of DNA are said to be anti parallel, as one strand of DNA runs in 5' to 3' and other strand runs in 3' to 5' direction

5. The offset B-form of DNA. Pairing of two strands creates a major groove and a minor groove.
6. The B-form of DNA is about 20A wide.
7. Helix rise per base pair is 3.4A. Each turn of helix is of 34A length with 10 base pairs.
8. The base pairs lie inside the helix, perpendicular to the sugar phosphate backbone, which lies outside the helix.
9. An important feature of double helix is specific complimentary in base pairing always occurs between adenine-thymine and guanine-cytosine.
10. Adenine pairs with thymine with two hydrogen bonds and Guanine pairs with cytosine with three hydrogen bonds.
11. Because of three hydrogen bonds GC pairing is stronger than AT pairing. The higher the G-C content of a DNA the greater will be the density and stability.

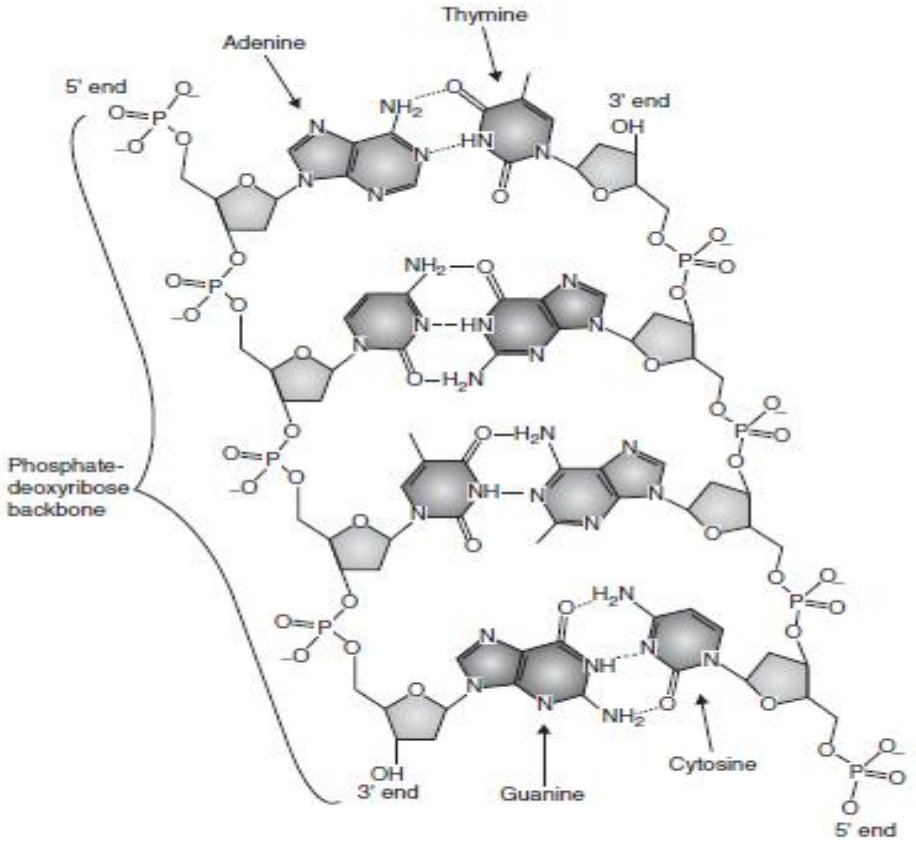
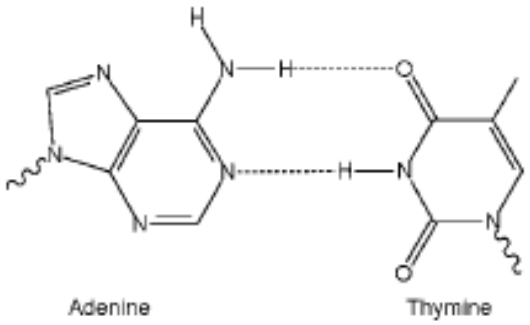
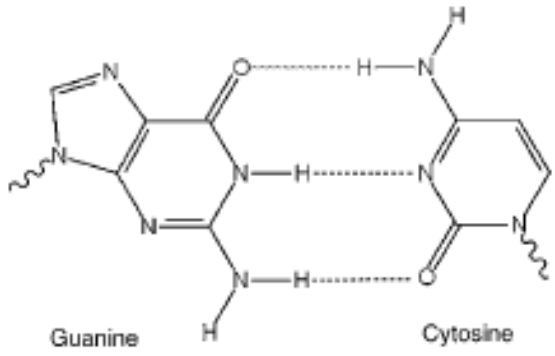


Figure 8-29 An illustration of laying two DNA strands end to end, with the concept of the 5′ end and the 3′ end. Note that the two strands run in opposite directions. (*Courtesy of Wikipedia.*)



Bonding between adenine and thymine in a DNA molecule involves two hydrogen bonds.



The bond between the base pairs guanine and cytosine in DNA involves three hydrogen bonds.

A. Other types of DNA :

The DNA can exist in different secondary conformations depending on their base composition and physical conditions under which it exists. Along with the above discussed B-form of DNA many other forms are proposed like A,C,D and Z.

(i) A-DNA :

When the DNA molecule is exposed to dry conditions or to high salt content the DNA molecule changes its helix nature. This new form of DNA is called A-DNA. The helix rise per turn to eleven. A-DNA is not present in vivo.

(ii) C-DNA:

When the DNA molecule is exposed to very low humidity levels in presence of Li^+ ions, the newly attained conformation is called C-DNA. The diameter and helix stoichiometry of C-DNA are different from that of B-DNA. The number of base pairs per turn are 9.33.

(iii) D-DNA:

If the DNA has very low content of guanine, that DNA forms a new type of structure called D-DNA. Only 8 base pairs per turn are present in D-DNA.

(iV) Z-DNA :

The very characteristic feature of Z-DNA is left –handed rotation whereas all other forms are right handed. The guanine cytosine rich DNA attains this form, in vivo. The phosphate backbone of DNA is of zigzag manner.

TERTIARY CONFORMATION OF DNA:

To get the very long length DNA into a small nucleus it must be packaged into a compact form. The genomic DNA of all organisms is highly supercoiled. Mostly negative (left-handed) supercoiling is seen. In eukaryotes histone proteins participates in organization of DNA. But these are absent in prokaryotes (bacteria)

In 1879 Walter Flemming found banded objects in the nuclei of stained eukaryotic cells. He called it as chromatin which is now known to consist of DNA and various proteins. The DNA and protein assemble to package the DNA to chromatin form in eukaryotes. The major proteins of chromatin are known as histones. Eukaryotic species contain five different histones called H1, H2A,H2B, H3 and H4. The histones are small basic proteins containing more lysine and arginine amino acids. Positive charge of these basic amino acids allow the proteins to bind to the negatively charged sugar – phosphate backbone of DNA.

Nucleosomes : When chromatin is treated with a solution of low ionic strength, it unfolds, giving rise to a structure that looks like beads on a string in an electron micrograph as shown in Fig. The beads are DNA histone complexes called nucleosomes, and the string is double stranded DNA.

Each nucleosome is composed of one molecule of histone H1, two molecules each of histones H2A, H2B, H3 and H4, and about 200 bp of DNA. The H2A,H2B,H3 and H4 molecules form a protein complex called the histone octamer. Around this octamer DNA is wrapped. About 146 bp of DNA are in close contact with the histone octamer. This forms core of a nucleosome. The DNA between core particles is called linker DNA. It is about 54 bp long. The histone protein H1 can bind to the linker DNA and to the core particle. H1 is responsible for higher – order chromatin structures.

Higher levels of chromatin structure : The packaging of DNA into nucleosides reduces the length of a DNA molecule about tenfold. Further reductions comes from higher levels of DNA packaging, the beads - on – a – string structure is itself coiled into a solenoid to yield the 30 nm fiber forms when every nucleosome contains a molecule of histone H1 and adjacent molecules of H1 bind to each other cooperatively, bringing the nucleosomes together into a more compact and stable form of chromatin. Condensation of the nucleosomes structure into a solenoid achieves a further fourfold reduction in chromosome length.

Finally 30nm fibers are themselves attached to an RNA – protein scaffold that holds the fibers in large loops. There may be as many as 2000 such loops on a large chromosome. The attachment of DNA loops to the scaffold accounts for an additional 200 fold condensation in the length of DNA. The loops of DNA are attached to the scaffold at their base. Because the ends are not free to rotate, the loops can be supercoiled.

Although histones are found only in eukaryotes, prokaryotic DNA is also packaged with proteins in a condensed form. Most of these prokaryotic proteins resembles the eukaryotic histone proteins. So they are called histone like proteins. There are no nucleosome like particles in prokaryotes and much of the DNA is not associated with protein. Bacterial DNA is attached to a scaffold in large loops of about 100 Kb. This arrangement converts the bacterial chromosome to a structure known as the nucleoid.

DENATURATION AND RENATURATION :

The double helical DNA can dissociate the two strands of DNA and reassociate again. In vivo the two DNA strands partially separated for replication, transcription and recombination. In vitro when the double helical DNA is exposed to high temperatures or extreme acidic or basic conditions, the two strands of DNA completely dissociate. This process is called denaturation. The denaturatin results in the increase of ultraviolet light absorption, called hyperchromic effect. Viscosity of DNA decreases with denaturation.

The process of reformation of double helical DNA is called renaturation. With the reversal of temperature and p^H conditions to normal DNA strands they reassociates.

TYPES AND FUNCTIONS OF RNA

Ribonucleic acid (RNA) is present in all living cells except in the DNA viruses. Usually RNA molecules are single stranded except in some viruses. RNA has a sugar-phosphate backbone with the sugar ribose (deoxyribose in DNA) moieties of nucleotides linked together by 3', 5' – phosphodiestser linkage. The nitrogenous bases are attached invariably to the C-1 of ribose. The RNA molecules contain the pyrimidine uracil instead of thymine in DNA.

Depending on their characteristics and biological role RNA is mainly divided into different types.

1. mRNA (messenger RNA or template RNA)
2. tRNA (transfer RNA or soluble RNA)
3. rRNA (ribosomal RNA or Insoluble RNA)
4. hnRNA (heterogeneous nuclear RNA)
5. dRNA (Small RNA)

mRNA (messenger RNA) :

Jocob and Monod (1961) proposed the term messenger RNA of the RNA carrying information for protein synthesis from the DNA to ribosomes (the organelle of protein synthesis). The mRNA varies greatly in length and molecular weight. Length of mRNA ranges from 100 to 12,000 nucleotides.

mRNA is always single stranded. It contains the nitrogenous bases adenine, guanine, cytosine and uracil. A few unusual bases can also found. The polypeptide mRNA strand will have no base pairing, as it needs to continuously participate in translation process. The base pairing in mRNA strand destroys its biological activity.

mRNA is synthesized by transcription of DNA strand of the cell. Transcription is the enzymatic process of copying RNA on one of the sequence of bases along one strand of DNA. This mRNA then directs the synthesis of polypeptide chain using the ribosomal machinery. As it acts as template for protein synthesis mRNA is also called template RNA.

Features of mRNA :

1. Normally each gene present in the genetic material of a cell transcribes its own mRNA. So the cell will have many types of mRNA molecules as there are genes. But among all the different typ0es of mRNA of a cell the difference arises only in length and base composition.
2. In most eukaryotes at the 5' end of mRNA molecule a common sequence of mGpp Nmp Np is found. This is called Cap. In this sequence m stands for methyl, G for Guanine, P for phosphate, and N for nay nucleotide. The cap of mRNA increases the binding efficiency of mRNA to rib0osome during translation.
3. In all the mRNA's after 10 to 100 nucleotides length there is a codon. The codon is a nucleotide triplet which codes for an aminoacid. AUG is the initiation codon for both prokaryotes and eukaryotes. In prokaryotes many initiation codons are found as they are polycistronic, coding for many proteins.
4. The translatin process started at the initiation codon continues throughout coding region till it reaches a codon which will not code for amino acids. These codons are called stop or nonsense or termination codons. The general termination codons are UAA, UAG or UGA.
5. The 3' end of most of eukaryotic mRNAs have 100 to 200 adenylic acid residues attached at their 3' ends, called poly (A) tails. The polyadenylatin occur after transcription has been completed. This tail increases the stability of mRNA. All eukaryotic mRNA except histone mRNA's have the poly(A) tail.

6. During translation mRNA binds to the ribosome. In case of prokaryotes a short purine-rich 5' AGGAGG3' consenses sequence known as 'Shine-Dalgarno sequence' binds to 16S r RNA of the ribosome.
7. mRNA is single stranded linear polymer. It can be easily degraded by cellular ribonuclease. In bacteria, mRNA may be so shors-lived that while one end is participating in translation process the other end may be degrading. In E. coli the average half-life of some mRNA is about two minutes.
8. In eukaryotes mRNA synthesis takes place in nucleus and then transported to cytoplasm for participating in translation.

tRNA :

The smallest polymeric form of RNA which transports the aminoacids to the site of protein synthesis is called tRNA. In a bacterial cell there are more than 70 types of tRNAs and in eukaryotic cells this number is even more, because there are tRNAs specific for mitochondria and chloroplasts. tRNAs are specific for aminoacids i.e., a particular type of tRNA can transport only a particular type of amino acid. But for one aminoacid there may be more than one tRNA.

(A) Structural features of tRNA :

After 7 years effort, Robert Holle discovered the base sequence of yease alanine tRNA. All organisms contain many species of tRNAs. The tRNAs are named based on the type of amino acid they are transporting, for example, alanine transporting aminoacid as $tRNA^{ala}$.

A. The primary structure of tRNAs is of 76 to 95 base long with phosphodiester linkage. The of 76 bases of yeast $tRNA^{ala}$ are modified bases like dihydrouridine (D), ribothymine (T) pseudouridine (ψ), Inosine (1).
B. tRNAs are schematically arranged in a secondary structure called cloverleaf model.
C. 5'end of tRNA is usually phosphorylated guanine.
D. The 5' end nucleotides along with its successive six nucleotides base pairs with its successive six nucleotides base pairs with the nucleotides nearer to 3' end forming a stem like structure called acceptor or amino acid stem. The terminal four nucleotides of 3' end are free stem loop. The 3' end have a common sequence 5'CCA3' which accepts amino acid, to be carried by it. Amino acid binds to the hydroxyl group of Adenine at 3' end.
E. The 3 – 4 base pairs stem ending in a loop of five to seven nucleotides is called Darm, as it contains the modifies bases dihydrouridine (D). The loop is called D loop. In case of mitochondrial $tRNA^{ser}$ D arm is entirely missing.

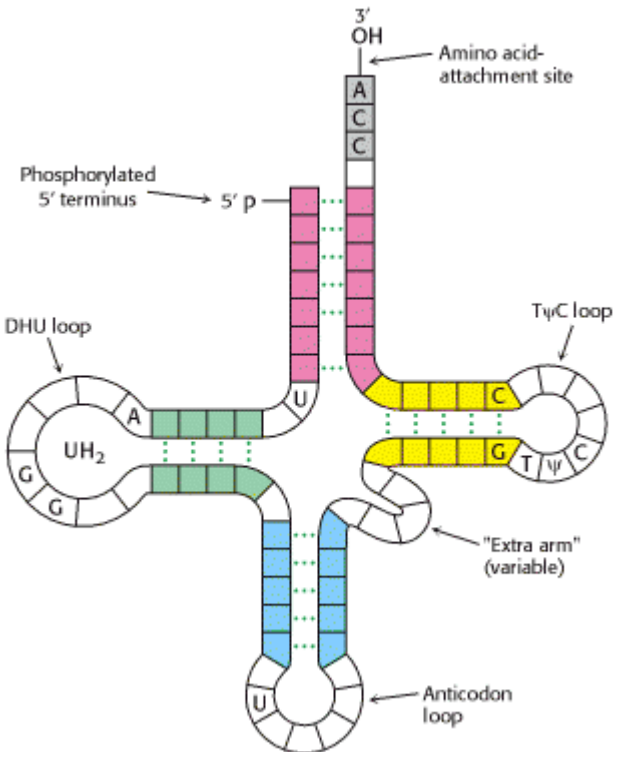


Fig. General structure of tRNA

F. A five base pairs stem ending in a loop that contains the anticodon is called anticodon arm. Anticodon is a triplet of bases that is complementary to codon bases specifying the tRNA.

G. A five base pairs stem ending in a loop, containing the nucleotides sequence ribothymine (T), pseudouracil (ψ), cytosine © is called T ψ C arm.

H. Between anticodon arm and T ψ C arm there is a region of greatest variability called variable arm. The nucleotide number of variable arm varies from 3 to 21.

I. In all the tRNAs 15 invariant positions and 8 semivariant positions were found.

J. The X-Ray Crystal structure of $tRNA^{phe}$ elucidated by Alexander Rich and Sung How Kim and Aaron Klug gives the idea of tertiary structure of tRNA. The cloverleaf secondary structure of tRNA is further folded to L – shaped tertiary structure by further base pairing. In this L – shaped conformation one end of L is with acceptor and T ψ C arm's. The other end is with D arm and anticodon arm

K. The acceptor site and anticodon site are 76 A apart in the L shaped conformation. The tRNA gains 20 to 25A narrow structure with this conformation. This makes it suitable to participate in biological functions.

(B) Role of tRNA in protein synthesis:

During protein synthesis the tRNA is loaded with activated amino acids by aminoacyl tRNA synthetases. The accurate recognition of tRNA by the enzyme is an essential part to prevent mistakes in protein synthesis. The activated tRNA always binds to the A site of ribosome except at the initiation of translation. This is because the first amino acyl tRNA (methionine in eukaryotes and formyl methionine in prokaryotes are first amino acids) binds in P site of ribosome

r RNA :

The most stable form of RNA found in ribosomes is called rRNA. Depending on their sedimentation rate the rRNA is of different types. In case of prokaryotes three types of rRNA are 5S, 16S and 23S and their molecule weights are 36,000, 55,000 and 1,20,000, respectively. The 5S and 23S rRNA present in 50S (larger) subunit and 16S rRNA present in 30S (smaller) subunit of prokaryotic ribosomes. Eukaryotes contain four types of rRNA. They are 5S, 5.8S, 18S and 28S rRNA. The eukaryotic ribosomal 60S (larger) subunit have 5S, 5.8S and 28S rRNA and the 40S (smaller) subunit have 18S rRNA.

Among different types of RNAs rRNA is about 80% of total cellular RNA. The G-C content of rRNA is more than A – T content and rRNA is a single unbranched polynucleotide strand.

The rRNA is necessary for the assembly of the machinery of translation. The 16S rRNA has a pyrimidine rich 5'CCCUCC3' sequence which is complementary to the purine rich sequence 5'AGGAGG3', called **Shine – Dalgarno** sequence in procaryotic mRNA. So the mRNA can perfectly bind to the ribosome using this complementary sequence of 16S rRNA.

hnRNA:

The precursor mRNA synthesized in eukaryotic cells is called heterogeneous nuclear RNA (hnRNA). The sedimentation coefficient of hnRNA is greated than 8S. The hnRNA containing both coding regions (exons) intervened by noncoding sequences (introns). So this will be processed to remove introns t become mRNA. Splicing is the mechanism to remove introns. The 5'end of hnRNA is capped with GPPP and 3' end is extended with poly (A) tail during the mRNA synthesis.

s RNA :

Eukaryotic cells have small RNAs serving a variety of functions located both in the nucleus and cytoplasm.

(a) Small nuclear RNAs (Sn RNAs) : Many of the Sn RNAs are associated with the proteins small nuclear ribonucleoprotein particles (SNRPs). The SNRPs participate in the splicing of introns in hn RNA to form mRNA.

(b) Small cytoplasmic RNAs (ScRNAs) : The major ScRNA is 7S RNA. This is of 294 nucleotides length, which is the RNA component of signal recongnition particle (SRP). The biological role of SRP is in tranlocation of proteins.

Lecture 6

Biological role of DNA in cell - control of metabolic activity of cell - maintenance and regulation of cell activity - DNA replication - semiconservative model - The Meselson stahl experiment

REPLICATION OF DNA

Cells do not live forever. In light of this, they must pass the genetic information to the new cells generated from it. In order to pass genetic information the copy DNA should be passed on to new cells. The process of accurate, efficient and rapid copying of the cellular genome is called DNA replication. When replicating the (Watson and Crick) double helix of DNA it unwinds, so that each strand of it can be exposed for replication. The free nucleotides pair with the nucleotides of the unwinded strand by hydrogen bonds and they will be linked by phosphodiester linkage. Adenine code for thymine and guanine code for cytosine and vice versa. In this way two new strands can form around each old strand.

MODE OF REPLICATION – SEMICONSERVATIVE :

Watson and Crick proposed that each strand of double helical DNA serves as template for synthesis of its complementary strand. Depending on whether the old strands (the template strands) are conserved in the original double helix or not, the possible modes of replication are

A. Conservative replication
B. Semiconservative replication
C. Dispersive replication

A. In the conservative replication the two newly synthesized strands come together to form a helix and the parental strands reassociate. This allows to conserve the original helix.
B. Semiconservative replication : Involves strand separation of parental DNA. After replication in the two DNA duplexes, one new strand and one old strand associates.
C. In the dispersive replication the parental DNA is broken down into smaller fragments. They are dispersed into two new double helixes following replication. Hence each strand of duplex DNA consists of both old and new DNA. This mode of replication cannot produce exact replicas.

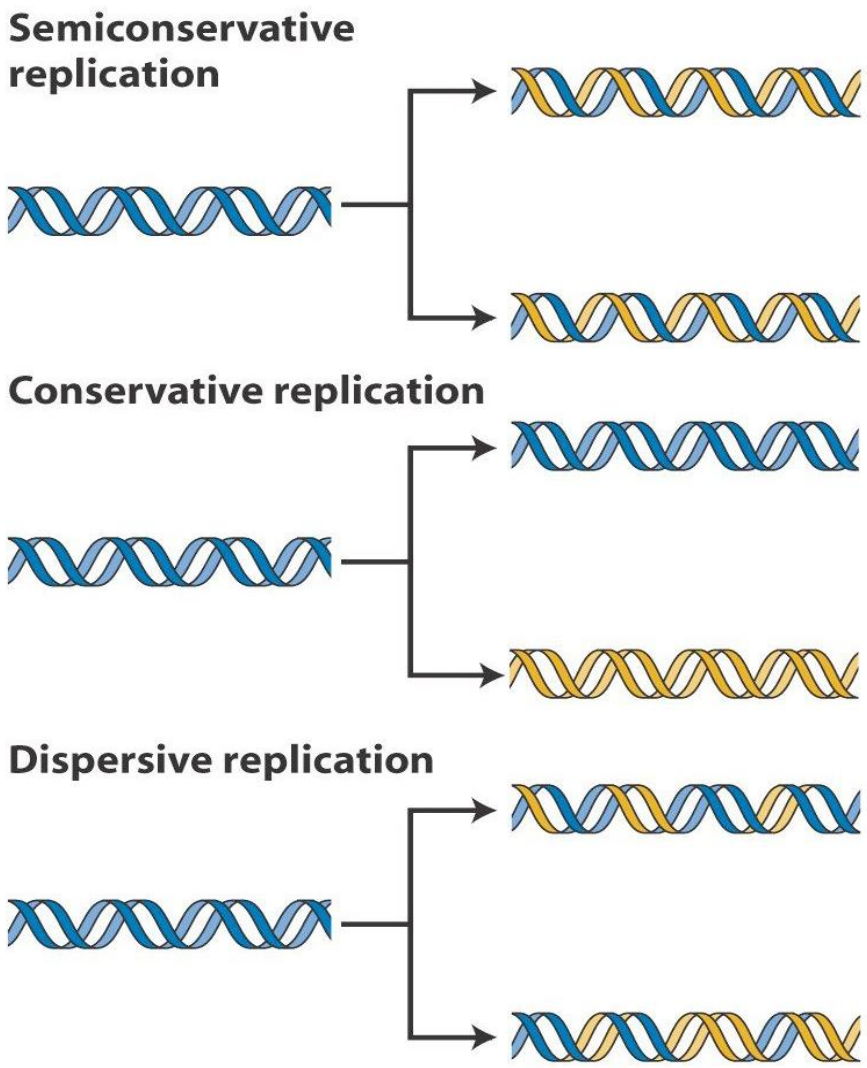


Fig. Representations of DNA replication models.

With in these three possible modes of replication semiconservative mode of replication is confirmed to be possible invivo by many experiments. The strong evidence is given by Meselson and Stahl.

THE MESELSON – STAHL EXPERIMENT:

Matthew Meselson and Franklin Stahl in 1958 provided the evidence for semiconservative mode of replication in bacteria. (For this experiment). They grew E.coli cells for many generations in a medium that had ^{15} N labeled ammonium chloride($^{15}NH_4CI$) as the only nitrogen source. The heavy isotope of nitrogen (^{15}N) contains one more neutron than the naturally occurring ^{14}N isotope unlike unstable radioactive isotopes, ^{15}N is stable. After many generations, all nitrogen containing

molecules, including the nitrogenous bases of DNA, E.coli cells contained the heavier isotope ^{15}N in the E.coli cells.

The uniformly labeled ^{15}N cells were transferred to a medium containing only $^{14}NH_4CI$. Thus the newly synthesized DNA contain only the lighter isotope ^{14}N of nitrogen. The time of transfer of bacteria to the new medium was considered as zero (t=0). The E.coli cells were allowed to replicate for many generations. The cell samples were removed after each replication cycle and DNA was isolated. Each sample is studied by using density gradient centrifugation. Sample is studied by using density gradient centrifugation. Samples are forced by centrifugation through a density gradient of cesium chloride. The more dense ^{15}N – DNA will reach equilibrium in the gradient at a point closer to the bottom. Where as lighter ^{14}N-DNA forms above that. This is because of their difference in buoyant density. The hybrid ^{15}N, ^{14}N-DNA forms the band in between ^{15}N-DNA and ^{14}N-DNA forms the band in between ^{15}N-DNA and ^{14}N-DNA, because of its intermediate density.After one generation the DNA isolated from bacterial sample form a single band. This is the expected result for semi conservative replication, as each replicated DNA is a hybrid with one new ^{14}N and one old ^{15}N-strands. This result confirms that conservative replication is not possible. Because of it is conservative mode, two bands one for ^{15}N helix and one for ^{14}N helix should have to appear with first generation sample.

The DNA samples collected after second cell division showed two density bands – one intermediated band and one lighter band. The lighter band is corresponding to the ^{14}N position in the gradient. The results of remaining generations are also confirming the semi conservative replication.

The results of first generation experiments supports the dispersive mode of replication to some extent. Meselson and Stahl disproved the dispersive mode of replication with their further studies. They continued experiment on first generation and also studied second generation.

(a) They further extended the experiment by heat denaturing the first generation hybrid DNA molecule. This separates the two strands of DNA. Then they are subjected to density gradient centrifugation. The strands exhibited either in ^{15}N profile or in ^{14}N profile, but not an intermediate density. This observation is supporting the semiconservative replication, but inconsistent with the dispersive replication.

(b) The DNA of second generation cells is collected and subjected to density gradient centrifugation. This showed two bands one is of lighter band ($^{14}N/^{14}N$) and other one intermediate band ($^{15}N/^{14}N$). This is also supporting semiconservative replication.

Lecture 7

Requriments for DNA synthesis - Substrate - Primer - proteins - DNA polymerase - I, II, III- Helicase - Topoisomerase - primase - ligase - ssb proteins - Mechanism of replication -Initiation - elongation – termination

BASIC REQUIREMENTS FOR DNA SYNTHESIS

A. **Substrates** : Deoxynucleoside triphosphated (dNTPs) are the substrate for DNA synthesis. The four types of deoxynucleoside triphosphate are deoxy adenosine triphosphate (dATP), deoxy guanosine triphosphate (dGTP), deoxycytidine triphposphate (dCTP) and deoxythymidine triphosphate (dTTP). During the polymerization of nucleotides α and ß phosphates are hydrolysed to get the energy.

B. **Template** : DNA replication cannot occur without a template. A macromolecular pattern for the synthesis of an informational macromolecule is called template. The template guides the addition of appropriate complementary nucleotide to the newly synthesized DNA strand according to Watson – Crick base pairing. In semiconservative replication each strand of parental DNA serves as template.

C. **Primer** : A primer is a segment of short nucleotides which is complementary to the template, with a free 3' – hydroxyl group to which nucleotides can be added. So that nucleotides are polymerized during DNA synthesis in 5' 3' direction. Most of the primers are RNA primers. For starting DNA synthesis primer is required. This is because DNA polymerases can only add nucleotides to a preexisting strand. Primers are generally oligonucleotides if RNA synthesized by primases. Primer will not be found in the final DNA. This is removed by polymerase I to add deoxynucleotides in place of ribonucleotides.

D. **Proteins** : Many enzymatic and nonenzymatic proteins are required for translation. They are polymerases, helicases, topoisomerases, ligases, SSB proteins etc.

   (i) DNA polymerases : Arthur Kornberg in 1955 characterized the DNA polymerase from E.coli cells. Later E.coli cell was found to contain five types DNA polymerases. They are I,II,III,IV and V. More than 90% of the DNA polymerase activity observed in E.coli extracts can be accounted for DNA polymerase I. It polymerises nucleotides specifically in 5' → 3' direction. Along with polymerization two types of hydrolytic activities in DNA polymerase I are as 5'→ 3' exonuclease and 3' → 5'exonuclease. → Protease subtilisin cleaves polymerase I into two fragments - the large fragment, called klenow fragment contains both the polymerase and 3' →5' exonuclease activity and the small fragment contains the 5' →3' exonuclease activity. 3' →5' exonuclease activity remove wrong paired nucleotide added in the replication. The activity is called proof reading. DNA polymerase II is involved in DNA repair.

DNA polymerase III is the principal replication enzyme of E.coli. The comparison of three polymerases is given in etc., DNA polymerases IV and V, identified in 1999 and are involved in an unusual form of DNA repair.

(ii) Helicases : The DNA synthesis process requires the separation of two parental strands. This is carried out by the enzyme helicase (Dna B protein). They move along the DNA and separate the stands using chemical energy from ATP.

(iii) Topoisomerases : The strand separation by helicases created topological stress in the helical DNA, which is relieved by the action of topoisomerases. DNA topoisomerase II also called DNA gyrase plays mojor role, not only removing supercoils but also in decatenating DNA.

(iv) Primases : Primers are short segments of nucleotides, generally of RNA, to initiate replication. These are synthesized by primases. Ultimately the RNA primers are removed and replaced by DNA by the DNA polymerase I with its 5' 3' exonuclease activity. After removal of RNA primer the gap is filled with DNA.

(v) DNA ligases : The nicks in the polymerizing DNA back bone are sealed by the DNA ligases. The nicks in okazaki fragments of lagging stand, the nick on circular DNA after leading strand synthesis and the nick after primer removal, all are sealed yb ligases. The energy required for sealing is obtained by the hydrolysis of NAD^+ or ATP.

(vi) SSB proteins : Single strand binding (SSB) proteins binds as tetramer to the separated DNA strands, to stabilize them. SSB proteins maintain DNA in unpaired form. The SSB proteins are stripped off from DNA just before it can be replicated by polymerase.

MECHANISM OF REPLICATION :

In prokaryotes DNA replicated in different mechanism like Ø replication, replication, rolling circle model of replication, looped rolling circle model of replication. The E.coli chromosome replicated by the bidirectional mode from a single replication origin. The DNA unit undergoing replication is called replicon. All the protein particles associated at the site of replication as a single unit, called replisome. The replication of DNA proceeds in three stages.

A. Initiation
B. Elongation
C. Termination

A. Initiation : Replication of the E.coli chromosome is initiated as its origin site of replication, called oriC. This oriC is a 245 bp sequence. With in the oroiC four 9-bp repeats and three 13-bp repeats play significant role in replication.

About nine proteins participates in the initiation of replication. They are DnaA, DnaB (helicase), DnaC, DnaG (primase), SSB (single strand binding proteins), RNA polymerase, DNA gyrase, histone like proteins (HU), Dam methylase. These separated the two strands of DNA at OriC and also making it ready for the elongation process.

DnaA protein is the initiation factor of replication. About 20 DnaA protein molecules as a single complex bind to the four 9-bp repeats at the origin site. Once the four 9-bp repeats are occupied, 20 to 40 additional DnaA monomers bind occupying the whole oriC region. This association results in negative supercoiled oriC around DnaA protein. The protein HU prevents the initiation at sites other than oriC.

With the help of ATP and HU the DNA sequences at three 13 bp reats is denatures. Thus region is rich in A = T base pairs. This results in the formation of 45 bp open complex.

Two DnaB proteins binds at the open complex, one on each separated DNA strand. The DnaB protein is a hexamer. The binding of DnaB to the DNA strands requires DnaC and ATP. The DnaB proteins unwind the DNA, each one moving in opposite direction to one another. This bidirectional moment creates two replication forks. The DnaB protein assembled form is called prepriming complex.

The unwinded DNA strands by DnaB are occupied by SSB proteins. Many molecules of SSB bind cooperatively to single stranded DNA, stabilizing the separated strands and preventing renaturation. DNA gyrase (topoisomerase II) removes the topological stress produced by the DnaB.

Addition of the enzyme primase to the prepriming complex completes the formation of primosome. Primase synthesizes the RNA primer essential to DNA synthesis. Two primosomes are formed at OriC, one for each replication fork. The point where the DNA is being synthesized is called replication fork.

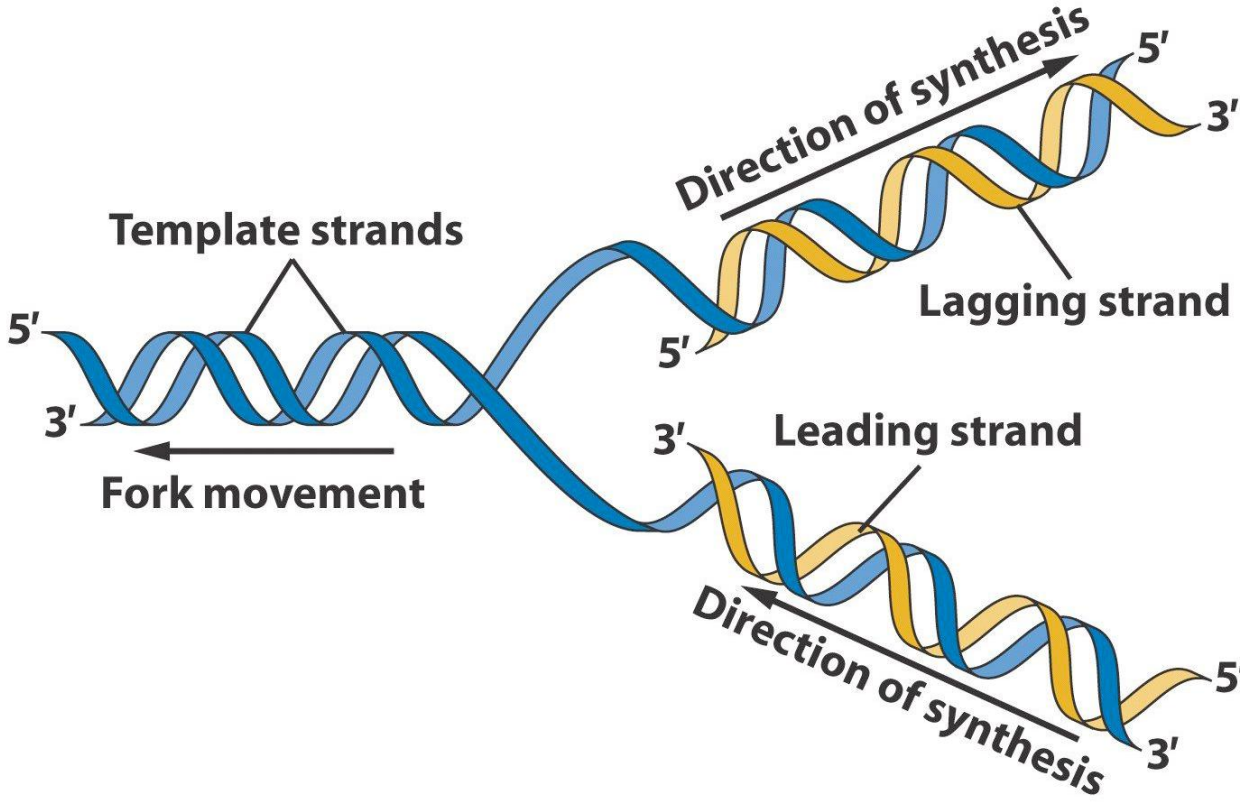


Fig. Representation of replication iniation

B. Elongation : In the elongation process the DNA strands are continuously copied with their complementary nucleotides. This stage of replication requires continuous unwinding by DNA helicase, removal of topological stress by topoisomerase, stabilization of single strands by SSB followed by polymerization of DNA strand by polymerase.

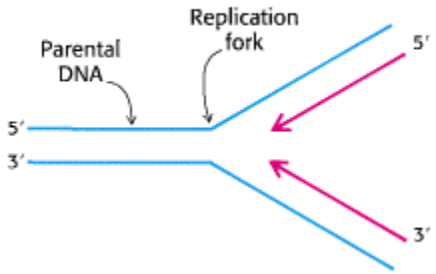


Fig. Representation of elongation at single replication fork.

Synthesis of two strands are different. The strand synthesized in 5' → 3' direction is called leading strand and strand appear to be synthesized in 3' →5' direction is called lagging strand. The two core enzymes of a single DNA polymerase III binds one on to each strand elongating the two strands.

The leading strand synthesis begins with the synthesis of a short RNA primer of 10 to 50 nucleotides at the replication origin. Then proceeds continuously with the addition of nucleotides by polymerase III.

The lagging strand synthesis is not a continuous process. It occurs in short fragments called Okazaki fragments of 1-2 kb long. Each fragment synthesis need the synthesis needs the synthesis of RNA primer by primase followed by binding of DNA polymerase III to elongate the fragment I 5' → 3'. The elongation of okazaki fragement is completed when it encounters the 5' –end of theprevious fragment. These fragments are joined to one another to form continuous DNA strand by DNA polymerase I and ligase. The DNA polymerase I removes the RNA primer and add deoxy nucleotides, finally the two successive fragments are joined by DNA ligase.

The coordination in the synthesis of two DNA strands in opposite directions by a single DNA polymerase III is accomplished by looping the DNA of the lagging strand. This bring the two polymerizing strands together and also into same direction.

C. Termination : Finally the two replication forks of the circular E.coli chromosome met at a region called terminus region. This region contain 20 bp sequences called "ter" sequences. A protein called Tus (Terminus utilization substance) binds to Ter sequences. This complex terminated the replication of E.coli. Sometimes the completely replicated two DNA circles are topologically linked.

The two DNAs linked topologically are called catenanes. The two double stranded DNAs are separated by topoisomerase II and IV. The separation of two DNA helices in catenanes is called decatenation.

Lecture 8

Genetic recombination - Gene transfer mechanism - Conjugation - Process - F plasmid -Hfr factor - Transformation - Competence - Gram positive transformation – Gram negativetransformation

GENE TRANSFER MECHANISMS IN BACTERIA

Transfer of genetic information to a cell from a donor cell by direct contact or in a free DNA form or through an agent is called gene transfer. It results in developing many new features to the transformed cell like pathogenicity, antibiotic resistance etc.,

Based on the mechanism gene transfer is of three types (1) Conjugation, (2) Transformation, (3) Transduction

CONJUGATION :

The break through of science, conjugation was announced by Joshua Lederberg at his 21 years old along with Edward Tatum in 1926. They had discovered that individual cells of E.Coli can exchange genetic information by a process that was subsequent next 10 years he demonstrated the gene transfer from F^+ to F^- involves tube like structures called sex pili.

The transfer of genetic information from a donor cell to a recipient cell through direct physical contact between cells is called conjugation. In the conjugation genetic information of either the free plasmid or present in the chromosome is transferred. The conjugative plasmid, F plasmid is the well studied example.

Factor : The fertility (F) plasmid of E.coli is the best example of conjugation. It is a circular DNA of about 100 kb length. It is an episome. Episomes are the plasmids which can exist either in the autonomous replicating extra chromosomoal state or in integrated for with chromosome. The bacteria with F plasmid are indicated as F^+ and the bacteria without F plasmid as F^-.

The F factor codes for a protein called pilin. It polymerizes to pilus. It is the surface appendage of E.coli. F pili are hair like, 2-3 µm long. A bacteria has 2-3 F pili. The bacteria that are F^+ are able to mate with bacteria that are F^-. This contact is followed by transfer of the F factor. The whole process is called conjugation.

A large region of about 33 kb of F plasmid, called the transfer region is required for conjugation. It contains about 40 genes that are required for transfer.

Process of Conjugation : Mating is initiated when the tip of the F^- pilus of F^+ cells is never permitted. This is because of surface exclusion proteins, that make the cell a poor recipient in such contacts.

After making contact between donor and recipient cells the F factor of donor cell is initiated at its origin of transfer site, called oriT. This site is located at one end of the transfer region. Then the covalently closed circular F factor is nicked at ori T. This follows the separation of two strands of DNA to about 200 bp length. The freed 5' end leads the way into the recipient bacteria. The entered F factor into the recipient cell synthesized its complementary strand and will be closed to form complete F factor. In the donor cell also the remaining single strand synthesizes concomitantly its complementary strand to regenerated its double stranded form.

Hfr Factor : Generally F factor exists autonomously as a plasmid. But in some cells the F factor exists as integrated with cellular chromosome called Hfr (High frequency of recombination) cell. They are son named because of high frequence of insertion at about 10^{-5} to 10^{-7} per generation. Among 100 F^+ cells one will be Hfr cell. The F factor of some cells can be converted to Hfr as shown in figure. Integration of F factor with chromosome involves homologous recombination between two covalentlyh closed circular DNA molecules the chromosome and the F plasmid. The insertion sequences (IS) present in the F plasmid and those in the hose chromosome serve as regions of homology for the insertional event. Once integrate, the F DNA is replicated along with the lost chromosome. This is because after integration it losses its autonomous replication capacity.

An integrated F factor still has active transfer functions. So an Hfr conjugates with F^-. The transfer of DNA starts with a short sequence of F DNA and continues until prevented by loss of contact between the bacterial. Since conjugal transfer of the host chromosome in an Hfr cell ios time dependent a gene can be mapped relative to the positin of the Hfr factor. For this the total time of E.coli chromosome transfer can be considered as 100 minutes. The gene can be mapped relative to the position of the Hfr factor simply by determining how long it takes for the gene to be transferred to a recipient.

F' Factor : In the conjugation process of Hfr factor the host chromosome restored to its original strate. However at a low frequency improper excision can occur forming a plasmid containing both F and bacterial DNA. This typ0e of factor is called F-prime (F) factor. It may result in lost of some F sequences or carrying of some host DNA.

TRANSFORMATION :

The transformation principle was already mentioned in the unit-III, when discussing "DNA is the genetic material". All types of bacteria are unable to transfer the genetic material by conjugation. Genetic material in them is transferred by other methods. Transfer of the cell free DNA into a recipient cell is called transformation. Griffith first discovered this phenomenon in 1928 in his investigations with Streptococcus pneumonia. The wild type S.Pneumoniae are with smooth (S) mucus coat virulent organisms. Certain mutant strain of S. pneumonia growing as rough ® colonies lost their virulence are called avirulent forms. Griffith found that heat-killed virulent S.pneumoniae will not cause infection in mouse. But a mixture of heat-killed virulent ad live avirulent causes infection in mouse. This medicates that some how the virulent trait transformed from the dead cells to the live avirulent forms.

Later, Averty, Macleod, Mc carty in 1944 demonstrated that the transformed trait from heat-killed virulent to live avirulent was DNA.

The transformation is possible in competent species only. Competence is a physiological stage that permits a cell to take up transforming DNA and can be genetically changed by it. Transformation occurs in many genera like Haemophilia, Neeisseria, Rhizobium, etc.

Transformation process in microorganisms is divided into two main types.

(i) Gram positive transformation.
(ii) Gram negative transformation.

(i) Gram positive transformation : This is also called Streptococcus –bacillus model. The gram positive bacteria are competent only in the exponential phpase of their growth. In S.pneumonia this state is induced by a protein, the competence stimulatin peptide.

(ii) Gram negative etransformation : the best studied gram negative systems are Neisseria gonorrheae and Haemophilus influenzae. In addition to gram positive membrane system in gram negative bacteria the DNA has to cross the outer membrane of them. They are competent through out their life cycle for transformation. But competence is highly induced in stationary phase.

In rENA technology the micrology the microbial cells are artificially transformed with purified DNA by using calcium chloride, micro injection or electroporation, etc.

TRANSDUCTION :

Transfer of bacterial genes from one cell to another through bacterial viruses (bacteriopphages) is called transduction. Many different bacteriophages are capable of transduction. Generally transduction is the result of an error in bacteriophage reproduction. In the life cycle of bacteriophages the coat proteins of phages assemble themselves, packing genetic material within it, to create a new viral particle. Each bacteriophage has a mechanism for packaging its genome into a capsid. Some bacteriophages occasionally make an error and package a piece of host cell's DNA. This event is normally random, so without showing specificity any bacter4ial gene will be transduced by bacteriophage.

Bacteriophages that contain bacterial DNA also are capable to attach to a new host cell and to inject DNA. Once inside the new cell, the transduced bacterial DNA can recombine with the resident genome, it can be expressed and transmitted to next generations. In lysogenic bacteria the bacterial genes become part of a bacteriophage chromosome.

Transduction is of different types :

1. Generalized transduction
2. Co-transduction
3. Abortive transduction
4. Specialized transduction

1. **Generalized transduction** : The process of transfer of any portion of a bacterial cell's genome into another bacterial cell by a bacteriophage is called generalized transduction. The transudcing bacteriophage contains only bacterial DNA, without phage DNA. Sometimes during loading of genetic material into viral protein heads instead of phage DNA, bacterial DNA can also be packaged. When this type of packed phage binds to another bacterial cell, the donor DNA is injected into bacterium and integrated into its genome by recombination. For example phages that can mediate generalized transduction are P^1 Phage in E.coli and P^{22} Phage in Salmonella. P^{22} bacteriophage of salmonella is the well studied example of generalized transduction. About one-one hundredth size of Salmonella chromosome can be trasduced by P^{22} phage.
2. **Co-transduction** : Transfer of two or more genes of a donor cell at a time by a bacteriophage ot a recipient bacteria cell is called cotransduction. The cotransduced genes must be close to each other.
3. **Abortive transduction** : In this type of transduction the transferred gene will not integrate with the recipient cell',s chromosome. So it is transmitted to only one of its two daughter cells.

4. **Specialized transduction** : Transfer of specific site of donor genetic material by bacteriophage is called specialized transuction. This is mediated by bacteriophages that integrate into specific site of bacterial chromoseome. This specificity limits the transfer of genetic material with in the vicinity of integrated site. Lambda (λ) phage is the well studied example for specialized transduction. The λ phage integrated (attachment) at site near gal (galactose) region of the chromosome. So mediate specialized transduction in that region only

Lecture 9

Micro injection - calcium chloride mediated - calcium phosphate mediated – electroporation Particle bombardment method- Transduction - Generalized transduction - Co-transduction - Abortive transduction - Specialized transduction

After construction of recombinant or chimeric DNA it should be transformed into host cells. The host cell may be bacterial, animal or plant cell. Depending on the nature of the all transformation of DNA varies. Transformation is the process of intro0ducing free DNA into a cell.

(A) Genetic transformation of prokaryotes
(B) Genetic transformation of eukaryotes

A. **Genetic transofrmatin of prokaryotes**:

E.coli is the main host cell ion r DNA technology. The uptake of DNA by E.coli cell follows three main ways.

(i) Calcium chloride ($CaCl_2$) treatment

(ii) Electroporation

(iii) Microinjection

(i) Calcium chloride treatment : The E. coli cells should be treated witnice-cold C_aCl_2 and then exposed to high temperatures (42^0C) for about 90 sec. Using this method 1 in 10^{-3} cells can be transformed.

It si assumed that the bacterial cell wall is broken down in localized regions,which allow the plasmid DNA to be transformed into bacterial cell

(ii) Electroporation : The bacterial cells are induced to uptake DNA by subjecting to a high strength electric field in the presence of DNA. This method of transformation of DNA is called electroporation.

For E.coli electroporation, the cells and DNA are placed in a chamber fitted with electrodes and a single pulse of 2.5 KV is administered for about 4.6 milliseconds. This treatment yields transformation efficiencies of 10^6 to 10^9 transformants per µg of DNA.

B. **Genetic transformation of eukaryotes** :

In animal cells DNA is injected into either the fertilized egg or into embryo stem cells. In plant cells protoplasts or embryonic tissues are used to inject DNA of interest. In case of animal cells the term transformation is replaced by transfection and fertilized eggs or embryos are transfected with whold nuclei or whold chromosome or DNA fragments. This transfection of fertilized eggs is by using cytochalasin-B or by microinjection. Cultured animal cells are transfected by coprecipitation with calcium phosphate or

electroporation or microinjection. Cultured animal cells are transfected by coprecipitation with calcium phosphate or electroporatin or microinjection.

In case of plant cells the gene transfer is by physical delivery or by using plasmids like Ti or Ri. The physical delivery is by using polyethylene glycol or by microinjection or by macroinjection or by electroporatin. Ti plasmid of Agrobacterium tumefaciena and Ri plasmid of A. rhizogenes have been effectiveloy used in gene transformation in plant cells.

The purposes of gene transfer to plant or animal cells are.

(i) To produce chemicals or pharmaceutical drugs or fertilizers.
(ii) Study of structure and function of genes.
(iii) Production of transgenic animals and plants with improved qualities.

Protoplast transformation :

The organisms like B. subtilis can be transformed naturally. But natural transformation is suitable best to linear DNA, but not to circular Plasmids. So,a method known as protoplast transformation was developed. Protoplasts can be prepared by enzymatic removal of bacterial cell wall. Enzymes like lysozyme in the presence of osmotic stabilizer such as sucrose, generates protoplasts, exposing cytoplasmic membranes. Addition of DNA solution together with polyethyolene glycol (PEG), causes the cells to take up the DNA. Then the cells are allowed to regenerate on an osmotically stabilized medium. If the correct conditions are used, a very high proportion of the resultant colonies will be transformed. The same procedure is used in as of eukaryotic organisms also.

The formation and regeneration of protoplasts is a key feature of an alli9ed procedure known as protoplast fusion, where the addition of PEG to a mixture of protoplasts of two different strains results in the formation of a fused cell that contains the complete genome of both parents. Protoplast fusion has been more widely used for eukaryotic cells.

With fungi, the initial stage in mating is the formation of a heterokaryon. A cell containing two different nuclei is called heterokaryon. This can be difficult to achieve by conventional means, as many commercially important fungi are devoid of sexual activity. However, if protoplasts can be produced the addition of polyethylene glycol will cause the protoplasts to aggregate, and the very close contact between the exposed membranes results in cell fusion. After allowing the cell walls to regenerate, heterokaryotic cells are produced which contain nuclei from both parents. Protoplast

fusion is an extremely powerful technique that is applicable to any cells as long as the cell wall can be removed and viable cells regenerated from the protoplasts.

Protoplast fusion has been used with bacteria, fungi, plant and animal cells. The animal cells do not have a cell wall, so the process of cell fusion is considerable simpler. Construction of hybridoma cells for monoclonal autibodies production is the best example for cell fusion

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Lecture 10

Strain Improvement - Importance of pure culture - Isolation and purification of pure cultures Alteration of genes by mutation - induced mutation - Frame shift mutations

STRAIN IMPROVEMENT

Several options are open to an industrial microbiology organization seeking to maximize its profits in the face of its competitors' race for the same market. The organization may undertake more aggressive marketing tactics, including more attractive packaging while leaving its technical procedures unchanged. It may use its human resources more efficiently and hence reduce costs, or it may adopt a more efficient extraction system for obtaining the material from the fermentation broth.

The operations in the fermentor may also be improved by its use of a more productive medium, better environmental conditions, better engineering control of the fermentor processes, or it may genetically improve the productivity of the microbial strain it is using. Of all the above options, strain improvement appears to be the one single factor with the greatest potential for contributing to greater profitability. While realizing the importance of strain improvement, it must be borne in mind that an improved strain could bring with it previously non-existent problems.

For example, amore highly yielding strain may require greater aeration or need more intensive foam control; the products may pose new extraction challenges, or may even require an entirely new fermentation medium. The use of a more productive strain must therefore be weighed against possible increased costs resulting from higher investments in extraction, richer media, more expensive fermentor operations and other hitherto non existent problems. This possibility not withstanding, strain improvement is usually part of the program of an industrial microbiology organization. To appreciate the basis of strain improvement it is important to remember that the ability of any organism to make any particular product is predicated on its capability for the secretion of a particular set of enzymes. The production of the enzymes, themselves depends ultimately on the genetic make-up of the organisms. Improvement of strains can therefore be put down in simple term as follows:

(i) regulating the activity of the enzymes secreted by the organisms;
(ii) in the case of metabolites secreted extra cellularly, increasing the permeability of the organism so that the microbial products can find these way more easily outside the cell;
(iii) selecting suitable producing strains from a natural population;
(iv) manipulation of the existing genetic apparatus in a producing organism;
(v) introducing new genetic properties into the organism by recombinant DNA technology or genetic engineering.

MUTATIONS

Genes are chemically the segments of DNA molecules except in some viruses, as some viruses are found to contain RNA as genetic material. They are normally transmitted with great exactness. But sometimes variations may be caused by physical

or chemical agents resulting in altered phenotype. The heritable changes in the genome of a cell are called mutations. Those mutations which occur in the somatic cells are called somatic mutations. These are not transmitted to next generation. Mutations occurring in the germ cells are called germinal mutations. These mutations influence the gametes and are passed to next generation, generating new variability and contributing to the process of evolution.

Mutations have both advantages as well as disadvantages. Increasing the microbial mutation rates bring out the genetic changes which have been put to many important uses in the laboratory and industries. For example mutations in some plants like tulips producing colourful flowers. Mostly mutations effect the normal existence of cells. For example in bactereia auxotrophs are developed from wild type because of mutations. In humans mutations leads to physiological abnormalities like sicklecell anaemia.

TYPES OF MUTATIONS

Mutations are classified in different ways on the basis of one or the other criterion. They may be depending on their origin, depending on the type of change in base composition, on the basis of type of the cell, on the basis of the nature of their effect, etc. Among all these classification criteria the significant one's are (A) Depending on their origin. (B) Depending on the type of change in base composition.

(A) Depending on their origin : Mutations are of two types
(i) Spontaneous mutations
(ii) Induced mutations.

(i) Spontatneous mutations : Mutations that occur naturally are called spontaneous mutations. Their origin is indeterminate and unknown. They are generally assumed to be random changes in the nucleotide sequences of genes. Spontatneous mutations are linked to normal chemical processes in the organism that alter the structure or the sequences of genes. For example all the four common bases of DNA have unusual tautomeric forms. Which are, however, rare. Tautomers are the mutually interconvertable structural isomeric forms. Normally nitrogenous bases in DNA present in the keto form. As a result of tautomeric rearrangement they can be transformed into the enol form.

The tautomeric rearrangement changes the hydrogen bonding characteristics of bases. Normally AU and GT base pairs. The tautomeric changes during replication substitutes nitrogenous bases with others. If a purine for purine and pyrimidine for pyrimidine are substituted the type of mutation is called transition mutation. If a purine for pyrimidine and pyrimidine for purine substituted the mutation is called transversion. The transtition

and transversion mutations are also termed point mutations. Spontaneous mutations also occurs by frame shifts of DNA.

Once an error is present in the genetic code, it may be reflected in the amino acid composition of the specified protein. If the changed amino acid is present in a part of the molecule, determining the structure or biochemical activity, functional alteration can occur. Many spontaneous mutations are reported. For example albinism and hares lip in man; a tobacco mutant producing seventy leaves all of a sudden in a normal progeny producing an average of twenty leaves.

(ii) Induced mutations : The mutations resulting from the influence of any artificial factor are considered to be induced mutations. Muller subjected drosophila to powerful x-rays and obtained a number of mutations. The chemicals or any other means that induce mu8tations are called as mutagens or mutagenic agents. The mutagens acts in different ways like incorporation of base analogs, specific mispairing and intercalation.

Base analogs are structurally similar to nitrogenous bases of DNA, and can be incorporated into the growing polynucleotide chain during replication. Specific mispairing is caused when a mutagen changes a bases structure, by that alters it s base pairing characteristics.

Lecture 11

Chromosomal mutation - Additions - Deletions - use of rDNA technology of alter the genes- better yield of metabolites - primary metabolites - secondary metabolites

The different types of mutations changing the nucleotide number or order of DNA are

(1) Frameshift mutations

(2) Chromosomal mutations

(1) Frameshift mutations : As pointed out in the third unit the genetic information in DNA is expressed first into mRNA by the transcription. mRNA is translated to proteins on reading triplet code from a fixed starting codon. If a single nucleotide is deleted or inserted in the normal sequence then the reading frame changes. The mutations leading to the change in reading frame are called frame shift mutation. These are two types.

(i) Deletion mutations

(ii) Insertion mutations

(i) Deletion mutations : The reading frame of mRNA does not have any punctuation. So if nucleotides deleted it changes the amino acid sequence of protein expressed by it.

Normal sequence :

DNA	AAA	GCT	ACC	TAT	CGG	TTA
MRNA	UUU	CGA	UGG	AUA	GCC	AAU
Protein	Phe	Arg	Trp	IIe	Ala	Asn

Addition mutation :

DNA	AAA	GCT	ACC	ATA	TCG	GTT
MRNA	UUU	CGA	TGG	TAT	AGC	CAA
Protein	Phe	Arg	Trp	Tyr	Ser	Gin

Deletion pmutation :

DNA	AAA	GCT	CCT	ATC	GGT
MRNA	UUU	CGA	GGA	UAG	

Protein Phe Arg Gly Stop

Fig. 4.2 Representation of Frame shift mutations

Deletion mutations are of variable length ranging in deletion of the number of nucleotides. Deletion of three successive nucleotides will not effect all the protein composition. It is with one amino acid less only, as the codon is triplet code.

Dyes like acridines can bring about deletion mutations. In heterozygous diploid eukaryotes, a deletion involving the dominant alleles amy result in the expression of the recessive phenotype.

(ii) Insertion mutations : Inserting nucleotides into a normal gene results in a mRNA, in which the reading frame is altered. This type of mutations causing insertion ofnucleotides are called insertion mutations.

2.Chromosomal mutations : The mutations effecting the number, size, shape and gene complements are chromosomal mutations. These are of different types like a chromosomal segment may be lost by deletion, or it may undergo inversion or it may be translocated to a different site or may be duplicated to tandem repeats.

MUTAGENS :

Mutations inducing agents are called mutagens. They create mutations in different ways. Depending on the nature of mutagens they are of two types

(A) Physical mutagens
(B) Chemical mutagens

(A) Physical mutagens : Mutations can be naturally or artificially induced by a variety of physical mutagens. H.J.Muller, founder of genetics, demonstrated in 1927 that mutations can be artificially induced by treating flies with x-rays. Similarly L.J.Stadler in 1928 demonstrated and increase in the rate of mutations due to x-rays in barely and maize. Besides x-rays gamma rays can also induce mutations.

The physical agents are broadly divided into two types :

(i) Ionizing radiation
(ii) Nonionizing radiation

(i) Ionizing radiation: X-rays and gamma (Y) rays are ionizing radiations. They have short wavelength and high penetration power. They can penetrate into deeper tissues causing ionization of the molecules along their way. When X-rays penetrate into cells, electrons are ejected from the atoms of molecules

encountered by the radiation. As a result the stable molecules and atoms change into free radicals and reactive irons. The radicals and ions can initiate a variety of chemical reactions, which can affect the genetic material, resulting in point mutations. i.e., affecting only one base pair in a given location. The rate of mutation increases with the increasing dose of X-rays administered.

(ii) Nonionizing radiation : Ultra Violet (UV) rays are nonionizing radiations. They have long wavelength and low penetration power. The purines and pyrimidines absorb UV radiation most intensely at about 260 nm. This property has been useful in the detection and analysis of nucleic acids. In 1934 it was discovered that UV radiation is mutagenic. The major effect of UV radiation is formation of pyrimidine dimmers, particularly between two thymines. Cytosine-cytosine and cytosine-thymine dimmers are less prevalent.

The dimers damage the DNA structure and effects normal replication.

B.Chemical mutagens : Charlotte Auerbach, author of “Science of Genetics” was the first to find that mutatins can also be induced due to certain chemicals. Chemical mutagens can remove, replace or modify DNA bases.

(i) Alkylating agents : Alkylatin of nitrogenous bases by the alkylating agents either removes the base or modifies it. Guanine residues can be alkylated by the methyl methane sulfonate and ethyl methane sulfonate. These agents alkylates guanine at N^7 and weakens the purine-deoxyribose linkage. This leads to deppurination creating gap at that site. N-methyl-N^1-nitro –N –nitrosoguanidine CH_3-N(NO)-C(NH)-NH-NO_2 is a powerful mutagen in E.coli. Some alkylating agents change the GC positin ina nucleotide to AT.

(ii) Intercalating agents : Intercalating agents produces frame shift mutatinos in bacteriophages like T_4. For example acridines are mutagenic to bacteriophphages but not to bacteria. As the acridines are unable to enter bacterial cell.

(iii) Base analogs : Base analogs are structurally similar to normal nitrogenous bases and can be incorporated into the growing polynucleotide chain during replication. These analogs will have base pairing properties direrent from the bases they replace. One of the first base analog formed to induce mutations in phage T_2 is 5 bromouracil (BU) an analog of thymine. In the normal keto form BU base pairs with adenine. But its tautomeric enol form pairs with guanine like cytosine.

Lecture 12

Industrial fermentation process - Fermentor - design and construction - Fermentation media- Physical properties of media - Types of fermentors - Stirred tank fermentors - Packed bedfermentors - Fluidized bed fermentors - Bubble column fermentor - Air lift fermentor -Cylindrical fermentors - Flocculated cell culture fermentor - Multi phase bioreactors - Tricklingbed bioreactors

Characteristics of an Ideal production medium.

1. Chemical composition: the production medium must have a suitable chemical composition. Medium should contain a source of carbon, a source of Nitrogen, growth factors and mineral salts.
2. Precursors: In certain fermentations, the medium should supply the required precursor for better yields of a desirable product.
3. Buffering capacity: Maintenance of the pH in the optimum range is necessary for making the process successful, since acidic and /or basic compounds depending on the nature of the fermentation process accumulate during the progress of the fermentation. To control the pH of the medium, buffers should be added to the medium (E.g. $CaCO_3$). Media containing considerable quantities of proteins, peptides, and amino acids possess good buffering capacity in the pH range near neutrality. Additional buffering capacity in this pH range also provided by phosphates (Mono and dihydrogen potassium or Sodium phosphates).
4. Avoidance of Foaming: Foaming is a serious problem in fermentation industry, since it may help in contaminating the fermentation medium and also causes other problems for the fermentation. Hence defoamers (e.g. hard oil mixed with octadecanol for penicillin fermentations) should be used for controlling foam. These defoamers are added to the production medium before sterilization or incorporated after sterilization or added during the fermentation.
5. Toxicity: the ideal production medium free from any toxic effect on culture or product formation.
6. Consistency: In aerobic fermentations, it is necessary to supply sterile air into the medium. Under such circumstances, liquid media allow the diffusion of air

throughout the medium under agitation. Fermentation media should not be viscous. Viscous nature of the medium creates difficulty in the penetration of the air interior of the medium. Air is not easily absorbed by the liquid medium.

7. Contamination: Certain conditions of the production medium are helpful to check the contamination. For example low pH values in citric acid production using *Aspergillus niger* avoids contamination. Media having low pH values may be sterilized at low temperatures.
8. Recovery: Recovery of the desired product is an important criteria. Components of the medium should be such that separation and extraction of the product becomes easy and cheaper.
9. Availability of raw materials: Raw materials required for designing of the production medium should be freely available in large quantities at a reasonable price.

Raw materials as Media:

Different types of raw material are used in different types of industrial fermentation processes. Usually crude nutritive sources are preferred, since they are economical. Mostly agricultural products are utilized as a source of raw material in fermentation industries.

1. **Saccharine Materials**: Sugar cane, sugar beets, molasses, and fruit juices may be included in this category.

Molasses: Molasses is a byproduct of the cane and beet sugar industry. It is recovered at any one of several stages in the sugar refining process. Chemical composition of sugarcane black strap is variable. It depends on the quality and variety of the cane but also on the process involved in the manufacture of sugar. About 95%of the total sugar in cane molasses is fermentable. It is particularly rich in biotin, pantothenic acid, thiamine, phosphorous and sulphur. The organic nitrogen content is less than beet molasses, since it does not contain betaine. Bu this substance is not assimilated by yeasts.

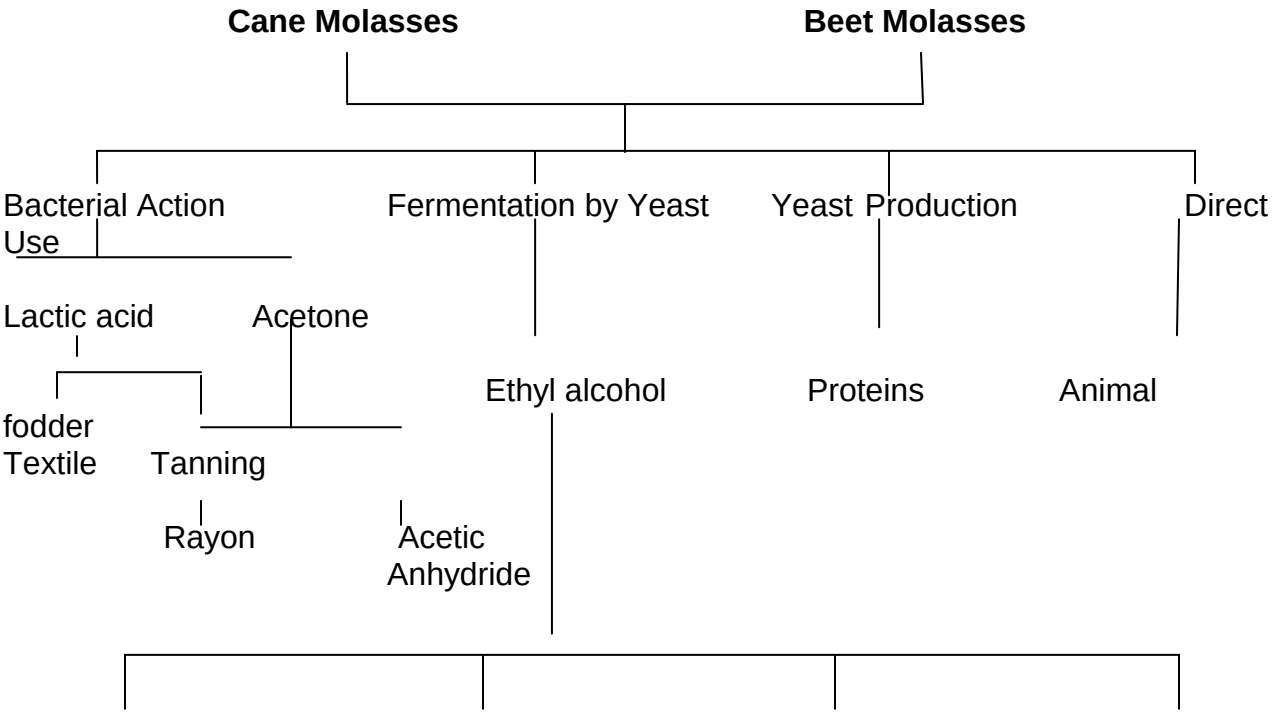
Beet molasses are produced by the same processes employed for cane molasses. Vitamins such as biotin, pyridoxine, thiamine, pantothenic acid and inositol are also present. Beet molasses have limited biotin. Therefore, in fermentations involving yeast

culture, a small amount of cane black strap molasses or other biotin supplying material should be incorporated in the production medium. Because yeast require biotin for their growth.

The largest utilization of cane black strap molasses in India is in the alcohol industry, which utilizes it for the manufacture of spirit, country liquors, rum, brandy, gin and whisky.

Fruit juices: Fruit juices contain soluble sugars. Grape juice contain glucose 7 fructose. Therefore, fruit juices can be used as a source of carbon in fermentation industries. Grapes are used in the production of wine.

Cheese way: The straw coloured liquid produced as a byproduct of cheese making is called cheese whey. It is a major waste product for the cheese industry. It cannot be disposed of without proper treatment. Therefore, it is desirable to use it for useful products. It is also used as pig feed. For lactic acid production and SCP production it is served as raw material because it contains lactose, nitrogenous substances including vitamins (e.g. vitamins) and inorganic salts.



Oils & Waxes Synthetic rubber Dyes Cellulose Products

2. Starchy Material: There are two main sources of commercial starches

1. Cereals (Wheat, rice, maize)
2. Roots & tubers (potatoes, tapioca e.t.c)

The moisture content of the grain is low where as that of roots and tubers are very high. Starches require pretreatment to bring about the conversion to fermentable sugars. This is done either by enzymatic or chemical agencies.

3. Cellulosic Materials:

Cellulosic materials are complex carbohydrate materials. The cellulosic molecule is made up of the repeating units of β-glucose. The formation of β-cellobiose requires two molecules of β-glucose, which are linked through α 1, 4-linkage.

1000 to 10,000 units of cellobiose are required to form a simple linear polymer called cellulose. Units of cellobiose are joined end to end through 1, 4-β-glucosidic linkages. For this reason cellulosic materials require some sort of pretreatment.

Cellulosic materials are Sulfite waste liquor, wood molasses and Rice straw.

Sulfite waste liquor:

In the manufacture of paper pulp, wood is subjected to hydrolysis which is brought about with the help of Calcium bisulfite under heat and pressure. This operation is called digestion process. At the end of this process, the spent liquid is left and it is referred to as sulfite waste liquor. It cannot be disposed of unless it is properly treated.

Sulfite waste liquor contains 10 to 12 percent solids, of which sugars make up about 20%. It contains sugars in the form of hexoses and pentoses.

It is used in the industrial production of ethyl alcohol using *Saccharomyces cerevisiae* and in the growth of *Torula utilis* cells for animal feed. *Saccharomyces cerevisiae* requires hexoses where as *Torula utilis* requires both hexoses and pentoses.

Sulfite waste liquor can not used directly as fermentation medium. It contains free sulfur dioxide or sulfurous acid which is toxic to microorganisms. These toxicants are removed by steam stripping or precipitation with lime.

Wood molasses:

It is produced by acid hydrolysis of wood cellulose itself. This may produce 65-85% fermentable sugars. Sulphuric acid of about 0.5% concentration is used at a temperature range of 150 to 185^{o}C. Using a continuous process a syrup may be obtained from saw dust. This syrup may contain 4 to 5% reducing sugars (a mixture of glucose and pentoses) with an overall yield of 45 to 55%. It may be subjected to concentration to give a kind of wood molasses.

Rice Straw:

Rice straw and related agricultural materials can serve as a good source of cellulose. It is a poor quality animal feed in its natural state because of its bulkiness, poor palatability, low protein content and low digestibility. Numerous microorganisms are capable of using cellulose for their growth. Rice straw has been used as a fermentation medium in the production of silage and single cell protein (SCP), mushroom cultivation e.t.c.

4. Hydrocarbons & Vegetable Oils:

Hydrocarbons used as fermentation substrates are usually mixtures of various hydrocarbon components. These fermentation raw materials are relatively cheap. However, purified hydrocarbon fractions or hydrocarbon compounds are more expensive.

Hydrocarbon substrates (e.g. gas oil and n-paraffins) are used to produce single cell protein (SCP) products. In this way biomass of yeasts (e.g. *Candida lipolytica, Candida kofuensis, Candida tropicalis*) can be produced on a significant scale under aerobic conditions.

Vegetable oils: Oils obtained by deoiling of vegetable seeds are called vegetable oils. On the basis of their degree of unsaturation, they may be grouped into following three major classes:

a. Oleic (or ‘non drying’ type): these include olive and groundnut oils.
b. Linoleic (or ‘semi drying’ type): these have a higher content of the double unsaturated fatty acid found in maize, sunflower and cotton seed oils
c. Linolenic acid (or ‘drying type): These include linseed and soya bean oils containing a fatty acid with three double bonds.

These oils may undergo drying if exposed to the atmosphere due to the oxidation of the unsaturated components. Commercial vegetable oils (e.g. maize oil) may be used in conjunction with surface active agent as anti foams or alone as a nutrient source of carbon.

5. Nitrogenous Materials:

Corn steep liquor(CSL): The used steep water results from the steeping of corn during the manufacture of starch, gluten and other corn products this by product is subjected to concentration to approximately 50% solids and this concentrate is called corn steep liquor. Corn steep liquor was originally found to be useful for penicillin production specifically. But, it is now recognized as valuable in many fungal antibiotic fermentation media. In addition to this, it is also used in the manufacture of food stuffs.

Soya bean oil: the material left after deoiling of the soya bean seeds is called soya bean meal. Soya bean meal contains approximately 8% w/w nitrogen. This differs from corn steep liquor, since soya bean meal is a much more complex nitrogenous source than corn steep liquor, and therefore not readily available to microbes. This is used as a ingredient for fermentation media in the production of streptomycin.

Pharmamedia: Pharmamedia is a clean, yellow, finely ground powder prepared from the embryo of cotton seed. It contains 56% w/w protein, 24% carbohydrate, 5% oil, and 5% ash. Ash, in turn, contains calcium, iron, chloride, phosphorous and sulfate. It is used as an ingredient for production media (e.g. Tetracycline production).

Distillers Solubles: In the manufacture of alcohol using grain or maize, alcohol is distilled from fermented grain or maize, leaving the residue (containing 6 to 8% w/v total solids). The suspended solids from the residue are eliminated by screening, leaving the effluent. Thereafter effluent is subjected to concentration, until the solid content reaches 35% w/v giving ‘evaporator syrup’. This syrup is then drum dried to yield ‘distillers solubles’. This may be used as a production medium component, since it supplies nitrogen, together with many accessory food factors (e.g. vitamin B complex).

Other natural organic nitrogenous materials are ground nut meal, fish meal, bacto peptone, Difco yeast extract.

Precursors & Inducers:

Certain substances, which generally improves the yield or quality of the product. These substances are known as the precursors. These precursors are incorporated without any major change in to the molecule of the fermentation product.

Ex: Phenyl acetic acid and Cobalt are being added for penicillin G and Vitamin B_{12}.

Corn steep liquor yields various pencillins but addition of phenyl acetic acid determines the penicillin G production.

Proteases for various proteins, α-amylases for starch, cellualse for cellulose, pectinase for pectin and penicillin acylase for phenyl acetic acid.

Repressors: The substances which are being employed for the repression of the industrial cultures are known as repressors.

Example 1. Media allowing restricted growth provides high product yield as major portion of carbon and other components of the medium are shunted to product formation rather than to growth.

2. Nitrogen sources such as soyabean meal and praline for streptomycin; production is probably due to their slow utilization, thus avoiding nitrogen metabolite repression.

3. *Aspergillus niger* for gluconic acid production is first grown on a medium that supports a rich growth as well as product formation, then the mycelium is separated and placed in a fresh medium high in carbon substrate (sugar) but lacking combined nitrogen so that additional growth cannot occur.

Antifoams: Antifoams are surface active agents, reducing the surface tension in the foams and destabilizing protein films by hydrophobic bridges between two surface, displacement of absorbed protein and rapid spreading on the surface of the film.

An ideal antifoam should have a fast action on the existing foam but should not be metabolized by the microorganisms. It should be cheap, heat sterilizable, non toxic, long acting and active at low concentrations.

Examples: Stearyl alcohol and octyl decanol, esters, fatty acids, cotton seed oil, linseed oil, castor oil, cod liver oil etc, silicones, sulphonates.

If the oxygen transfer rate is severely affected by antifoam addition, then mechanical foam breakers may have to be considered as a possible alternative.

Lecture 13

Types of fermentations - Batch fermentation - Feed batch fermentation - Sub batch fermentation - Continuous fermentation - Multiple fermentations - Multistage fermentations

Before discussing the different types of fermentors individually, it is essential to consider the general features of an ideal industrial fermentor. They are:

(1) Material used in the fabrication of a fermentor should be strong enough to withstand the interior pressures due to the fermentation medium. It should be resistant to corrosion, and free from any toxic effect for the microbial culture.

The nature of the construction material to be selected depends upon the particular fermentation that is to be carried out in the vessel. For example, wooden tanks are used in carrying out some fermentations, such as those producing ale and lactic acid. On the other hand, materials in the fabrication of or lining of fermentation vessels include copper, stainless steel, iron and glass. Stainless stool or glass is not used for lining of fermentors except in certain cases, since they are costly materials.

(2) A fermentor should permit easy control of contaminating microbes.

(3) The fermentation tank should be provided with the inoculation point for aseptic transfer of inoculum.

(4) In case of aerobic submerged fermentations, the tank should be equipped with the aerating device.

(5) A fermentor should be provided with a stirring device for the uniform distribution of air, nutrients and microbes.

(6) Baffles should be present to avoid vortex formation.

(7) The fermentation vessel should be equipped with a sampling valve for withdrawing a sample for different laboratory tests.

(8) There should be a provision for controlling temperature and pH of the fermentation medium.

(9) There should be a facility for the intermittent addition of an antifoam agent. Similarly, there should be provision for feeding certain medium components during the progress of fermentation (e.g. in penicillin fermentation).

(10) A drain at the bottom is essential for the removal of the completed fermentation broth for further processing.

(11) A man-hole should be provided at the top for access inside the fermentor for different purposes (e.g. repairing and thorough cleaning between fermentation runs).

Important types of industrial fermentors are stirred, baffled and aerated tanks types. These are provided with systems of temperature, pH and foam formation control. Most of the commercial fermentors have mechanical agitation system which is design to get proper mixing and oxygen transfer, while a few are designed in such a way that it depends on very high gas inputs without mechanical agitation. The design of a fermentor (bioreactor) also depends on the type of environment required for an organism, its structure and the product. Therefore, following are the major types of industrial fermentors: (1) mechanically agitated fermentor (2) Non-mechanically agitated fermentor and (3) Non-agitated fermentor.

1. Batch Fermentor
2. Deep jet Fermentor
3. Cyclone column fermentor
4. Air lift Bioreactor and Modified Air lift Bioreactors
5. Novel Seesaw Bioreactor
6. Stirred tank Fermentor (CSTF)

Batch Fermentor

Batch fermentors are used to carry out micro-biological processes on a batch basis. There are a number of steps involved. These are associated with the development of micro-organisms from a stock culture, and include agar slope and shake-flask stages. Thereafter, this is followed by 'seed' and production stages.

Size: Batch fermentors are available with varying capacities. The capacity of the tank may range from a few hundred to several thousand gallons. The capacity of the fermentor is usually stated on the basis of the total volume capacity of the same. Thus, small laboratory fermentors, pilot-plant fermentors and larger or production fermentors

may be available. Small laboratory fermentors are in the size range of 1 to 2litres with a maximum upto 12 to 15 litres. Pilot plant fermentors have a total volume of 25 to 100 gallons upto 2000 gallons total volume. Larger fermentors range from 5,000 or 10,000 gallons total volume to approximately 1, 00,000 gallons. Still larger sized fermentors are rarely employed. These are spherical (Horton spheres) with a size range of 2, 50,000 to 5, 00,000 gallons total capacity.

Actually, the working volume in a ferment or is always less than that of the total volume. In other words, a 'head space' is left at the top of the fermentor above the aqueous medium. The reason for keeping a head space is to allow aeration, splashing and foaming of the aqueous medium. This head space usually occupies a fifth to a quarter or more of the volume of the fermentor.

pH Control: pH Control is achieved by acid or alkali addition, which is controlled by an auto-titrator. The auto-titrator, in turn, is connected to a pH probe.

Temperature Control: Temperature control is achieved by a water jacket around the vessel. This is often supplemented by the use of internal coils, in order to provide sufficient heat-transfer surface.

Agitation: The agitating device consists of a strong and straight shaft to which impellors are fitted. An impeller, in turn, consists of a circular disc to which blades are fitted with bolts. Different types of blades are available, and are used according to the requirements. The shaft passes through a bearing in the lid of the fermentation tank. It is rotated with the help of an electric motor mounted externally at the top of the tank. Usually, the speed of the agitator is varied with the help of adjustable pulleys and belts connected with the motor. In some cases, where the agitator is directly driven, impeller action is varied by the use of different types of impeller blades. Recently, the impeller of small fermentors is moved by a magnetic coupling to a motor mounted externally at the bottom of the tank. Moreover, the height of the impeller blades above the bottom of the fermentor is adjustable according to one's desire. The liquid medium is thrown up towards the walls of the fermentor while rotating the impeller blades at a high speed. This results in the formation of a vortex which is eliminated, usually, by four equally spaced baffles attached to the walls of the fermentor.

Aeration: Usually, the aerating device consists of a pipe with minute holes, through which pressurised air escapes into the aqueous medium in the form of tiny air bubbles. This aeration device is called a sparger. The size of the holes in a sparger ranges from 1/64 to 1/32 of an inch or larger. Holes smaller than this require too high an air pressure for economical bubble formation. One should always remember that the smaller the air bubbles, the greater is the bubble surface area. Subsequently, it is more likely that the oxygen of that air would pass across the bubble boundary and dissolve in the aqueous medium. However, small air bubbles require higher air pressure for their formation through the fine holes. It is desirable to adjust the size of the air bubbles to give the greatest possible aeration without greatly increasing the overall cost of the fermentation process. The reason for this is that sterile air is a costly item for large scale fermentation.

When working with mycelium-forming microbial fermentations with heavy mycelia and involving long fermentation times, clogging problems can become serious. This renders aeration completely ineffective. Such a problem has been encountered by some workers in the laboratory in the case of cellulose fermentation to produce cellulases with an LKB Ultroferm laboratory fermentor. The second reason for the clogging of the orifices is occasional power failures, when the cellulose particles settle down by gravity to plug the orifices.

These problems have become by a simple modification by changing the direction of the orifices by a full 1800 .

As a result, the orifices now face downwards, and fermentations run successfully. Admittedly, the efficiency of aeration is reduced, because now the air bubbles do not impinge on the impeller directly. However, the important problem of clogging has been solved.

The application of the latter sparger arrangement is more likely in the small laboratory fermentors where relatively narrow orifices are used.

There are various ways for introducing air into the fermentation vessel. The principal modes of injecting air are:

1. Impeller air injection; air is fed to the impeller by means of a hollow drive shaft, and injected into the medium through holes in the impeller.

2. Two-phase injection; a two-phase mixture of air and nutrient medium is fed into the fermentor in the form of a foam or suspension..

3. air-lift fermentor; air is used to circulate the' contents of the fermentor, either through tubes external to the vessel or internally by using a draft" tube.

4. Sparger air injection; the most widely used fermentor is the stirred, baffled, upright cylinder with air sparger.

It is almost a universal practice to introduce the air through a sparger placed close to the underside of the impeller. Single jet, multi-jet and porous spargers are used, though the differences as regards oxygen transfer are minimal, at least in stirred vessels. This is because the shear rates in the region of the impeller are generally sufficient to break up a jet of air into bubbles. This, in turn, provides an extensive area for oxygen transfer. Porous spargers are unpopular owing to their tendency to be blocked by micro-organisms. But they are still a feature of the waste-water treatment industry, where they are employed in processes that are not otherwise agitated.

Time: The time required for a batch fermentation varies from hours to weeks, depending up on the conversion being attempted, and the conditions used (Rhodes and Fletcher, 1966). Throughout this time, contamination must be avoided and the vessel contents must be agitated and their temperature controlled.

Designing: The design problem associated with a deep tank fermentor lies in the specification of the size of the vessel, the process time, the initial reactant (substrate) concentrations required, the holdup volume of microbial mass per unit volume of a fermentor, of micro-organisms, the power and aeration requirements and the area of heat-transfer surface.

The process of 'sludge digestion' in the waste-water treatment industry is an example of a batch fermentation.

Continuous Stirred Tank fermentor:

A continuous stirred-tank fermentor (CSTF) need not be basically different from the batch fermentor, except insofar as feed and overflow devices are added. The fundamental difference lies in the fact that the contents of the vessel are at a steady

state. In other words, contents of the vessel no longer vary with time, this applies to the hold-up of micro-organisms and the concentration of the components of the medium in the fermentor.

Steady-state conditions can be achieved by operation on either 'chemostatic' or 'turbidostatic' principles. The former involves adjusting of the flow-rate to the fermentor to an appropriate and constant value and allowing the micro-organism, substrate and biochemical product concentrations to attain their natural levels. The 'Turbidostat' requires an experimental determination of the turbidity (i.e. an indirect measurement of the microbial concentration). This is then used to control the flow-rate. Both these methods have been employed in practice, though the former is obviously the simpler from every view point. Consequently, it is the one used in operations other than those on a laboratory scale.

The immediate consequence of the steady state condition is, that for satisfactory economic operation the environmental conditions selected for the fermentor have to lead to acceptable yields of microbial and biochemical products. This single feature demands depth of knowledge of the physiological and biochemical factors (which influence the microbial activity) far in excess of that demanded by batch fermentations (where detailed empirical procedures for fermentor operation are usually developed over a period of years). Once a small yield of product has been achieved in a batch fermentation, this can be enhanced on a developmental basis. In contrast, for continuous operation detailed knowledge is demanded of all the relationships between the rate of reaction and the operating variables. A further complication arises in that 'natural' media, media obtained from complex natural sources, in particular farm products or other fermentations e.g. molasses and corn steep-liquor, as opposed to specially prepared chemical substrate solutions, are the norms m the fermentation industries. Therefore, the determination of the effects of all the concentration variables is scarcely feasible. The use of such media results in a further restraint, since the concentrations, on a relative basis, are fixed and this makes it difficult to consider a variety of inlet 'compositions as part of the design exercise. Detailed application of batch

data to a continuous system that presents significant difficulties, and these are scarcely made easier by the physiological and biochemical changes known to occur in the micro-organisms themselves during the period of a batch fermentation.

The most successful continuous systems to date have been those employing yeasts and bacteria, in which the desired products are the cells or primary metabolites, compounds that form the chemical 'inventory' of a microbe, (e.g. enzymes and amino acids), or some product clearly associated with growth or energy producing mechanisms (e.g. the production of alcohol). A comment made by Righelato and Elsworth (1970) is worth noting, if they wish to adopt continuous methods, industrial researchers must be prepared to carry out their own empirical research as they have done with batch cultures, perhaps developing both new strains and media'. In fact, there appears to be no reason why any fermentation cannot be achieved in a continuous process, provided that the economic justification can be made, and the will to achieve it exists. The most widely used continuous process based on the CSTF is the activated sludge process (Ainsworth, 1970). It is used in the waste water treatment industry

In continuous processing the autocatalytic (a reaction in which one of the products of the reaction increases the overall rate of a reaction) nature of microbiological reactions takes on a further significance. This is because the presence of one of the products, additional micro-organisms, enhances the overall rate of reaction. In the absence of micro-organisms no reaction can take place. Therefore, it is essential to retain at least a portion within the fermentor. It follows that if the flow rate is raised to a high value, then all the micro-organisms will be swept from the fermentor, and the conversion will cease. This phenomenon is commonly known as 'Wash-out'. Obviously, if micro-organisms are fed to the fermentor simultaneously with the substrate feed, the problems associated with wash-out are abated, and the reaction proceeds normally. Operation under such conditions requires a continuous flow of micro-organisms identical with those within the fermentor. The logical source of these is the effluent stream, as this contains micro-organisms and nutrients in the same biochemical and physiological conditions, as those within the fermentor. The effluent may be passed through either a centrifuge or a

sedimentation tank. This operation produces a concentrated microbial suspension for recycling to the fermentor .

Both these methods have been successfully applied under industrial conditions for the production of beer. The latter method is a normal feature of' the activated sludge process. A constant inflow of microorganisms obviates the wash-out problem. It also results in increased productivity from the higher total hold-up of micro-organisms within the fermentor.

CSTF are normally operated at high substrate conversions. Therefore, the substrate concentration in the effluent stream is quite low, and loss of substrate is not a major feature. However, if a number of fermentors are used in a train, it means that an additional substrate has to be added to each of them. A further characteristic of a fermentor train IS that, if micro-organisms occur in the first fermentor washout cannot occur, in spite of the fact that the flow-rate through succeeding fermentors increases in the direction of the flow as a result of substrate addition. Thus, the productivity of each succeeding fermentor is greater than the previous one. Such a train of fermentors has been used successfully for the production of bakers' yeast.

Multistage systems for microbial reactions are particularly useful when, in the equivalent batch process, it is necessary to vary the environmental conditions over the course of the reaction. In a multistage system this is equivalent to providing different environmental conditions at the various stages.

An alternative form of CSTF has been described by Kitai and Yamagata (1970). This fermentor consists basically of a tube divided into equal sections by horizontal perforated plates. The fermentor is intended for use under aerobic conditions with the medium and air fed concurrently to the base of the column. In operation the gas liquid ratio can be adjusted, so that a pool of liquid may be maintained on each plate with an air space above. It is claimed that the liquid volumes are completely mixed, that the transport of liquid up the column is by entrainment, and that no back flow occurs. Under these conditions the fermentor performs exactly like a CSTF with each section or stage acting as one of a train.

Tubular Fermentor

The microbial mass in a fermentor can exist in two geometric states:
(i) Freely suspended in the fermentor, or
(ii) Adhering to the surfaces of the fermentor.

The state that contributes most significantly to the performance of a given fermentor configuration largely depends upon the fraction of the total microbial mass present in a particular state.

Freely suspended micro-organisms often occur in groupings or flocks with a characteristic size. This size is of many orders of magnitude greater than a single microbe. Factors affecting the pr04ucction of a particular floc size are largely unknown. The enhancement of the flocculation reaction can be achieved by flocculating agents (e.g. aluminium and calcium chloride). Reversely, the floc size can also be decreased (e.g. by increased shear or surface active agents). At present, it is reasonable~ to suggest that all microbes can occur either as individual cells or in multicells groups. However, some microbes have a tendency to form larger flocs than others under apparently the same environmental conditions.

Although the basic mechanism of adhesion is not fully understood (Munson, 1970), microbes ‘attached’ to an 'inert' surface, develop film) a microbial film that covers the surface. The ‘thickness' of this film presumably depends upon similar factors to those contributing to floc formation.

The performance of the batch fermentor aid the CSTF is dominated by the suspended microbes. They can, and often do, occur as large flocs. In contrast to this, the performance of the tubular fermentors can largely be dominated by either the flocs or the films, depending upon the details of the configuration and the flow rates used.

In a tubular fermentor containing microbial flocs, individual microbes are exposed to different environments. This may be an advantage in that the conditions can be compared with the batch fermentation, if the; distance along the tubular fermentor is related to the time under batch conditions. These fermentors are analogous, since the conditions at a particular position in a tubular fermentor are characterized by the time the fluid passing that position has been in the fermentor.

In the case of the CSTF, the substrate concentration in the outflow from a CSTF is generally very small (i.e. the bioconversion is large). Because of the progressive fall in substrate concentration along the length of a tubular fermentor the outlet concentration is likely to be of measurable proportions, resulting in some loss of the raw material.
Processes with micro-organism adhering to a support surface are chiefly used in the waste water treatment industry under the name, 'Trickling Filter'.

Fluidised Bed Fermentor:

This is a characteristic of beds of regular particles suspended in an up flowing liquid stream.
If an additional gas phase is involved, there is a tendency for the particles in the bed to become less evenly distributed.
There are two important features of the beds of mixed particle sizes:
(i) The increase in porosity from the bottom to the top of the bed, and
(ii) The decreased particle movement when compared with beds containing particles of constant size.
Since porosity or voidage is a measure of the local free space within a bed, it also represents a measure of the microbial hold-up when expressed as wet volume per unit bed volume. Thus, a variation in microbial hold-up is to be expected within a 'fiuidised bed' fermentor on fiuidisation, the smaller particles rise relative to the larger particles, and produce a situation where the smaller particles are at the top and the larger particles are at the bottom of the bed. As the smaller particles have the lowest settling velocity, the bed arranges itself, so that the smaller particles may be in the region of the highest porosity and the lowest linear velocity.

The tower fermentor (developed for the continuous production of beer) is based upon these general principles (Ault et al, 1969). In this process yeast flocs are maintained in suspension by the upward movement of the nutrient medium. Moreover, any entrained particles are returned by means of a sedimentation device at the top of the tower. Essentially, the fermentor consists of a vertical cylinder with an aspect ratio (length to diameter) of approximately 10:1

At the top of the tower a separator is provided to induce the gas bubbles produced by th,e reaction, to coalesce and escape from the liquid phase. Within the separator there is a quiescent lone, free of the rising gas, so that the yeast may settle and return to the main body of the tower, and clear beer can be removed.

A flocculent yeast (i.e. a yeast capable of achieving relatively large floc sizes) is essential for an alcoholic fermentation in a PBP at acceptable flow rates, otherwise a large proportion of the yeast would be washed out. As a result of this, an insufficient yeast concentration is maintained. A mean yeast concentration of 25 % by weight (expressed as centrifuged wet weight) is typical with values as high as 30-35% by weight at the bottom of the tower, and as low as 5-10% by weight at the top. A significant feature of the tower is the progressive and continuous fall in the specific gravity of the nutrient medium between the bottom and the top of the tower. There is an initial rapid fall at the bottom of the tower. It is followed by a slower fall over the middle and the top of the tower. This gradual fall in the specific gravity is due to the fermentation of the sugars.

The Deep-jet Fermentor

The necessary mechanical power input is achieved with a pump to circulate the liquid medium from the fermentor through a gas entrainer nozzle and back to the fermentor. The injector and the ejector are the gas entrainer nozzles that are used. In an injector a jet of medium is surrounded by a jet of compressed air. The gas from the outlet enters the large tube with a nozzle velocity of 5 to 100 ms^{-l} and expands in the tube to form large bubbles which are dispersed by the share of the water jet. In an ejector, the liquid jet enters into a large converging diverging nozzle and entrains the gas around the jet. The gas which is sucked into the converging-diverging jet is dispersed in that zone.

The Cyclone Column Fermentor

Dawson (1974) developed the cyclone column particularly for the growth of filamentous culture. Important characteristics of cyclone column are good gas exchange, lack of foaming and limited wall growth. The culture liquid is pumped from the bottom to the top through a closed loop. The descending liquid runs down the wall in a relatively thin film. Air was fed along with nutrients near the base of the column while the exhaust gases escape from the top.

There are several problems in this kind of fermentor. The agitator shaft rotates and there is a micro clearance between the shaft and stuffing box. This is an important factor in its design. It cannot be neglected and overtime it increases due frictions. To ensure aseptic condition in the fermentor special arrangements are needed to avoid contamination and which needs additional expenses. It has high shearing and tearing effect of impeller blades. Mechanically agitated stirred tank reactor is not suitable for mycelial fungal strains or for tissue cultures. Since it is made of a very large capacity vessels, therefore, it is difficult to achieve uniform mixing. To overcome this problem, various other types of bioreactors have been developed. They are: (1) air-lift bioreactors, (2) air-lift fermentor with external loop, (3) continuous fermentation stirred tank, (4) cyclone type bioreactor, (5) deep jet fermentor, (6) fluidized bed bioreactor, (7) hollow fiber bioreactor, (8) ICI Air-lift fermentor with internal loop, (9) membrane recycle bioreactor, (10) modified air lift bioreactor, with both internal and external loop type (TIT Kharagpur), (11) packed bed bioreactor.

Air lift Bioreactor and Modified air lift Bioreactors

This kind of fermenter works on principle of an air lift pump. It is of two kinds: (1) internal loop type (2) external loop type. The reactor's volume is determined by its capacity, kinetic data, and specific growth rate of the organism used. The rate of airflow of the reactor depends on the volumetric mass transfer coefficient in the reactor system. Most of the industries are using bioreactor developed by IITTKharagpur. It is a uniform

cylindrical cross type and has an internal loop or external loop riser configuration, diverging converging. The external loop riser configuration is adjustable and the change in the configuration improves the O_2 transfer rate vis-a-vis mass transfer coefficient for a particular rate of airflow. This helps provide required particular dissolved O_2 concentration for specific microbial system. This reactor reduces the operating cost for pumping air through the bioreactor.

Novel Seesaw Bioreactor

Foam formation occurs while aeration and agitation in the bioreactor, which disturbs the physical system and blocking the flow passages and the cell mass attaches to the foam, thereby reduces the cell density in the broth. Cells in contact with bubbles may be destroyed. In such cases aeration is to be done from outside the reactor. This increases the cost of the system. Novel Seesaw bioreactor is design in such a way to consider certain inherent problems. In this bioreactor aeration of broth is done by surface diffusion which avoids bubbling and reduces the cause of foam formation. Shear effect is also negligible.

The reactor has minimum two bioreactor chambers and two gas chambers. Each bioreactor chamber is connected to gas chamber, which controls the gas pressure inside the chamber by means of valve and blower. The gas is alternately forced into each bioreactor chamber. Gas forces the liquid levels inside the bioreactor for alternate up and down ward movement. The system is a closed circuit. Inflow of gas is from outside, and supply is controlled. It has the provision to remove excess of gas. The bioreactor has additional solid surfaces for improving the aeration efficiency. The reactor has also controlling device to control pH, dissolved 0_2, CO_2

Lecture 14

Regulation of gene expression - Induction- repression - LAC operon- The operon model -promoter - operator- Structural genes - Lac Z gene - Lac Y gene - Lac A gene - regulation oflac operon - negative regulation - positive regulation

REGULATION OF GENE EXPRESSION

All organisms contain numerous genes in their genomes to synthesize various proteins. Expression of these proteins varies at various stages of activities depending on their needs. Certain proteins which may be synthesized during embryonic development are not synthesized in adult hood, similarly certain proteins which may be synthesized during adulthood are not synthesized during embryonic development. But every cell will have same set of genes in the genome, even though their expression is different. Therefore, it is necessary that there should exist a definite mechanism, so that specific genes will only be expressed, with their switch on or switch off.

In bacteria expression of several genes depends on the nature of the environment. Absence of a normal growth substrate of a bacteria may force the cell to develop the ability to utilize alternate substrate. This is by the expression of a new set of enzymes required to utilize the alternate substrate. This process of expression is called induction. On the other hand a compound normally synthesized by the bacteria, if provided to it, bacteria may stop synthesizing enzymes of this particular metabolism. This is called repression.

The regulatory mechanisms of gene expression can operate either at the stage of transcription or at translation. What is the need of regulation of gene expression? The reason is that gene expression is expensive process. It needs lot of energy to produce RNA and protein. The organism will not be efficient if all the RNA and protein are unnecessarily synthesized.

LAC OPERON:

In bacteria a gene control serves mainly to allow a single cell to adjust to change sin nutrients of media. So its growth and division can be regulated and maintained. Among the different bacteria E.coli gene expression regulation is well studied. The source of carbon and energy of E.coli can be obtained from either glucose or other sugars such as the disaccharide lactose. When E.coli cells are grown in a medium containing glucose, the activity of lactose metabolizing enzymes is called repression. But when the

same cells are transferred to a medium containing lactose, without glucose, the activity of lactose metabolizing enzymes is more needed by the cell. The process of increasing the expression of lactose metabolizing enzymes is called induction.

The operon model : Jacob and Monod proposed the operon hypothesis to explain regulation of galactoside in E.coli. An operon is an expression unit including structural genes and control elements I DNA recognized by the reguolator gene products. The lax operon includes three structural genes, which are preceeded by regulating unit. The three structural genes of lac operon are lac Z, lac Y and , lac A. The lac Z gene codes for B – galactosidase. This enzyme hydrolyses the disaccharide lactose ot its monosaccharides glucose and galactose.

Lac Y gene codes for the enzyme lactose permease. It transports lactose into the cell. The lac A gene codes for the enzyme thiogalactosid etransacetylase. Function of this enzyme is not well understood.

The three structural genes are transcribed together to produce one mRNA, called polycistronic mRNA. Their expression strats under the control of single regulation unit from a single promoter region. This region is the site of binding of RNA polymerase under different regulators like cAMP –CRP complex. The promoter site is followed by an operator site. The control rate of gene expression through regulation of RNA polymerase binding. The inducer (lac I gene product) binds to the operator site. Lac I gene codes for a repressor protein which is inducer of negative regulation. The repressor binds as a tetramer to the operator site inducing negative regulation.

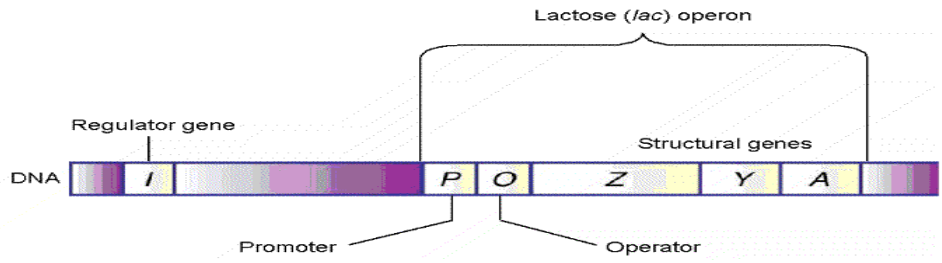


Fig. Lac operon representation.

REGULATION OF LAC OPOERON :

The three genes lac Z, lax Y and lac A are transcripted as a single polycistronic mRNA. But the final concentration of three proteins-galactose : permease : transacetylase is 100:50:20. How this difference in concentrations is maintained in a cell even though they are transcrpted together ?. This is by the gene expression control mechanisms.

The regulation of lac operon transcription is by dual control with both negative regulation and positive regulation.

A. Negative regulation:

In the absence of lactose in the medium the lac operon genes are repressed. The negative regulation was dixcovered by Jacob and Monod using mutants. Mutation in the operator region or lac I gene results in constitutive synthesis of lac operon products. The lac I gene codes for a lac repressor. This tetramerizes to bind to the operator. The operator to which the repressor binds most tightly is the transcription start site. The lac I gene is transcribed from its own promoter (P) independent of the lac operon genes. To repress the operon lac repressor bind to the operator, by blocking transcription initiation.

Binding of lac repressor to operator reduces the rate of transcription ignition 1000 folds. Even in the repressed state the E.coli cells has a few molecules of galactosidase and galactoside permease. These may be synthesized rarely, when the repressor transiently dissociates from operator. This minimal level of transcription called constitutive level is essential to operon regulation.

When the E.coli cells are provided with a lactose containing medium, the lac operon is induced. Inducer molecules bind the repressor and changes repressor's conformation, weakening the bond between operator and repressor. This results in dissociation of repressor from operator

Positive regulation:

When E.coli is transferred to a medium containing lactose as the source of carbon and energy the lac operon is induced for its expression. An inducer molecule binds to the repressor on the operator site, making it to be released from operator. The inducer of lac operon is not lactose itself, but an isomer of lactose called allolactose. After entry into the E.coli cell lactose is converted to allolactose by the few ß – glactosidase existing in the cells. Release of the lac repressor by allolactose allows the lac operon

genes to be expressed and leaves to 1000 fold increase in the rate of expression of lac genes.

ß – galactosides that are structurally related to lactose are inducers of the lac operon. One very effective and nonmetabolizable inducer of the lac operon is isopropylthiogalactoside. (IPTG).

What happens to the lac operon expression, when both glucose and lactose aree present in the medium ? Then the lac operon is regulated by another regulatory mechanism called catabolite repression. This regulatory mechanism prevents the expression of genes of lac operon, ara (arabinose) operon and other sugar catabolic operons in the presence of glucose. The catabolite repression is by cAMP (cyclic adenosine monophosphate) and a protein called cAMP receptor protein (CRP or catabolite gene activator protein, CAP). This CRP is a dimer with binding sites for both DNA and cAMP.

When glucose is absent, CRP cAMP complex binds to a site near the promoter consensus regions and stimulates transcription of lac genes with 50 fold increase in expression.

The effect of glucose is mediated by cAMP. In the presence of glucose the cAMP synthesizing enzyme, adentlate cyclase is inhibited. So the CRP – cAMP complex is not formed and thereby decreasing the expression of lac operon. In the absence of glucose cAMP synthesis isnot affected and CRP – cAMP complex binds to promoter site inducing lac operon expression.

Strong induction of the lac operon requires both lactose and a lowered concentration of glucose. Binding of CRP – cAMP complex is at, a little upstream of RNA polymerase binding site. The RNA polymerase and promoter interaction is increased by CRP – cAMP complex. However, RNA polymerase can proceed to transcript the lac genes, only if the operator is free and not occupied by repressor. Therefore, lac operon is switched on only when lactose present and glucose is absent. So both positive and negative regulation of lac operon are essential for regulating its expression.

Lecture 15

Gene Manipulation tools - Different enzymes used - Helicases - premases - topoisomerases- RNA polymerase I,II, - Holoenzyme - sigma factor - DNA Gyrase - DNA polymerase - I, II,III

RNA Polymerase

(1) RNA polymerase required all the four ribonucleoside 5' triphosphpate (ATP,GTP,CTP, and UTP) as precursors of the nucleotide units of RNA.
(2) Required Mg^{2+} as cofactor.
(3) The purified enzyme also contains zinc as an essential part of its active group or active site.
(4) RNA polymerase elongates on RNA strand by adding ribonucleotide units to the 3' OH end of the RNA chain and this builds RNA chains in the 5' → 3' direction.
(5) RNA polymerase requires preformed DNA for activity.
(6) RNA polymerase does not require a primer strand

Prokaryotic RNA polymerase.

Chamberlin & Berg isolated RNA polymerase from E.coli in prokaryotes. A single RNA polymerase enzyme controls all the different types of cellular RNA (mRNA ,rRNA, tRNA).

RNA polymerase in it's active form (Holoenzyme) sediments at about 15S and contains 5 different polypeptide chains. The holoenzyme consists of a core enzyme and a sigma (σ) factor. In E.coli and Anacystis (blue green alga) the core enzyme consists of four polypeptide chains ß, ß, α and α (ß ß $\alpha_1\alpha_2$). In Bacillus subtilis there are seven polypeptides chain ßßαα SW_1W_2 in the core enzyme. The chain is present twice. Covalent bonds join the various chains. The aggregation results from the formation of secondary bonds. The enzyme has a elongated shape. The attachment of to the other chains is not very firm so it is relatively easy to isolate. The factor just recognizes the start signal on the DNA template and directs the binding of RNA polymerase to the promoter region.

Core enzyme RNA polymerase

When E.coli were infected with Bacteriophage T_4, another RNA polymerase factor called T_4 factor was isolated from E.coli cells. This factor stimulates the core enzyme to transcribe T_4 DNA and not the bacterial DNA.

Another factor called the Psi. factor isolated from E.coli cells causes the transcription of DNA coding for rRNA. The P si factor requires the entire RNA polymerase or the holoenzyme for it's action.

Components in the holoenzyme of RNA polymerase in E.coli and Bacillus subtilis (Prokaryotes)

Sub unit	Mol.wt. E.coli	B.Subtilis	Function
ß'	1,60,000	1,60,000	Binding of RNA polymerase to DNA
ß	1,50,000	1,50,000	Binding with σ factor
d (ø)	40,000	45,000	Promoter recognition
S	--	21,000	?
W_1	--	11,000	Not essential for normal activity
W_2	--	9,500	Not essential for normal activity
σ	90,000	55,000	Recognition of start signals (Initiation)

Eukaryotic RNA Polymerases :

In eukaryotic cells different forms of DNA dependant RNA polymerase are responsible for the synthesis of cellular RNA.

There are three different RNA polymerases in all eukaryotic cells. They are RNA pol I,II,III. These 3 enzymes differ in structure, function and localization. These 3 enzymes differ in structure, function and localization. Like prokaryotic RNA polymerases these are large and complex enzymes. They contain about 10 subunits.

RNA pol I or class A RNA polymerases :

(i) They are present in Nucleolus
(ii) They can be further classified as A I (RNA pol I) and A II or (RNA polymerase I_B)
(iii) These enzymes are insensitive to Amanitin. They are not affected even by high cone of α Amanitin.
(iv) A I enzyme has been purified from calf thymus mouse myeloma and rat liver
(v) Main function is to synthesise rRNA. (5.8 S, 18 & 28 S)

Class B or RNA polymerases or RNA pol II

(1) They are present in nucleoplasm and chromatin
(2) They are further classified into B O (RNA pol II_0), BI (RNA pol.iii A) and B II (a+b) (RNA pol II B)
(3) These enzymes are sensitive to low concentrations of aminitin. These enzymes are affected by 10^{-9}. To 10^{-8} M concentration of Amanitin.
(4) These enzymes have been purified from Calf Thymus, Rat liver, Mouse myeloma and Chick liver.
(5) They help in the synthesis of hnRNA and therefore help in the synthesis of mRNA, because hn RNA is the precursor of m RNA.

Class C Polymerases or RNA pol. III

(1) They are present in Nucleoplasm, chromatin, Cytoplasm.
(2) They are further classified into C I, C II, C III a (RNA pol.III A) and C III b (RNA Pol III b (RNA pol III B).
(3) These enzymes are sensitive to high concentrations of amanitin. They affected at 10^{-5} to 10^{-4} M concentration of amanitin.
(4) They were isolated form mouse myeloma cells.
(5) They help in the synthesis of t-RNA and 5S RNA.

All the RNA polymerases requires a DNA template and ribonucleoside triphposphates (ATP,GTP,CTP,UTP).

DNA LIGASE

DNA ligases are those enzymes involved in DNA replication in prokaryotes and eukaryotes. They heop in sealing the nick or gap between DNA sequences. They catalyse the formation of a phospho0diester bond between a 3' OH group at the end of one DNA strand and 5' phosphate group at the end of another strand.

(1) **Prokaryotic DNA ligase** :

DNA ligases from E.coli and B. subtilis used NAD a coenzyme and energy source for the synthesis of the phosphodiester bond. DNA ligases induced by phage T_4 and T_7 infection use ATP.

Properties :

(i) The E.coli enzyme is 75 kd.
(ii) It is a single polypeptide chain.
(iii) It has elongated shape and hence shows low sedimentation coefficient of 3.9 S.
(iv) The enzyme is destroyed by proteolytic cleavage by Trypsin.
(v) The proteolytic product which is much smaller in size than the intact enzyme can still form the first stage covalent enzyme AMP intermediate normally but it cannot transfer the AMP group to DNA for phosphodiester bond formation.
(vi) Phage T_4 encoded ligase is 60 KD.

It is a single polypeptide and it's sedimentation coefficient is 3.5 S and has elongated structure. The enzyme shows marked inhibition by salt being virtually inert at 0.2 M. KCI spermine inhibits by raising the K_m for DNA substrate.

Enzyme Mechanism :

Ligase utilizes the energy of NAD^+ or ATP to form phosphate diester bond between Nucleic acid chains. The reaction occurs in 3 steps.

(i) Formation of an enzyme intermediate by transfer of the adenylate group of the coenzyme to the NH_2 of a lysine residue in the enzyme. The other product is FMN when NAD is cofactor or PPI when ATP is the cofactor.
(ii) Adenylyl activation of the 5' – phosphoryl terminus of the DNA by transfer of the adenylyl group from the enzyme.
(iii) Phosphodiester bond formation by attack of the 3' OH terminus of the DNA on the activated 5' phosphoryl group with the release of free AMP.

(2) **Eukaryotic DNA ligases** :

They are activated by adenylyl transferrase by the same mechanism as prokaryotic ligases. Unlike prokaryotes, the mammalian cells have at least two antigenically distinct ligases named I & II. Ligase I is about 200 KB but may be converted to an active form only half that size on incubation in crude extracts during isolation.

DNA ligase II is 25 KD and less stable than ligase I is a dominant form in proliferating cells such as regenerating rat liver and mitogenically stimulated lymphocytes.

Ligase II is the principle enzyme in resting cells.

Functions of DNA ligase :

1. It helps to repair single strand nicks in duplex DNA
2. It helps to link the neds of linear DNA duplexes to yield circles.
3. To join segments of DNA in the process of recombination which occurs during genetic transformation, transduction and Lysogenisation in bacteria and in Meiosis (crossing over) in eukaryotes.
4. To cooperate with DNA polymerase in replication of DNA.

DNA polymerase

In 1955 Arthur Kornberg and his collegues discovered an enzyme called DNA polymerase (now called DNA polymerase I) from E.coli. They found that this enzyme was responsible of polymerising the deoxyribonucleoside triphosphates on DNA template to form a new complementary strand. Briefly they considered DNA polymerase I as the DNA synthesis enzyme. Later on DNA polymerase II and DNA polymerase III were also discovered and were also found to have DNA synthesizing (or polymerising) activity.

DNA Polymerase I :

(1) It is a single polypeptide chain having about 1000 amino acid residues.
(2) It has a molecular weight of 109 K.D.
(3) It is a metallo enzyme having one or more atoms of zinc per chain at the active site.
(4) It has a non essential SH group and also has an intra chain disulphide linkage.
(5) The shape is roughly spherical.
(6) The enzyme is attached at regular intervals to the DNA chains.
(7) Enzyme Trypsin splits the polypeptide chain of polymerase I into a large fragment (M.W.75000) and a small fragment (M.W = 36000).
(8) The large fragment has ploymerse activity as well as 3' →5' exonuclease activity while smaller fragment has 5' → 3' exonuclease activity.

(9) Thus DNA polymerase I in prokaryotes have three activities i.e., 5' ⟶ 3' polymerizastion activity, 3' ⟶ 5' exonuclease activity and 5' ⟶ 3' exonuclease activity.

Activities of DNA polymerase I :

1. 5' ⟶ 3' polymerization activity

The synthesis of a new DNA chain from its nucleotide pieces, in 5' ⟶ 3' direction is called polymerization. DNA pol I polymerises nucleotides at a rate of about 1000 molecules perminute at 37^0 C in E.coli though DNA pol. I has 5' ⟶ 3' polymerization activity. It is not the essential enzyme for DNA replication or polymerization. The main polymerization enzyme is DNA pol III. Even in the absence of DNA pol I, DNA replications occurs. It is now believed that DNA pol I can synthesise only short segments of DNA and thus takes part mainly in repair activity.

Mechanism of 5' 3' ~~pol~~ymerization activity:

$D(NMP)_n + dNTP \leftrightarrow d(NMP)_{n+1} + PPi$

Growing strand lengthened DNA

The overall reaction for the addition of a single nucleotide residue is shown as above. The reaction requires Mg^{2+} and does not occur in the absence of preformed DNA.

DNA pol I catalyses the addition of mononucleotide units to the free 3' OH end of DNA chain, the direction of DNA syntheisis is thus 5' 3'. The fundamental reaction is a nucleophilic attach by the 3' OH group of the nucleotide at the 3' end of growing strand on the 5'- α – phosphorus of the incoming deoxy nucleotide 5' triphosphate. Inorganic phosphate is released in the reaction.

A phosphodiester linkage is formed between the 3' OH group of the incoming nucleotide. The energy required to form the new phosphodiester linkage is provided by pyprophosphate cleavage from dNTP. The free energy charge for the above reaction is negative. The net free energy charge for the above reaction is A 60 = -28 KJ/mol.

Requirements for the above reaction:

(1) **Primer site** : It has a short (~ 100 nucleotides) complementary segment of RNA on which the newly synthesized DNA strand grows i.e.,. The primer has the 3' OH group to which new nucleotides can be added.

(2) **A primer terminus site** : It is tip pregion of the primer, which has a terminal 3' OH group.

(3) **A template site** : All DNA polymerases require a template strand. The polymerization reaction is guided by a template strand according to base-pairing rules of Watson and Crick. For eg. If a guanine is present in template strand then a cytosine is added in the new strand, if a thymine is present then adenine is added in the new strand and so on. This arrangement proves semiconservative replication of DNA.

(4) A triphosphate site is where an incoming nucleoside triphosphate matches a complementary nucleotide on the DNA template and is bound to the 3' OH position of the primer.

II. **3' ⟶ 5' exonuclease activity of DNA polymerase I**

DNA polymerase I helps in removal of nucleotides (hydrolysis of DNA) in 3' 5' direction. Since nucleotides are removed sepuentially in a direction opposite to that of polymerisatio, DNA pol I is also called 3' → 5' exonuclease. DNA pol I acts as a "proof reader" and it "edits" mis matched nucleotides at the primer terminus i.e., it hydrolysis nucleotides. Only at the 3' OH terminus. In other words nucleotides which are removed by DNA polymerase I should have a free 3' OH terminus.

Thus errors made during polymerization are corrected by DNA pol I. It thus acts in repair process and helps in resynthesis of the correct nucleotide pairs. The 3' ⟶ 5' exonuclease activity of DNA pol I is present in its larger fragment.

III. **5' → 3' exonuclease activity of DNA pol I**

DNA pol I can also remove nucleotides in the 5' ⟶ 3' direction. This is called the 5' → 3' exonuclease activity of DNA pol I. This is called the 5'→ 3' exonuclease activity of DNA pol I. This activity helps in the removal of Thymine dimmers formed in DNA after exposure of DNA to U.V.light. When DNA strand is exposed to U.V.light a covalent linkage is formed between adjacent pyrimidines (eg T = T). This causes the formation of pyrimidine dimmers or (Thymine dimmers). Such dimer formation is very harmful because it blocks replication and hence should be removed.

Another important role of 5' → 3' exonuclease activity of DNA pol. I is to remove the short RNA primer during replication of DNA, one strand of daughter DNA is continuously synthesized in 5' → 3' direction. Another strand of daughter DNA is discontinuously synthesized. i.e., synthesis of DNA takes place in small fragments (okazaki fragments). To each of these fragments a primer RNA segment is attached. The 5' →3' exonuclease activity of DNA polymerase I is responsible for removing this RNA primer segments and then helps in filling the gap by deoxyribonucleotides. When DNA pol I

→

moves forward it cuts off ribonucleotides in front and adds deoxyribo nucleotides from behind. This 5' → 3' exonuclease activity of DNA pol I is found in it's smaller fragments.

TYPES OF DNA POLYMERASES :

In E.coli, three types of DNA polymerases have been identified.

1. **DNA polymerase -1** : It is also known as Kornberg's enzyme or Pol-I. It acts as 5' – 3' polymerase. It also acts as 5' -3' – exonuclease.
2. **DNA polymerase –II** : It is also called as pol-II. It acts as 5' – 3' – polymerase and also acts as 3' – 5' –exonuclease. However, it lacks 5'-3'-exonuclease activity.
3. **DNA-polymerase –III :** It is also called as Pol-III. It acts as a polymerase and 3' – 5' – exonuclease. It lacks 5'-3' – exonuclease activity.

In E.coli, DNA polymerase III is the main enzyme which is needed for DNA replication. DNA polymerase-I is needed for DNA repair and for exonuclease activity.

II. **DNA polymerases of oEukaryotic cells** :

In the Eukaryotic cells such as hepatocytes, fibroblastes, thymocytes, five other DNA polymerases are found.

(1) **DNA polymerase α (alpha**) : This enzyme is found in the cell nucleus and forms the main enzyme responsible for nuclear DNA replication. Its concentration rises during synthetic phase in the cell cycle. The enzyme does not possess nuclease activity.

(2) **DNA polymerase β (beta)** : This enzyme also exists in the cell nucleus. Its concentration rises during synthetic phase of cell cycle. It brings about nuclear DNA synthesis only by repair mechanism.

(3) **DNA polymerase γ(gamma)** : This polymerase exists in the mitochondria and catalyses replication of the mitochondrial DNA.

(4) **DNA polymerase δ (delta) :** DNA polymerase delta is responsible for replication on the leading strand of DNA and it possesses proof reading activity.

(5) **DNA polymerase ε (epsilon) :** This DNA polymerase is involved in DNA synthesis on the lagging strand and it also possesses proof reading capacity

Lecture 16

Restriction enzymes - restriction endonucleases - Nomenclature of enzymes - three lettercode - Molecular scissors - nature of cutting ends - Blunt ends - Sticky ends - Isoschizomers- Recognition sites - Star activity - neoisoschizomers - Cleavage - mechanism of action -uses of restriction enzymes

RESTRICTION ENDONUCLEASES

The term restriction endonuclease was coined by Lederberg and Meselson (1964) to describe the nuclease enzymes that destroy (restrict) any foreign DNA entering the host cell. These enzymes have been classified into three different types viz. Type I, Type II and Type III. In gene manipulation technology, restriction endonucleases are popularly called molecular knives or molecular scissors or molecular scalpels.

Naming of Restriction Endonucleases :

About 350 restriction endonucleases have been isolated from than 200 bacterial strains and large number of these enzymes has called for a uniform nomenclature. A system based on the proposals of Smith and Nathans (1973) has been followed for the most part. This includes :

(1) Each enzyme is named by a three-letter code.
(2) The first letter of this code is derived from the first epithet of the genus name.
(3) The second and the third letters are from the species name.
(4) This is followed by the strain number. If a particular strain has more than one restriction enzyme, these will be identified by Roman numerals as I,II,III etc.
(5) A general name endonuclease R may be added. For example,EcoRI enzyme is derived from E.coli carrying antibiotic resistance plasmid (RI).

Nature of the Cut Ends : The restriction enzymes cut DNA molecules by cleavage. This action occurs in two styles, (i) blunt end style and (II) sticky or cohesive end style.

(i) **Blunt –end Style** : Certain restriction endonucleases (e.g. Hae III, Sma I) make cuts across both strands of DNA at the same position. The utility of this style during the joining of DNA fragments is that any pair of ends may be joined together irrespective of sequence. This is especially useful for those

researchers who are interested to join two defined sequences without introducing any additional material between them.

(ii) **Sticky or Cohesive end Style** : In this style the restriction enzymes (e.g. Eco RI, Bam HI, Hind III) make single-strand cut. Some nucleotide pairs apart in the opposite strands of DNA, and so generate fragments with protruding termini (ends). These DNA fragments can associate by hydrogen bonding between overlapping termini, or the fragments can circularize by intramolecular reaction, and for this reason the fragments are said to have sticky-or cohesive ends. Shows the diagrammatic representation of sticky – or cohesive-end cut by the restriction enzyme.

Isoschizomers : Isoschizomers are restriction enzymes which are isolated from different organisms but recognize identical base sequences in the DNA. For example, Asp 718 and Kpn I have identical recognition.

G T T A C C

C C A T G G

Source of Asp 718 is Achromobacter species 718, Source of Kpn I is Klebsiella pneumonia OK 8. Some pairs of isoschizomers cut their target at different place (e.g. Sma I, Xam I).

(1) Restriction enzymes are endonucleases and recognize, cleave the foreign DNA at the same specific sequence of bases that in the host cell DNA has been modified by methylation.
(2) Restriction enzymes are present only in prokaryotes.
(3) Restrictionf enzymes make double strand breaks within recongnition site, which has two fold symmetry.
(4) Three major types of Restriction enzymes are known.

(i) Types I Restriction endonuclease recognize of specific nucleoside sequence and double strand cut in the molecule some where near by, but without any specificity as to the nucleotide that is cut.
(ii) Type II restriction enzymes recognize a specific sequence and make a double strand cut at a fixed point within that sequence have symmetric recognition site.
(iii) Type III Restriction enzymes recognize a sp sequence and make a double strand cut at a fixed point. Some number of nucleotide pairs to the side of that sequence have symmetric recognition site.

(5) **Nomenclature of Restriction enzymes** :

(i) Each Restriction enzymes has a symbol and is named after the bacterium from which it is isolated.

Eg: ECORI.E = Genera; CO = Species ; RI = Strain designation

(6) **Recognition site** :

(i) Each Restriction enzymes recognize a unique sequence of o4 or 6 groups of nucleotide and some even recognize group of 18 nucleotides.

(ii) Recognized sequence may be continuous or interrupted

(iii) Most recognize sites have predominant bases with a exception of Aha 111 tt AAAAAA TTT

(iv) If Restriction enzymes recognize G nucleotide pairs the propability is G^4 = 250 or in other words once in few hundred nucleotides and for 6 G^6 = Once in few thousand bp for eg: Sv 40 genome I has 5243 bp. ECOR, recog site is GAATTC. This occurs only once in SV 40, for Hac 111 the recog site in GGCC and occurs 18 times.

(v) Recognition seg present in one DNA can be absent in other DNA. Phage T_7 does not have recog site for ECOR

Enzyme	Source	Recog seg And CI site	No. of recog Seg perch
			Phageλ SV 40
Ecor R 1	E coil ky 13	GAA TTC CTT AAG	5 1
Hind 11	Hemphilus intwenzar R_4	GTPy PoAC CAPo	34 7
Hind 111	"	AAG CTT TTC GAA	6 6
Hpa 11	Hemophilus pariant wenzac	GTT AAC CAA TTG	11 4
Hpa 11	"	CC GG GG CC	50 1
Hac 111	Hemophiolus aegytus	GG CC CC GG	50 18

Bam H	Sacilluar amyloto-queracin	GGD TCC CCT AGG	

Gemone size 8140 = 5243 sp λ = 48.502nn

7) Cleavage :

Type –II Restriction enzymes has site of cleavage within the Recognition site cut can be (i) blunt ends or flash ends. (ii) staggered or sticky ends

(i) Blunt end

Hpal

GTT ACC GTT + AAC ⟶

CAA TTG CAA TTG

(ii) Staggered end

Bam N1

CGA TCC G + GATTCG ⟶

CAA TTG CCTAG

1. **Mechanism of Action :**
   (i) Restriction enzymes are dimmers and bind to both the strands at recognition site.
   (ii) Polypeptide subunits of ECOR, wraps itself on to the double helix making complement attachment at the major grove.
   (iii) The central kink unwinds on binding of ECOR.
   (iv) Unwinding is by 25^0 and bends DNA 12^0. The process result in enlargement of major grove.
2. **Uses of Restriction Enzyme** :

In recombinant DNA technology studies enzymes are used

(1) For sequence analysis of DNA
(2) For Restriction mapping
(3) For making chimeric DNA
(4) Shot gun experiment
(5) Chromosome walking
(6) Site specific mutagenesis

(7) RFLP

Lecture 17

Recombinant DNA technology - Selection of DNA - Selection of suitable vehicle - cloningvector - Selection of suitable enzyme - Introduction of rDNA - Screening of host cells -selection based on antibiotic resistance - complementation of nutritional defects - assay ofbiological activity - immunochemical method- colony hybridization - Expression of targetgene in the host cell

RECOMBINANT DNA TECHNOLOGY

Manipulating the genetic material is a natural process leading to the development of diverse cells in the evolution. Laboratory manipulation technologies of nucleic acids emerged in 1970s. Using these technologies, varieties of DNA molecules are constructed with nucleic acids from different sources. The products of these manipulation technologies are called recombinant DNA molecules. The technology used for manipulation of DNA and transfer of DNA from one cell to other cell is termed as recombinant DNA technology. Application of this technology is called genetic engineering.

The recombinant DNA experiment always starts with isolation or synthesis of DNA. Isolation of a gene from a large chromosome requires methods for cutting DNA and of ligation DNA fragments to another DNA (vector). All these steps requires the enzymes like restriction endonucleases, polymerases and ligases. The vectors like viral vectors, plasmid vectors with autonomous replicatin capacity are used to insert gene of interest. Recombinant ENA (rDNA) is now introduced into host cells, like D.coli, by using methods like micro injection, electroporation. Etc., Then use the identifying techniques to identify the cells containing DNA of interest. They can be propagated to form clones, called cloning. Advances in the techniques of rDNA technology are revolutionizing medicine, agriculture, and other industries.

ENZYMES USED IN r DNA TECHNOLOGY :

Many enzymes are used in r DNA technology as an important biological tool. They are restriction endonucleases, ligases, reverse transcriptases, DNA polymerases, alkaline phosphatases, topoisomerases, etc. Now we see about.

(A) Restriction endonucleases, (B) Polymerases (C) Ligases.

A. Restriction endonucleases : Nucleases degrade DNA molecules by hydrolyzing the link between the nucleotides (phosphodiester bond). Nuclesases are of two kinds.

(i) Exonucleases : The nucleases hydrolyzing the nucleotides one at a time from the end of a DNA molecule. Ex: Bal 31

(ii) Endonucleases : The nucleases hydrolyzing the internal phosphosphodiester bonds with in a DNA molecule.

Ex: Eco RI

Special group of endonucleases called restriction endonucleases cleave double-starnded DNA only at a limited number of specific recognition sites. Depending on their specificity to cut DNA restriction endonucleases are of three main types

(i) Type I restriction endonucleases

(ii) Type II restriction endonucleases

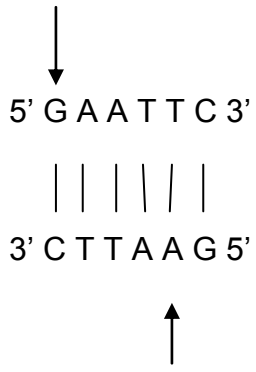
(iii) Type III restriction endonucleases

(i) Type I restriction endonucleases : These are large, multisubunit complexes having endonuclease and also methylase activities. They cut the DNA at about 1000 base pair away from their recognition sequence. It requires energy from ATP for catalysis.

(ii) Type II restriction enconucleases : These are simpler, require no ATP for their action and cleave the DNA with in the recognition sequence itself. The widely used type of restriction endonucleases in rDNA technology are of type II. They were first isolated by Hamilton Smith and their significance was first demonstrated by Daniel Nathans.

The recognition sequence of restriction endonucleases are usually four to six base pairs length and palindromic. The sequences having rotational symmetry about an axis are called palindromes.

Ex: ECORI restriction site is



Nomenclature of restriction endonucleases : Because many restriction enconucleases are discovered and still some in progress a particular protocol is followed to name them. The first capital letter indicated the genus of organism from which it was isolated. This is followed by two small letters. These are first two letters of the species name. Roman numbers are used to designate the order of characterization of different restriction endonucleases from the same organism.

Ex : Eco RI

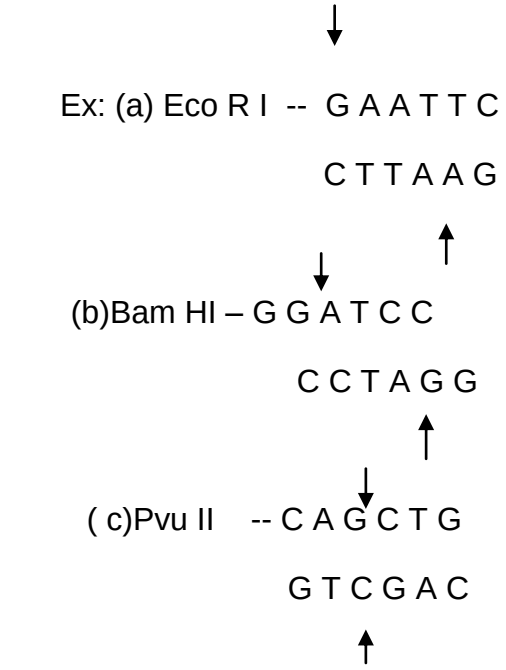
E : indicates the genus Escherichia

Co :indicates the species coli

R : indicates the strain

I : indicates it is first characterized restriction endonuclease from E.coli strain R.

Catalytic mechanism of type II restriction endonucleases : The significant feature of restriction endonucleases is that each enzyme hydrolyse the DNA at a specific recognition sequence.

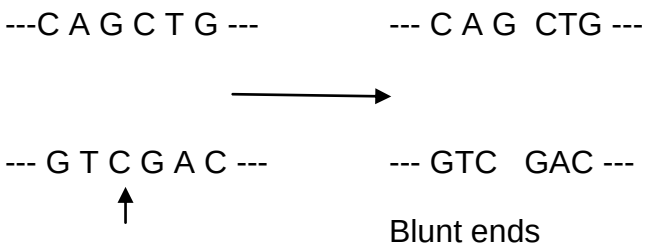


↑ Indicates the site of restriction by the endonuclease.

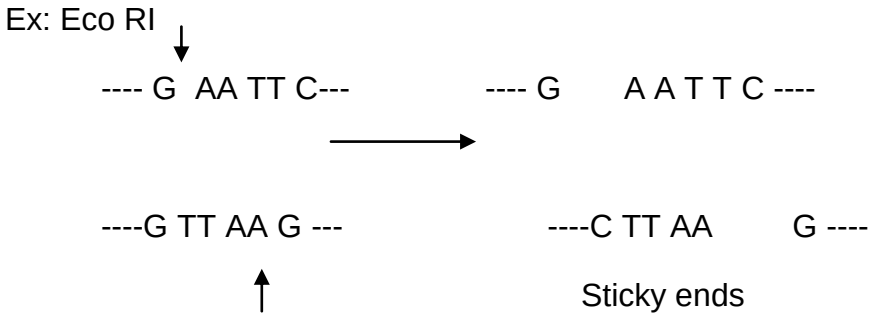
Some restriction endonucleases make a simple double stranded cut in the middle of recognition site. This is called blunt or flush end cut.

Ex : Pvu II

↓



However, a large number of other restriction endonucleases cut DNA in a slightly different way. In this the two strands of DNA are not cut at exactly the same position resulting in the short single stranded over hangs at each end. This type of ends formed are called stickly or cohesive ends.



(iii) Type III restriction endonucleases : These are like type I endonuclease with large, multisubunit complexes containing both the endonuclease and methylase activities. Type III restriction endonucleases cleave the DNA at about 25 base pairs fro the recognition sequences. It requires ATP for catalysis.

B. Polymerases : Polymerases are the enzymes which synthesize the nucleotide polymers by joining them with phosphodiester linkage. Depending on the type of nucleotide polymerases are two types.
   1. DNApolymerases
   2. RNA polymerases

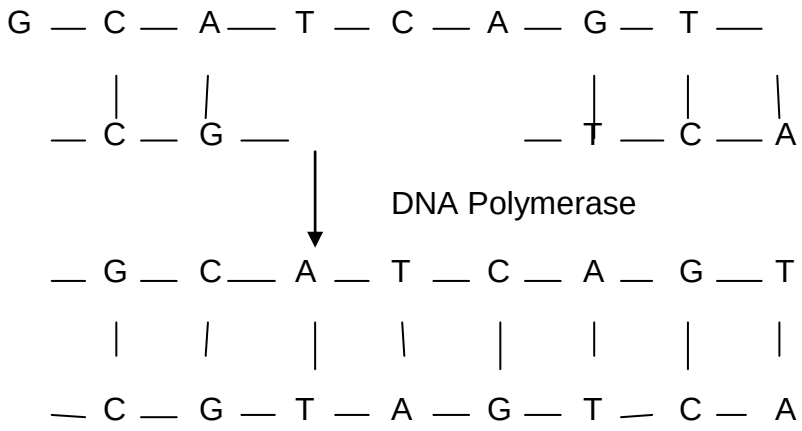
1. DNA polymerases : The enzyme synthesizing DNA strands from deoxyribonucleotides are called DNA polymerases. These require a template, to direct the polymerization and a primer to provide initial binding site for polymerizing

nucleotides. All these are discussed in detail in the unit-III. Based on the template DNA polymerases are of two types.

(i) DNA dependent DNA polymerases
(ii) RNA dependent DNA polymerases

(i) DNA dependent DNA polymerases : These polymerases copy DNA from a DNA strand (template). They synthesize a single new strand that is complementary to the template strand adding nucleotides to the 3 end of the primer attached to template. Synthesis proceeds as more bses are added to the primer. DNA polymerases are of E.coli are five types-type I, typeII, typeIII, type IV and type V. With in these type I, is used in rDNA technology. This enzyme attaches to a short single stranded region (At nick site) in a double-stranded DNA molecule, and then synthesizes a completely new strand, (polymerases) degrading the existing strand (nuclease) as it proceeds. This process is just similar to joining of okazaki fragments of lagging strand replication. The DNA polymerase here acts both as polymerase and nuclease. The segment of DNA polymerase carrying out polymerization is called klenow fragment. This alone also used in rDNA technology.

Fragtment. This alone also used in rDNA technology.



(ii) RNA dependent DNA polymerase (reverse transcriptase) :

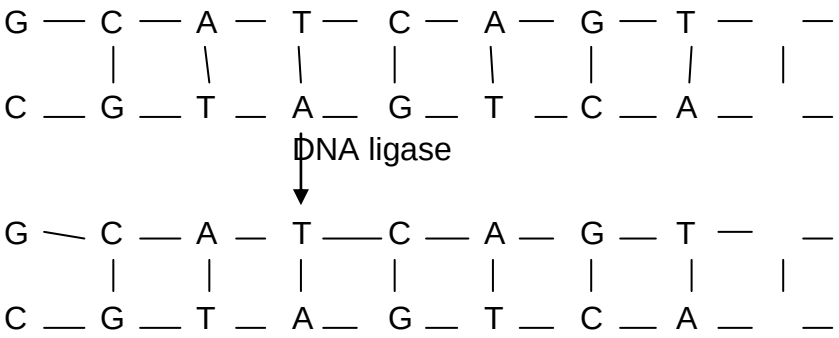
Reverse transcriptase is used to synthesize the copy of DNA by using mRNA as a template. The synthesized DNA is called copy DNA or complementary DNA (cDNA). RNA containing viruses will code for this specific enzyme. The RNA of viruses after entry into host is copied to its DNA form by reverse transcriptase to integrate into the host genetic material. Then DNA is used for synthesis of many copies of viral RNA in a

cell. For example HIV (Human Immuno deficiency Virus), the agent of AIDS containing the enzyme reverse transcriptase.

The ability of this enzyme to synthesize a DNA strand complementary to an RNA template is central to the technique called cDNA technology.

2. RNA polymerases : The enzymes synthesizing RNA molecule using DNA sequence are called RNA polymerases. These are discussed in detail in the unit-III (see transcription.) RNA polymerases require a special sequence of bases on the DNA template, called promoter. Beginning site of transcription is signaled by the promoters.

(C) DNA ligases : After selecting and isolating gene of interest and the vector into which it is going to be recombinated, the next step of rDNA technology is joining them together. The process of joining DNA fragments is called ligation. The enzymes catalyzing ligatin are called ligases. All the living cells produce DNA ligase, as they are required during replication and repair. But the enzymes used in rDNA technology are usually from T_4 bacteriophage infected E.coli. The T_4 DNA ligase is very significant in repairing any discontinuities in one of the strands of double stranded DNA. It joins the two adjacent nucteotides, where a phosphodiester bond is missing.



The purified ligases repair single strand discontinuities or join together individual DNA molecules or the two ends of same molecules also. The sticky end DNA strands can be easily ligated in rDNA technology. But the blunt end ligation takes the help of linkers or adaptors or ligated by homopolymer tailing.

VECTORS IN r DNA TECHNOLOGY :

The DNA molecules carrying a gene of interest, when inserted into it, providing specific features like autononus replication, high expression rate are called vectors. These are said to be cloning vehicles.

Features of vectors :

1. They must be able to replicate with in the host cell.
2. Numerous copies of rDNA molecules should be produced.
3. It must be heritable to next generation cells.
4. It must be smaller than 10 kilobases.

Based on the nature and source vectors are classified into o

(A) Plasimid vectors
(B) Viral vectors
(C) Other vectors

A. Plasmid vectors :

Plasmids are extrachromosomal, self replicating, closed, circular, double stranded DNA molecules. These plasmids are present in bacteria and yeast. Plasmids possess one origin of replication site and one or more genes. These genes code for charactreristics like antibiotic resistance. These are used as selecting markers of plasmids. The very significant characteristics of plasmids making them suitable for genetic engineering are small size and high copy number. The size of plasmids reanges from 1 to 250 kilobases. The number of plasmids in a single bacterial cell is called copy number. The copy number of plasmids varies from one to 50. Plasmids of less si9ze and more copy number are suitable as cloning vectors.

Features of plasmid vectors :

1. Autonomous replicatin
2. Less size
3. High copy number
4. Presence of markers like ampicillin resistance
5. Heritable to next generations

Construction of recombinant plasmid :

Construction of recombinant plasmid requires joining of foreign DNA (DNA of interest) into plasmid DNA. In this process first the plasmid is cut using restriction endonuclease. Then the plasmid will be opened. The restriction cuts of plasmid and foreign DNA must generate complementary ends, so that they can be easily ligated. The plasmid DNA then base pairs with foreign DNA, Joining the two molecules together. Finally DNA ligase seals the gaps.

Plasmid vector PBR 322 is widely used plasmid vector. It is constructed form the plasmids of E.coli, PBR 318 and PBR 320. The origin site of PBR 322 is col E1 plasmid derived. It possesses genes conferring resistance to ampicillin and tetracycline.

Recognition sites of 20 restriction endonucleases are present in this. So this is suitablef for a wide variety of foreign DNA.

B. Viral vectors :

The plasmid vectors are suitable for small size foreign DNA molecules transfer only. So for large DNA molecules viral vectors are developed. Bacteriophages like λ phage are commonly used as viral vectors because of their simple structure. Animal viruses like SV 40 and adenoviruses; and insect virus baculo viruses received most significant interest in using as vectors.

The genome of λ phage is a 48.5 kbp linear DNA molecule. It is packaged in the preteinaceons head. The middle one third of its genome is not essential for phage to infect host cells. So λ phage DNA has been used as vector for about 16 kbp foreign DNA molecules ; by inserting it in the unnecessary region. Then the recombinant DNA is packaged into the head followed by tail assembly. Bacteira infected with these recombinant phages can be transformed with foreign DNA and expressed

(C) Other vectors:

Apart from plasmid and viral vectors many other vectors are constructed.

(i) Shuttle vectors : Shuttle vectors are the plasmids capable of propagatin and transferring genes between two different organisms. One of which is typically a prokaryotes and the other a eukaryotes.
(ii) Cosmids : A cloning vector consisting of the λ cos site inserted into a plasmid and used to clone DNA fragments upto 40 Kb in size are called cosmids.
(iii) Phasmids : The vector constructed by insertion of plasmid into phage λ genome is called phasmid.
(iv) Artificial chromosomes : Bacterial artificial chromosomes (BACs) and yeast artificial chromosomes. (YACs) are developed to transform DNA molecules of about 2 megabase pairs in length. The artificial chromosomes include not only origin of replication but also a centromere and telomeres.

DNA LIBRARIES :

A particular gene of interest to recombinate is only a small part of any genome. So for isolating gene first a DNA library is constructed with fragments of all its genes. A DNA library is a set of aloned fragments that collectively represents the genes of a particular organism. The DNA fragment containing the gene of interest can be isolated from DNA libraries. This is similar to getting book of interest from your library. Depending on source DNA libraries are of two types.

(A) Genomic DNA libraries

(B) cDNA libraries

A. Genomic DNA libraries:

The DNA libraries produced by the genome of a cell are called genomic DNA libraries. In construction of these the genomic DNA is extracted, broken into fragments of reasonable size by a restriction endonuclease, for example Sau 3A1, and then inserted into a cloning vector to generate a population of recombinant vectors. The group of all the recombinant vectrs is called genomic library.

In the preparation of genomic libraries a number of restriction endonucleases are used. But at a time only one restriction endonuclease is used. These restriction endonucleases cut at different places of genome producing fragments of varying sizes. Some times a single gene can be fragmented.

In order to overcome this difficulty we have to use restriction endonucleases with short recognition sequences and genomic DNA need to obe only partially digested. Following these strategies long fragments without having any breaks on recognition sites in a gene can be easily obtained.

The cloning vector should also be cleaved with the same restriction endonuclease. The genomic fragments are then ligated with vector. The recombinated fragments packaged into bacteriophage particles or transformed into bacteria. All the genomic DNA will be represented in the library. For a probability level of 99% that all sequences are present in the library we may need 1, 500 cloned fragments for E.coli.

B. cDNA libraries : Complementary DNA (cDNA) are DNA molecules copied from mRNA templates. The DNA libraries. Constructed by synthesizing cDNA from purified cellular mRNA are called cDNA libraries. The enzyme reverse transcriptase catalyses the synthesis of cDNA from mRNA. This results in DNA-RNA hybrid form. The mRNA is removed and complementary DNA strand is synthesized to get double helical DNA.

Because most eukaryotic mRNAs carry 3' poly-(A) tails, mRNA can be selectively isolated from celolular RNA by using oligo (dT) –cellulose chromatography. Then to the isolated mRNA oligo (dT) chains are annealed to poly (A) tails. These oligo (dT) serves as primers for reverse transcriptase to carry out DNA synthesis. DNA polymerase then copies DNA to form double helical DNA. Using linker cDNA is cloned into a vector. cDNA library is prepared by constsructing cDNA recombinant vectors for all mRNA. cDNA can also be used as probes for screening genomic libraries. For cloning isolation of globin genes was done by firsts generating cDNA library from erythrocyte precursor cells. Half the mRNAs of these cells codes for globin proteins.

CLONING STRATEGIES :

After construction of recombinant or chimeric DNA it should be transformed into host cells. The host cell may be bacterial, animal or plant cell. Depending on the nature of the all transformation of DNA varies. Transformation is the process of intro0ducing free DNA into a cell.

(C) Genetic transformation of prokaryotes
(D) Genetic transformation of eukaryotes
C. Genetic transofrmatin of prokaryotes:
E.coli is the main host cell ion r DNA technology. The uptake of DNA by E.coli cell follows three main ways.

(i) Calcium chloride ($CaCl_2$) treatment

(ii) Electroporation

(iii) Calcium chloride treatment : The E. coli cells should be treated witnice-cold C_aCl_2 and then exposed to high temperatures (42^0C) for about 90 sec. Using this method 1 in 10^{-3} cells can be transformed.

It si assumed that the bacterial cell wall is broken down in localized regions,which allow the plasmid DNA to be transformed into bacterial cell

(iv) Electroporation : The bacterial cells are induced to uptake DNA by subjecting to a high strength electric field in the presence of DNA. This method of transformation of DNA is called electroporation.

For E.coli electroporation, the cells and DNA are placed in a chamber fitted with electrodes and a single pulse of 2.5 KV is administered for about 4.6 milliseconds. This treatment yields transformation efficiencies of 10^6 to 10^9 transformants per µg of DNA.

D. Genetic transformation of eukaryotes :
In animal cells DNA is injected into either the fertilized egg or into embryo stem cells. In plant cells protoplasts or embryonic tissues are used to inject DNA of interest.

In case of animal cells the term transformation is replaced by transfection and fertilized eggs or embryos are transfected with whold nuclei or whold chromosome or DNA fragments. This transfection of fertilized eggs is by using cytochalasin-B or by microinjection. Cultured animal cells are transfected by coprecipitation with calcium phosphate or electroporation or microinjection. Cultured animal cells are transfected by coprecipitation with calcium phosphate or electroporatin or microinjection.

In case of plant cells the gene transfer is by physical delivery or by using plasmids like Ti or Ri. The physical delivery is by using polyethylene glycol or by microinjection or by macroinjection or by electroporatin. Ti plasmid of Agrobacterium tumefaciena and Ri

plasmid of A. rhizogenes have been effectiveloy used in gene transformation in plant cells.

The purposes of gene transfer to plant or animal cells are.

(iv) To produce chemicals or pharmaceutical drugs or fertilizers.
(v) Study of structure and function of genes.
(vi) Production of transgenic animals and plants with improved qualities.

Protoplast transformation :

The organisms like B. subtilis can be transformed naturally. But natural transformation is suitable best to linear DNA, but not to circular Plasmids. So,a method known as protoplast transformation was developed. Protoplasts can be prepared by enzymatic removal of bacteriall cell wall. Enzymes like lysozyme in the presence of osmotic stabilizer susch as sucrose, generates protoplasts, exposing cytoplasmic membranes. Addition of DNA solution together with polyethyolene glycol (PEG), causes the cells to take up the DNA. Then the cells are allowed to regenerate on an osmotically stabilized medium. If the correct conditions are used, a very high proportion of the resultant colonies will be transformed. The same procedure is used in cas of eukaryotic organisms also.

The formation and regeneration of protoplasts is a key feature of an alli9ed procedure known as protoplast fusion, where the addition of PEG to a mixture of protoplasts of two different strains results in the formation of a fused cell that contains the complete genome of both parents. Protoplast fusion has been more widely used for eukaryotic cells.

Protoplast fusion has been used with bacteria, fungi, plant and animal cells. The animal cells do not have a cell wall, so the process of cell fusion is considerable simpler. Construction of hybridoma cells for monoclonal autibodies production is the best example for cell fusion

SELECTION OF RECOMBINANTS :

After construction of recombinant DNA and transformation into a host cell, it is necessary to find out the recombinants. This selection of ecombinants is by using.

(A) General methods
(B) Special methods.

(A) General methods :

The selection of recombinants can be generally done based on the vector features and manner of introducing DNA of interest into vector. The general methods are

(i) Selection based on antibiotic resistance

(ii) Selection based on protein activity.

(i) Selection basesd on antibiotic resistance : If a DNA fragment is introduced within antibiotic resistance coding region of vector, the recombinant cells are without having resistance for that antibiotic. When the transformed cells are plated on media containing the corresponding antibiotic, the antibiotic sensitive colonies signal the presence of foreign DNA in the vector.

(ii) Selection of recombinants based on protein activity : This method is suitable, when the foreign DNA is inserted into an enzyme coding region of vector. For example if a foreign DNA is inserted into the lac galactosidase coding region then the selection is based on the enzyme activity. The cells should be plated on agar plates containing the enzyme inducer and a substrate for example Isopropyl thiogalactosides is as inducer and X gal a chromogenic substrate of galactosidase. The recombinant cannot express the enzyme, so there is no colour development in colonies. So the white colonies indicates the presence of foreign DNA in the vector.

(B) Specific methods :

Specific recombinated cell can be selected by methods like

(i) Colony hybridization

(ii) Immunological assay

(i) Colony hybridization : The process of selection of recombinant with a probe is colony hybridization probe is a DNA sequence, used to detect the presence of complementary sequence by hybridization with a DNA sample. The hybridization depends on the formation of stable base pairs between the probe and the target sequence.

In the hybridization the target DNA is denatures and the single strands are irreversibly bound to a matrix of nitrocellulose. Then the labeled single strands of a DNA probe are incubated with the denatured, bound DNA sample. The probe will bind to target DNA at its complementary site. The hybridization can be detected by autoradiography.

(ii) Immunological assay : the method is applicable to identify the genes synthesizing particular polypeptide. This is a very powerful method, as it is based on the its suitable antibody for the polypeptide. The cells transformed can express the polypeptide, (antigen). Then using antibodies the expressed antigen can be identified by that recombinants can be selected.

Lecture 18

Plasmids - occurrence - extra chromosomal DNA - cloning vectors - Plasmids as vectors -Bacteriophages - Lambda phage vector - Bacteriophage M13 vectors - Cosmids as vectors- Eukaryotes as vectors - Plant viruses - pBR322 - Insertion vector- Replacement vector -Shuttle vectors - Phasmids - Artificial chromosomes - Bacterial artificial - yeast artificial(YAC)

PLASMIDS AND TRANSPOSONS

Plasmids and transposons are the accessory genetic elements present in both prokaryotes and eukaryotes.

PLASMIDS :

Plasmids are extrachromosomal hereditary molecules with circular double stranded DNA. These are found in bacteria and also in some eukaryotes like yeast in addition to main chromosome (DNA).

(A) **Properties of Plasmids** :

1. Plasmids are the genetic elements made up of small circular double stranded DNA.
2. They exists separately from main chromosome. Some plasmids can integrate with chromosome and exists along with it. This type of plasmids are called episomes. For example F factor of E.coli is an episome.
3. Plasmids replicate on their own without depending on chromosomal replication called autonomous replication. They have an origin of replication and replicated in ø mode as main chromosome.
4. In a single bacterial cell many copies of a single type plasmid cells are maintained, called copy number. The copy number varies from plasmid to plasmid. F factor is a low – copy number plasmid and col E 1 is a high – copy number plasmid. The Col E1 plasmid can be present at 50 to 100 copies per bacterial cell.

5. Plasmids along with the above mentioned common properties they code for specific features of cell like bacterial conjugation, antibiotic resistance.

(B) **Types of Plasmids** :

Depending on the character carried by them to the bacterial cell plasmids are divided into three main types.

(1) Resistance plasmids(R plasmids)
(2) Conjugative plasmids (F or sex plasmids)
(3) Colcinogenic plasmids (col plasmids)

(1) **R Plasmids** :

R Plasmids were discovered in late fifties in antibiotic resistand strains of Shigella, isolated from dysentery patients. These plasmids exhibit resistance to one or more antibiotics like ampicillin (Amp^R), streptomycin (Sm^R), tetracycline (Tet^R) and to drugs like sulfonamides. One of the better known R plasmids is R 100. So named because of 100 kilobasepairs in it. It has molecular weight of 6.5×10^6. The R100 plasmids code for resistance to streptomycin, tetracycline and chloramphenicol. The antitiotic resistance coding plasmids can be transferred from one type of bacteria to other. For example from Shigella to E.Coli and vice versa. The mode of transfer is some what like bacterial conjugation mediated by F factor. But the transfer is less efficient. When an R plasmid is introduced into a F factor, the activity of F factor is suppressed although the R factor's transmissibility is not affected. The R factors are transferred from normal flora to pathogenic organisms under natural conditions. Under wide spread and indiscriminate usage conditions of antimicrobial agents the further spread of plasmids is fetched.

(2) **F plasmids** :

This is discussed in detail later in unit –IV. The bacteria having F plasmid (F^+) comes in contact with the bacteria without F plasmid (F^-) and transfer the F factor from $F^+$to F^-. This transfer results in conversion of F^- to F^+. A copy of F factor retains in F^+ and another complementary copy of it is transferred to F^- . This typ0e of transfer of DNA is called conjugation. Sometimes the F factor along with it transfers a segment of bacterial chromosome also if it is inserted in the chromosome the. F factor can exists freely as a separate plasmid or sometimes as integrated factor with main chromosome also.

(3) **Col plasmids** :

The plasmids which provide the ability to synthesize bactericidal proteins are called col plasmids. These bactericidal proteins are produced by bacteria and kill bacteria other than those produced them. Some col plasmids also exists as episomes.

Col plasmids are transmitted from one bacteria to another. But not as R factors, the transfer of some col plasmids is independent of F factors with exceptions like col E1 plasmid, which require help of F factors for its transfer.

Colicins are of different types. The col E series of plasmids code for colicins which kill E. coli cells. The Col E plasmids are with molecular weight of about 5×10^6. Their copy number is 10 to 20 per cell. The colicin col E3 kills E.coli by inactivating the ribosomes. This inactivation is due to cleavage of one of ribosomal component 16S rRNA.

Plasmids as vectors :

Small plasmids present in many copies in a single cell. They also possess autonomous replicating sequences. So plasmids are ideal cloning vectors in recombinant DNA technology. The other features of plasmids, preferring them to be, as cloning vectors are easier to handle, readily isolated, resistant to damaging by shearing, specify resistance to antibiotics.

In the cloning circular plasmid genome is cut with a restriction enzyme with in one of the antibiotic resistance genes to generate a linear plasmid. Then the ends of the plasmid are joined with the foreign DNA to form a chimeric plasmid.

$P^{BR\ 322}$: The best example of plasmid, which are used in cloning is P^{BR322}. In this p stands for plasmids, BR stands for the creators of specific plasmid Bolivar and Rodrignes. It is a multicopy plasmid. It contains 4.3 Kbp. The plasmid codes for tgwo antibiotics resistance ampicillin (amp^r) and tetracycline (tet^r). It has an origin of replication, unique restriction sites for many restriction endonucleases are present in P^{BR322}.

TRANSPOSONS :

The mobile DNA sequences, which can be transposed into a new site in the genome are called transposons. The process by which these sequences are copied and inserted into a new site in the genome is called transposition. Transposons were first studied in maize by Barbara McClintock pin 1950 in her studies on the genetics of maize. She was awarded the Noble Prize in physiology or medicine for her studies. The term transposons was first used by R.W. Hedges and A.E.Jacob in 1974.

A. **Transposons in prokaryotes** :

James Shapiro and Peter Starlinger visualized specific type of mutations affecting different genes in various bacterial strains. For example, the expression of related genes, involved in galactose metabolism in E.coli was repressed as a result of one of

this type of mutation is heritable, but it was found not to be caused by normal type of mutations. Instead of that a short specific DNA segment has been inserted into the bacterial chromosome at the beginning of the galactose gene cluster. When the inserted DNA segment is removed, the mutant can be reversed to wild type. The small segment of DNA causing these effects is called an Insertion sequences.

The transposons in prokaryotes are of two types.

(i) Simplest transposons.
(ii) Complex transposons.

(i) **Simplest transposons**:

The simplest transposons of bacteria are called insertion sequences (IS). They contain only the elements necessary for transposition. They are short, not exceeding 2000 bp. The first insertion sequence characterized in E.coli is ISI. It is about 800 bp long. Later IS2, IS3,IS4 and IS5 were discovered. The insertion sequences present in main chromosome and also in plasmids.

The nucleotide sequences of IS contain inverted terminal repeats. These are the sequences repeated on the two ends of IS running in opposite directions. The inverted repeats may be 18 to 40 base pairs length.

(ii) Complex transposons :

The insertion sequences replicate utilizing its requirements from host genome and proving nothing useful in return to host. But some transposons carrying genes, that are useful to host are called complex transposons. The most familiar genes carried by complex transposons are of antibiotic resistance coding.

B. **Transposons in Eukaryotes**:

Eukaryotic transposons base on their size and nature of terminal repeats divided into four types

(i) With long terminal direct repeats
Ex: Ty in yeast and IAP in mice

(ii) With long terminal inverted repeats
Ex.TE in Drosophila

(iii) With short terminal inverted repeats

Ex.AC/DS in maize and Tam I in snapdragon

(iv) Without terminal repeats.
Ex: Alu in mammals.

In eukaryotes the transposition may be directly DNA to DNA transposition or through an RNA intermediate. Barbara Mc Clintock was awarded noble prize for her studies on AC – DS system transposons in maize.

In most Drosophila species 5-10 percent of the genome consists of about 40 distinct families of sequences having the features similar to transposable elements. Copia is the well studied transpons among all those. It contains about 5000 bp. A direct repeat of 267 bp is present at the ends. As insertion sequence copia also has an inverted repeat of 17 bp. Copia has been found in both integrated and free forms. Transposition of copia affects the activity of certain genes.

Lecture 19

Gene cloning- Production of identical cells - Isolation and purification of insert DNA - Isolation of vector DNA - Construction of recombined DNA - Introduction of recombined DNA into host cell - Identification and selection of cells containing cloned genes

GENE CLONING

A clone means an exact duplicate. Cloning is the creation of genetically identical cells or organisms from a single ancestral cell. Gene cloning refers to production of multiple copies of a desired gene. The gene cloning technique involves five main steps. They are

(1) Isolation and purification of insert DNA.
(2) Isolation of vector DNA.
(3) Construction of recombinant DNA.
(4) Introduction of recombinant DNA into host cells.
(5) Identification or selection of cells containing cloned genes.

1) **Isolation and purification of Insert DNA** : The gene of interest or insert DNA can be isolated either form bacterial cells or from plant cells or from animal cells. The insert DNA can be iolated by rupturing the bacterial cells by mechanical forces or by chemical treatment. Then proteins are separated from DNA by treating with 1:1 phenol, chloroform mixture. The DNA is taken out and estimated by measuring the absorbance at 260 nm. It the value of light absorbance at 260 nm is 1.0, it corresponds to 50.4g. of double stranded DNA per cell. The purity of the sample is tested using absorbance at 260 nm and 280 nm. If the ratio (A260/A280) is 1.8, it is taken as pure sample. Less than 1.8 indicates contamination with phenol or protein.
2) **Isolation of vector DNA**: The vector DNA is used to carry the insert DNA to the host cell for cloning. The vector DNA can be isolated by CaCI density centrifugation

method. Bacterial cells containing vector DNA that is plasmids are placed in test tubes containing differential concentrations of cscl. It is centrifuged at high speed. The DNA with different densities will form bands at different regions. Proteins will float and RNA will pellet out during this process. The band of plasmid DNA is collected from the tube and used in further experiment.

3) **Construction of recombinant DNA in vitro** : The next step in gene cloning is to insert the DNA sequence of interest into the plasmid to produce the recombinant DNA. For achieving this the insert DNA and plasmid DNA are cut with same type of restriction endonuclease enzymes. The restriction enzymes make two types of DNA fragments. Some enzymes make fragments with sticky ends and some enzymes make fragments with blunt ends. In gene cloning, it is desirable to have sticky or cohesive ends on the DNA molecules to be joined together. If blunt ended molecules are produced, they can be converted to sticky ends by introducing either linkers or adaptors or by homopolymer tailing.

Linker : Linkers are short pieces of double stranded DNA of known nucleotide sequences. The linkers have blunt ends, but has restriction site for a particular restriction endonuclease enzyme. The linkers are joined to either end of the blund ended DNA fragment. Now the DNA fragment with linkers and plasmid vector are treated with the same restriction endonuclease enzyme which produces staggered cuts. Then these two are joined together by ligase enzyme which producd recombinant DNA.

Adaptor : Adaptor is very similar to linkers, but it differs in having one sticky end, another blunt end. The blunt end of the adaptor is joined to the blunt end of the DNA sequence so that the DNA sequence now becomes sticky ended. The vector is treated with restriction enzyme to produce sticky ends and the DNA fragment and vector are joined to produce rDNA.

Homopolymer tailing : A homopolymer tailing involves tailing or attaching a homopolymer to the blunt ends of the DNA sequence. A homopolymer contains many subunits, which are all same. For example poly dA or poly dT. By using poly dA and poly dT, sticky ends can be produced in the presence of the enzyme terminal transferase.

This enzyme adds a series of nucleotides on to the 3' – OH termni of a double stranded DNA molecule. The DNA fragment is added with poly dA or poly dC and the vector is added with poly dT or poly dG tails. The sticky ends of plasmid DNA and the insert DNA are then joined by the enzyme DBA ligase. The joining of DNA fragments is called DNA ligation. This produce recombinant DNA.

4) **Introduction of recombinant DNA into host cells** : Introduction of recombinant DNA into the host cells is called transformation. This is done to produce many number of copies of insert DNA. Generally E.coli cells are used as host cells.

Plants and animals are also used as host cells. The rDNA is introduced into bacterial cells by treating bacterial cells with calcium chloride at low temperatures. It makes the cells competent to take up DNA molecules. After pretreatment with C_sCI_2 the rDNA is added. Then a mild heat shock is applied to the cells. Then bacterial cells readily take up rDNA. The efficiency of transformation depends on the size of the insert. The lesser the size of the insert, the more the efficiency.

The DNA is introduced into plant or animal cell by use of viral vectors or microinjection into eggs or calcium phosphate mediated DNA uptake or liposome mediated gene transfer or by electrophoration.

Lecture 20

Cell and Tissue culture- Animal cell culture- primary cell lines - secondary cell lines -Minimal essential medium - Amino acid assay medium - plant cell culture- Plasticity -Totipotency - MS medium - microprogation - callus formation - Organ development – tissue transformation - uses of tissue culture

Animal cell culture

A wider range of ingredients needed to support survival and proliferation or differentiation. *Invitro* animal cell cultivation requires a complex combination of nutrients, considering glucose and glutamine as main carbon, energy and nitrogen sources. Mineral salts, amino acids and vitamins are also required; other essential nutrients, like growth factors, hormones, and receptor and transport proteins are required in small quantities as well. The pH was maintained at 7.4 and often it includes pH indicator phenol red (red at 7.4, yellow at 6.5, purple at 7.8). A typical media may or may not comprise of serum. The latter is called a serum-free media. Some of the common sources of serum can be fetal bovine serum, equine serum, calf serum etc. both the types of media have their own set of advantages and disadvantages.

The culture media is prepared in such a way that it provides

1. The optimum conditions of factors like pH, osmotic pressure, etc.

2. It should contain chemical constituents which the cells or tissues are incapable of synthesizing. Generally the media is the mixture of inorganic salts and other nutrients capable of sustaining cells in culture such as amino acids, fatty acids, sugars, ions, trace elements, vitamins, cofactors, and ions. Glucose is added as energy source—its concentration varying depending on the requirement. Phenol Red is added as a pH indicator of the medium.

Basic Components in the Culture Media

Most animal cell culture media are generally having following 10 basic components and they are as follows:

1. Energy sources: Glucose, Fructose, Amino acids

2. Nitrogen sources: Amino acids

3. Vitamins: Generally water soluble vitamines B & C

4. Inorganic salts: Na+, K+, Ca2+, Mg2+

5. Fat and Fat soluble components: Fatty acids, cholesterols

6. Nucleic acid precursors

7. Antibiotics

8. Growth factors and hormones

9. pH and bufferig systems

10. Oxygen and CO2 concentration.

Animal cell culture media vary in their complexity but most contain:

Amino acids 0.1-0.2 mM

Vitamins ca. 1 µM

Salts NaCl 150 mM

KCl 4-6 mM

CaCl 1 mM

Glucose 5-10 mM

CULTURE MEDIA

A cell culture medium is composed of a number of ingredients and these ingredients vary from one culture medium to another. The nutrient media used for culture of animal cells and tissues must be able to support their survival as well as growth, i.e., must provide nutritional, hormonal and stromal factors

The various types of media used for tissue culture may be grouped into two broad categories:

1. Natural media

2. Artificial media.

The choice of medium depends mainly on the type of cells to be cultured (normal, immortalized or transformed), and the objective of culture (growth, survival, differentiation, production of desired proteins).Non transformed or normal cells (finite life span) and primary cultures from healthy tissues require defined quantities of proteins, growth factors and hormones even in the best media developed so far. But immortalized cells (spontaneously or by transfection with viral sequences) produce most of these factors, but may still need some of the growth factors present in the serum.

In contrast, transformed cells (autonomous growth control and malignant properties) synthesize their own growth factors; in fact, addition of growth factors may even be detrimental in such cases. Buteven these cultures may require factors like insulin, transferrin, silenite, lipids, etc.

Natural Media

These media consist solely of naturally occurring biological fluids and are of the following three types:

1. Cagula or clots

2. Biological fluids

3. Tissue extracts.

The natural biological fluids are generally used for organ culture. For cell cultures, artificial media with or without serum are used.

Clots

The most commonly used clots are plasma clots, which have been in use for a long time. Plasma is now commercially available either in liquid or lyophilized state. It may also be prepared in the laboratory, usually from the blood of male fowl, but blood clotting must be avoided during the preparation.

Biological Fluids

Of the various biological fluids used as culture medium, serum is the most widely used. Serum is one of the very important components of animal cell culture which is the source of various amino acids, hormones, lipids, vitamins, polyamines, and salts containing ions such as calcium, ferrous, ferric, potassium etc. It also contains the growth factors which promotes cell proliferation, cell attachment and adhesion factors. Serum may be obtained from adult human blood, placental cord blood, horse blood or calf blood (foetal calf serum, newborn calf serum, and calf serum); of these foetal calf

serum is the most commonly used. Serum is the liquid exuded from coagulating blood. Different preparations of serum differ in their properties; they have to be tested for sterility and toxicity before use.

Tissue Extracts

Tissue or organ extracts and/or hydrolysates (e.g., bovine pituitary extract (BPE), bovine brain extract,

chick embryo extract and bovine embryo extract), and animal-derived lipids and fatty acids, peptones, Excyte, sterols (e.g., cholesterol) and lipoproteins (e.g., high-density and low-density lipoproteins(HDLs and LDLs, respectively) are used in culturing of animal cells. Tissue extracts for example,

Embryo extracts—Other biological fluids used as natural media include amniotic fluids, ascetic and pleural fluids, aqueous humour (from eye), serum ultra filtrate, insect haemolymph etc. Chick embryo extract is the most commonly used tissue extract, but bovine embryo extract is also used. Other tissue extracts that have been used are spleen, liver, bone marrow, etc. extracts. Tissue extracts can often be substituted by a mixture of amino acids and certain other organic compounds.

Artificial Media

Different artificial media have been devised to serve one of the following purposes:

1. Immediate survival (a balanced salt solution, with specified pH and osmotic pressure is adequate),

2. Prolonged survival (a balanced salt solution supplemented with serum, or with suitable formulation of organic compounds),

3. Indefinite growth

4. Specialized functions.

The various artificial media developed for cell cultures may be grouped into the following four classes:

(*i*) Serum containing media

(*ii*) Serum free media

(*iii*) Chemically defined media

(*iv*) Protein free media.

SERUM

Liquid yellowish, clear content left over after fibrin and cells are removed from the blood is known as serum. Calf (bovine), foetal bovine, or horse are used, in some cases human. Fetal bovine serum (FBS)(10-20% v/v) is the most commonly applied supplement in animal cell culture media. Normal growth media often contain 2-10% of **serum.** These supplements provide carriers or chelators for labile or water-insoluble nutrients; bind and neutralize toxic moieties; provide hormones and growth factors, protease inhibitors and essential, often unidentified or undefined low molecular weight nutrients; and protect cells from physical stress and damage. Thus, serum and/or animal extracts are commonly used as relatively low-cost supplements to provide an optimal culture medium for the cultivation of animal cells. The role for all constituents (more than 200) is not clear proteins, peptides, special factors released during platelet aggregation e.g., PDGF, TGF-β, lipids, lipid transport proteins, carbohydrates, micronutrients such as minerals, etc.

Chemically Defined Media: These media contain contamination free ultra pure inorganic and organic constituents, and may contain pure protein additives, like insulin, epidermal growth factor, etc. that have been produced in bacteria or yeast by genetic engineering with the addition of vitamins, cholesterol, fatty acids and specific amino acids. The CHO cell lines are widely used for being highly stable expression systems for heterologous genes (those from a different organism), and for its relatively simple adaptation to adherence-independent growth in serum and protein free media.

Protein-Free Media: In contrast, protein free media do not contain any protein; they only contain non-protein constituents necessary for culture of the cells. The formulations MEM, DME, RPMI-1640,etc. are protein free; where required, protein supplementation is provided. Compared with serum

APPLICATIONS OF ANIMAL CELL CULTURE

The animal cell cultures are used for a diverse range of research and development. These areas are:

(*a*) Production of antiviral vaccines, which requires the standardization of cell lines for the multiplication and assay of viruses.

(*b*) Cancer research, which requires the study of uncontrolled cell division in cultures.

(*c*) Cell fusion techniques.

(*d*) Genetic manipulation, which is easy to carry out in cells or organ cultures.

(*e*) Production of monoclonal antibodies requires cell lines in culture.

(*f*) Production of pharmaceutical drugs using cell lines.

(*g*) Chromosome analysis of cells derived from womb.

(*h*) Study of the effects of toxins and pollutants using cell lines.

(*i*) Use of artificial skin.

(*j*) Study the function of the nerve cells.

(*k*) Many commercial proteins have been produced by animal cell culture and there medical application is being evaluated. Tissue Plasminogen activator (t-PA) was the first drug that was produced by the mammalian cell culture by using rDNA technology. The recombinant t-PA is safe and effective for dissolving blood clots in patients with heart diseases and thrombotic disorders.

Plant tissue culture

Most methods of plant transformation applied to genetically modified crops require that a whole plant is regenerated from isolated plant cells or tissues that have been genetically transformed. This regeneration is conducted *in vitro* so that the environment and growth medium can be manipulated to ensure a high frequency of regeneration. In addition to this, the regenerable cells must be accessible to gene transfer by whatever technique is chosen (gene transfer methods are described in Chapter 3). The primary aim is therefore to produce, as easily and as quickly as possible, a large number of regenerable cells that are accessible to gene transfer. The subsequent regeneration step is often the most difficult step in plant transformation studies

Plasticity and totipotency

Two concepts, plasticity and totipotency, are central to understanding plant cell culture and regeneration. Plants, due to their sessile nature and long life span, have developed a greater ability to endure extreme conditions and predation than have animals. Many of the processes involved in plant growth and development adapt to environmental conditions. This plasticity allows plants to alter their metabolism, growth, and development to best suit their environment. Particularly important aspects of this adaptation, as far as plant tissue culture and regeneration are concerned, are the abilities to initiate cell division from almost any tissue of the plant and to regenerate lost organs or undergo different developmental pathways in response to particular stimuli. When plant cells and tissues are cultured *in vitro* they generally exhibit a very high degree of plasticity, which allows one type of tissue or organ to be initiated from another type. In this way, whole plants can be subsequently regenerated. This regeneration of whole organisms depends upon the concept that all plant cells can, given the correct stimuli, express the total genetic potential of the parent plant. This maintenance of

genetic potential is called **totipotency**. Plant cell culture and regeneration do, in fact, provide the most compelling evidence for totipotency. In practical terms though, identifying the culture conditions and stimuli required to manifest this totipotency can be extremely difficult and it is still a largely empirical process.

The culture environment

When cultured *in vitro*, all the needs of the plant cells, both chemical (see Table 2.1)and physical, have to met by the culture vessel, the growth medium, and the external environment (light, temperature, etc.). The growth medium has to supply all the essential mineral ions required for growth and development. In many cases (as the biosynthetic capability of cells cultured *in vitro* may not replicate that of the parent plant), it must also supply additional organic supplements such as amino acids and vitamins. Many plant cell cultures, as they are not photosynthetic, also require the addition of a fixed carbon source in the form of a sugar (most often sucrose). One other vital component that must also be supplied is water, the principal biological solvent. Physical factors, such as temperature, pH, the gaseous environment, light(quality and duration), and osmotic pressure, also have to be maintained within acceptable limits.

Element	Function
Nitrogen	Component of proteins, nucleic acids, and some coenzymes; element required in the greatest amounts
Potassium	Regulates osmotic potential; principal inorganic cation
Calcium	Cell-wall synthesis, membrane function, cell signalling
Magnesium	Enzyme cofactor, component of chlorophyll
Phosphorus	Component of nucleic acids; energy transfer; component of intermediates in respiration and photosynthesis
Sulphur	Component of some amino acids (methionine, cysteine) and some cofactors
Chlorine	Required for photosynthesis
Iron	Electron transfer as a component of cytochromes
Manganese	Enzyme cofactor
Cobalt	Component of some vitamins
Copper	Enzyme cofactor; electron-transfer reactions
Zinc	Enzyme cofactor; chlorophyll biosynthesis
Molybdenum	Enzyme cofactor; component of nitrate reductase

Plant cell culture media

Culture media used for the cultivation of plant cells *in vitro* are composed of three basic components:

1 essential elements, or mineral ions, supplied as a complex mixture of salts;

2 an organic supplement supplying vitamins and/or amino acids; and

3 a source of fixed carbon; usually supplied as the sugar sucrose.

For practical purposes, the essential elements are further divided into the following categories:

1 macroelements (or macronutrients);

2 microelements (or micronutrients); and

3 an iron source.

Complete plant cell culture medium is usually made by combining several different components,

Media components

It is useful to briefly consider some of the individual components of the stock solutions.

Macroelements

As is implied by the name, the stock solution supplies macroelements required in large amounts for plant growth and development. Nitrogen, phosphorus, potassium, magnesium, calcium, and sulphur (and carbon, which is added separately) are usually regarded as macroelements. These elements usually comprise at least 0.1% of the dry weight of plants.

Nitrogen is most commonly supplied as a mixture of nitrate ions (from KNO_3) and ammonium ions (from NH_4NO_3). Theoretically, there is an advantage in supplying nitrogen in the form of ammonium ions, as nitrogen must be in the reduced form to be incorporated into macromolecules. Nitrate ions therefore need to be reduced before incorporation. However, at high concentrations, ammonium ions can be toxic to plant cell cultures and uptake of ammonium ions from the medium causes acidification of the medium. For ammonium ions to be used as the sole nitrogen source, the medium needs to be buffered. High concentrations of ammonium ions can also cause culture problems by increasing the frequency of verification (the culture appears pale and ‘glassy’ and is usually unsuitable for further culture). Using a mixture of nitrate and ammonium ions has the advantage of weakly buffering the medium as the uptake of nitrate ions causes OH^- ions to be excreted. Phosphorus is usually supplied as the phosphate ion of ammonium, sodium, or potassium salts. High concentrations of phosphate can lead to the precipitation of medium elements as insoluble phosphates.

Microelements

Microelements are required in trace amounts for plant growth and development, and have many and diverse roles. Manganese, iodine, copper, cobalt, boron, molybdenum, iron, and zinc usually comprise the microelements, although other elements such as nickel and aluminium are found frequently in some formulations. Iron is usually added as iron sulphate, although iron citrate can also be used. Ethylene diamine tetra-acetic acid (EDTA) is usually used in conjunction with iron sulphate. The EDTA complexes with the iron to allow the slow and continuous release of iron into the medium. Uncomplexed iron can precipitate out of the medium as ferric oxide.

Organic supplements

Only two vitamins, thiamine (vitamin B1) and myoinositol (considered a B vitamin),are considered essential for the culture of plant cells *in vitro*. However, other vitamins are often added to plant cell culture media for historical reasons. Amino acids are also commonly included in the organic supplement. The most frequently used is glycine (arginine, asparagine, aspartic acid, alanine, glutamic acid,glutamine, and proline are also used), but in many cases its inclusion is not essential. Amino acids provide a source of reduced nitrogen and, like ammonium ions, uptake causes acidification of the medium. Casein hydrolysate can be used as a relatively cheap source of a mix of amino acids.

Carbon source

Sucrose is cheap, easily available, readily assimilated, and relatively stable, and is therefore the most commonly used carbon source. Other carbohydrates (such as glucose, maltose, galactose, and sorbitol) can also be used and in specialized circumstances may prove superior to sucrose.

Gelling agents

Media for plant cell culture *in vitro* can be used in either liquid or ‘solid’ forms, depending on the type of culture being grown. For any culture types that require the plant cells or tissues to be grown on the surface of the medium, it must be solidified(more correctly termed **gelled**). Agar, produced from seaweed, is the most common type of gelling agent, and is ideal for routine applications. However, because it is a natural product, the agar quality can vary from supplier to supplier and from batch to batch. For more demanding applications, a range of purer (and in some cases, considerably more expensive) gelling agents are available. Purified agar or agarose can be used, as can a variety of gellan gums.

Plant growth regulators

Plant growth regulators are the critical media components in determining the developmental pathway of the plant cells. The plant growth regulators used most commonly are plant hormones or their synthetic analogues.

Classes of plant growth regulator

There are five main classes of plant growth regulator used in plant cell culture, namely:

(1) auxins;

(2) cytokinins;

(3) gibberellins;

(4) abscisic acid;

(5) ethylene.

Each class of plant growth regulator will be looked at briefly below.

Auxins

Auxins promote both cell division and cell growth. The most important naturally occurring auxin is indole-3-acetic acid (IAA), but its use in plant cell culture media is limited because it is unstable to both heat and light. Occasionally, amino acid conjugates of IAA (such as indole-acetyl-l-alanine and indole-acetyl-l-glycine), which are more stable, are used to partially alleviate the problems associated with the use of IAA. It is more common, though, to use stable chemical analogues of IAA as a source of auxin in plant cell culture media. 2,4-Dichlorophenoxyacetic acid (2,4-D) is the most commonly used auxin and is extremely effective in most circumstances. Other auxins are available and some may be more effective or 'potent' than2,4-D in some instances.

Cytokinins

Cytokinins promote cell division. Naturally occurring cytokinins are a large group of structurally related purine derivatives. Of the naturally occurring cytokinins, two have some use in plant tissue culture media zeatin and*N*6-(2-isopentyl)adenine (2iP). Their use is not widespread as they are expensive(particularly zeatin) and relatively unstable. The synthetic analogues kinetin and6-benzylaminopurine (BAP) are therefore used more frequently. Non-purine-based chemicals, such as substituted phenylureas, are also used as cytokinins in plant cellculture media. These substituted phenylureas can also substitute for auxin in someculture systems.

Gibberellins

There are numerous, naturally occurring, structurally related compounds termed gibberellins. They are involved in regulating cell elongation, and are agronomically important in determining plant height and fruit-set. Only a few of the gibberellins are used in plant tissue culture media, GA3 being the most common.

Abscisic acid

Abscisic acid (ABA) inhibits cell division. It is most commonly used in plant tissue culture to promote distinct developmental pathways such as somatic embryogenesis

Ethylene

Ethylene is a gaseous, naturally occurring, plant growth regulator most commonly associated with controlling fruit ripening in climacteric fruits, and its use in plant tissue culture is not widespread. It does, though, present a particular problem for plant tissue culture. Some plant cell cultures produce ethylene, which, if it builds up sufficiently, can inhibit the growth and development of the culture. The type of culture vessel used and its means of closure affect the gaseous exchange between the culture vessel and the outside atmosphere and thus the levels of ethylene present in the culture.

Plant growth regulators and tissue culture

Generalizations about plant growth regulators and their use in plant cell culture media have been developed from initial observations made in the 1950s. There is, , some considerable difficulty in predicting the effects of plant growth regulators: this is because of the great differences in culture response among species, cultivars, and even plants of the same cultivar grown under different conditions., some principles do hold true and have become the paradigm on which most plant tissue culture regimes are based. Auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture and are usually used together, the ratio of the auxin to the cytokinin determining the type of culture established or regenerated A high auxin to cytokinin ratio generally favours root formation, whereas a high cytokinin to auxin ratio favours shoot formation. An intermediate ratio favours callus production.

Culture types

Cultures are generally initiated from sterile pieces of a whole plant. These pieces are termed **explants**, and may consist of pieces of organs, such as leaves or roots, or maybe specific cell types, such as pollen or endosperm. Many features of the explant are known to affect the efficiency of culture initiation. Generally, younger, more rapidly growing tissue (or tissue at an early stage of development) is most effective. Several different culture types most commonly used in plant transformation studies are as follows.

Callus

Explants, when cultured on the appropriate medium, usually with both an auxin and a cytokinin, can give rise to an unorganized, growing, and dividing mass of cells. It is thought that any plant tissue can be used as an explant, if the correct conditions are found. In culture, this proliferation can be maintained more or less indefinitely, provided that the callus is sub cultured on to fresh medium periodically. During callus formation, there is some degree of dedifferentiation (i.e. the changes that occur during development and specialization are, to some extent, reversed), both in morphology (a callus is usually composed of unspecialized parenchyma cells) and metabolism. One major consequence of this dedifferentiation is that most plant cultures lose the ability to photosynthesize. This has important consequences for the culture of callus tissue, as the metabolic profile will probably not match that of the donor plant. This necessitates the addition of other components—such as vitamins and, most importantly, a carbon source—to the culture medium, in addition to the usual mineral nutrients. Callus culture is often performed in the dark (the lack of photosynthetic capability being no drawback) as light can encourage differentiation of the callus. During long term culture, the culture may lose the requirement for auxin and/or cytokinin. This process, known as **habituation**, is common in callus cultures from some plant species(such as sugar beet).

Callus cultures are extremely important in plant biotechnology. Manipulation of the auxin to cytokinin ratio in the medium can lead to the development of shoots, roots, or somatic embryos from which whole plants can subsequently be produced. Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant transformation studies.

Protoplasts

Protoplasts are plant cells with the cell wall removed. Protoplasts are most commonly isolated from either leaf mesophyll cells or cell suspensions, although other sources can be used to advantage. Two general approaches to removing the cell wall (a difficult task without damaging the protoplast) can be taken: mechanical or enzymatic isolation. Mechanical isolation, although possible, often results in low yields, poor quality, and poor performance in culture due to substances released from damaged cells. Enzymatic isolation is usually carried out in a simple salt solution with a high osmoticum, plus the cell-wall-degrading enzymes. It is usual to use a mix of both cellulase and pectinase enzymes, which must be of high quality and purity.

Protoplasts are fragile and damaged easily, and therefore must be cultured carefully. Liquid medium is not agitated and a high osmotic potential is maintained, at least in the initial stages. The liquid medium must be shallow enough to allow aeration in the absence of agitation. Protoplasts can be plated out on to solid medium and callus

produced. Whole plants can be regenerated by organogenesis or somatic embryogenesis from this callus. Protoplasts are ideal targets for transformation by a variety of means.

Root cultures

Root cultures can be established *in vitro* from explants of the root tip of either primary or lateral roots and can be cultured on fairly simple media. The growth of roots *in vitro* is potentially unlimited, as roots are indeterminate organs. Although the establishment of root cultures was one of the first achievements of modern plant tissue culture, they are not widely used in plant transformation studies.

Shoot tip and meristem culture

The tips of shoots (which contain the shoot apical meristem) can be cultured *in vitro*, producing clumps of shoots from either axillary or adventitious buds. This method can be used for clonal propagation. Shoot meristem cultures are potential alternatives to the more commonly used methods for cereal regeneration as they are less genotype dependent and more efficient (seedlings can be used as donor material).

Embryo culture

Embryos can be used as explants to generate callus cultures or somatic embryos. Both immature and mature embryos can be used as explants. Immature, embryo-derived embryogenic callus is the most popular method of monocotyledon plant regeneration.

Lecture 21

Expression of foreign genes - Transformation - calcium chloride mediated - calciumphosphate mediated - microinjection - liposome mediated gene transfer – electrophoration

Genetic transofrmatin of prokaryotes:

E.coli is the main host cell ion r DNA technology. The uptake of DNA by E.coli cell follows three main ways.

(i) Calcium chloride ($CaCl_2$) treatment

(ii) Electroporation

(iii) Microinjection

(iv)Calcium chloride treatment :

The E. coli cells should be treated with ice-cold $CaCl_2$ and then exposed to high temperatures (42^0C) for about 90 sec. Using this method 1 in 10^{-3} cells can be transformed. It is assumed that the bacterial cell wall is broken down in localized regions, which allow the plasmid DNA to be transformed into bacterial cell

(v) Electroporation :

The bacterial cells are induced to uptake DNA by subjecting to a high strength electric field in the presence of DNA. This method of transformation of DNA is called electrophoration. For E.coli electrophoration, the cells and DNA are placed in a chamber fitted with electrodes and a single pulse of 2.5 KV is administered for about 4.6 milliseconds. This treatment yields transformation efficiencies of 10^6 to 10^9 transformants per µg of DNA.

Genetic transformation of eukaryotes :

In animal cells DNA is injected into either the fertilized egg or into embryo stem cells. In plant cells protoplasts or embryonic tissues are used to inject DNA of interest.

In case of animal cells the term transformation is replaced by transfection and fertilized eggs or embryos are transfected with whole nuclei or whole chromosome or DNA fragments. This transfection of fertilized eggs is by using cytochalasin-B or by microinjection. Cultured animal cells are transfected by coprecipitation with calcium phosphate or electroporation or microinjection. Cultured animal cells are transfected by coprecipitation with calcium phosphate or electroporatin or microinjection.

In case of plant cells the gene transfer is by physical delivery or by using plasmids like Ti or Ri. The physical delivery is by using polyethylene glycol or by microinjection or by macroinjection or by electroporatin. Ti plasmid of Agrobacterium tumefaciena and Ri plasmid of A. rhizogenes have been effectiveloy used in gene transformation in plant cells.

The purposes of gene transfer to plant or animal cells are.

I. To produce chemicals or pharmaceutical drugs or fertilizers.
II. Study of structure and function of genes.
III. Production of transgenic animals and plants with improved qualities.

Protoplast transformation :

The organisms like B. subtilis can be transformed naturally. But natural transformation is suitable best to linear DNA, but not to circular Plasmids. So,a method known as protoplast transformation was developed. Protoplasts can be prepared by enzymatic removal of bacterial cell wall. Enzymes like lysozyme in the presence of osmotic stabilizer such as sucrose, generates protoplasts, exposing cytoplasmic membranes. Addition of DNA solution together with polyethyolene glycol (PEG), causes the cells to take up the DNA. Then the cells are allowed to regenerate on an osmotically stabilized medium. If the correct conditions are used, a very high proportion of the resultant colonies will be transformed. The same procedure is used in as of eukaryotic organisms also.

The formation and regeneration of protoplasts is a key feature of an alli9ed procedure known as protoplast fusion, where the addition of PEG to a mixture of protoplasts of two different strains results in the formation of a fused cell that contains the complete genome of both parents. Protoplast fusion has been more widely used for eukaryotic cells.

With fungi, the initial stage in mating is the formation of a heterokaryon. A cell containing two different nuclei is called heterokaryon. This can be difficult to achieve by conventional means, as many commercially important fungi are devoid of sexual activity. However, if protoplasts can be produced the addition of polyethylene glycol will cause the protoplasts to aggregate, and the very close contact between the exposed

membranes results in cell fusion. After allowing the cell walls to regenerate, heterokaryotic cells are produced which contain nuclei from both parents. Protoplast fusion is an extremely powerful technique that is applicable to any cells as long as the cell wall can be removed and viable cells regenerated from the protoplasts.

Protoplast fusion has been used with bacteria, fungi, plant and animal cells. The animal cells do not have a cell wall, so the process of cell fusion is considerable simpler. Construction of hybridoma cells for monoclonal autibodies production is the best example for cell fusion

TRANSDUCTION :

Transfer of bacterial genes from one cell to another through bacterial viruses (bacteriopphages) is called transduction. Many different bacteriophages are capable of transduction. Generally transduction is the result of an error in bacteriophage reproduction. In the life cycle of bacteriophages the coat proteins of phages assemble themselves, packing genetic material within it, to create a new viral particle. Each bacteriophage has a mechanism for packaging its genome into a capsid. Some bacteriophages occasionally make an error and package a piece of host cell's DNA. This event is normally random, so without showing specificity any bacter4ial gene will be transduced by bacteriophage.

Bacteriophages that contain bacterial DNA also are capable to attach to a new host cell and to inject DNA. Once inside the new cell, the transduced bacterial DNA can recombine with the resident genome, it can be expressed and transmitted to next generations. In lysogenic bacteria the bacterial genes become part of a bacteriophage chromosome.

Transduction is of different types :

1. Generalized transduction
2. Co-transduction
3. Abortive transduction
4. Specialized transduction

Generalized transduction : The process of transfer of any portion of a bacterial cell's genome into another bacterial cell by a bacteriophage is called generalized transduction. The transudcing bacteriophage contains only bacterial DNA, without phage DNA. Sometimes during loading of genetic material into viral protein heads instead of phage DNA, bacterial DNA can also be packaged. When this type of packed phage binds to another bacterial cell, the donor DNA is injected into bacterium and

integrated into its genome by recombination. For example phages that can mediate generalized transduction are P^1 Phage in E.coli and P^{22} Phage in Salmonella. P^{22} bacteriophage of salmonella is the well studied example of generalized transduction. About one-one hundredth size of Salmonella chromosome can be trasduced by P^{22} phage.

Co-transduction : Transfer of two or more genes of a donor cell at a time by a bacteriophage ot a recipient bacteria cell is called cotransduction. The cotransduced genes must be close to each other.

Abortive transduction : In this type of transduction the transferred gene will not integrate with the recipient cell,s chromosome. So it is transmitted to only one of its two daughter cells.

Specialized transduction : Transfer of specific site of donor genetic material by bacteriophage is called specialized transuction. This is mediated by bacteriophages that integrate into specific site of bacterial chromoseome. This specificity limits the transfer of genetic material with in the vicinity of integrated site. Lambda (λ) phage is the well studied example for specialized transduction. The λ phage integrated (attachment) at site near gal (galactose) region of the chromosome. So mediate specialized transduction in that region only

Lecture 22

Selection of cells containing cloned genes - selection based on antibiotic resistance -complementation of nutritional defects - assay of biological activity – immunochemical method - colony hybridization - Expression of target gene in the host cell - Shot gun method- DNA libraries - Genomic DNA libraries - cDNA libraries - Protoplast transformation

Identification or selection of cells containing cloned genes : After transformation, the recombinant DNA gets multiplied along with the multiplication of the host cells. The next step in gene cloning is to identify those transformed cells which actually carry the gene of interest. In bacteria, the cells carrying the recombinant plasmids are identified by any one of the following methods

(a) Selection based on antibiotic resistance genes.
(b) Selectin through complementation of nutritional defects
(c) Selection through assay of biological activity.
(d) Selection by immunochemical methods
(e) Selection through colony hybridization.

When plant or animal cells are used for cloning, the selection of recombinants is done by production of transgenic plants or animals.

The recombinants are identified most commonly by colony hybridization method. This method is as follows. The transformants are allowed to form colonies in an agar plate. A nitrocellulose filter paper is laid over the colonies in the agar plate, to prepare a replica of the master plate. Then it is incubated to form colonies on the filter paper. The filter paper is then taken out, and colonies are lysed by treating with O.J.N sodium hydroxide. This denatures their DNA and is fixed to the filter paper. In this way the pattern of colonies is replaced by an identical pattern of bound denatured DNA. The filter paper containing the bound denatured DNA is then incubated in a solution of a labeled DNA probe.

In this treatment the labeled oligonucleotide probe hybrizes with complementary nucleotides in the denatures DNA. This is known as colony hybridization. The unbounded probe is washed off. The filter paper is autoradiographed to located the hybridized coloies. This is made possible by theprsence of labeled probe. The cells in

the colonies that hybridize with the probe is picked up from the master plate. These cells contain the recombinant DNA. These cells are taken out and used for further studies.

Methods or strategies of Gene cloning?

A clone means and exact duplicate. Cloning is the creation of genetically identical cells or organisms from a single ancestral cell. Gene cloning refers to production of multiple copies of a desired gene. There are three methods of gene cloning. They are (1) Shot –gun cloning

(2) cDNA cloning and

(3) Cloning by gene synthesis

These methods mainly depend upon the nature of target DNA, the availability of screening methods and the selection scheme. Cloning may involve a specific fragment of DNA or an entire genome.

(1) Shot gun cloning : Cloning of entire genome of an organism is called shot gun cloning. By this method genomic library is created. In this experiment, the entire genome of an organism is isolated. In this experiment, the entire genome of an organism is isolated or extracted. Then the genome is broken into fragments of reasonable size. This is done either by mechanical shearing or by using restriction endonucleases.

The cloning vector or E.coli plasmid is also cut with the same restriction endonuclease enzyme and the DNA fragments of the genome are spliced into it. It produces a population of chimeric vector DNA molecules. The recombinant plasmids or vectors are introduced into E.coli cells to facilitate replication of DNA. The E.coli becomes transformed. The transformed E.coli is cultured to produce a large number of colonies each carrying a different piece of DNA. This collection of clones constitute a agene bank or genomic library or gene library.

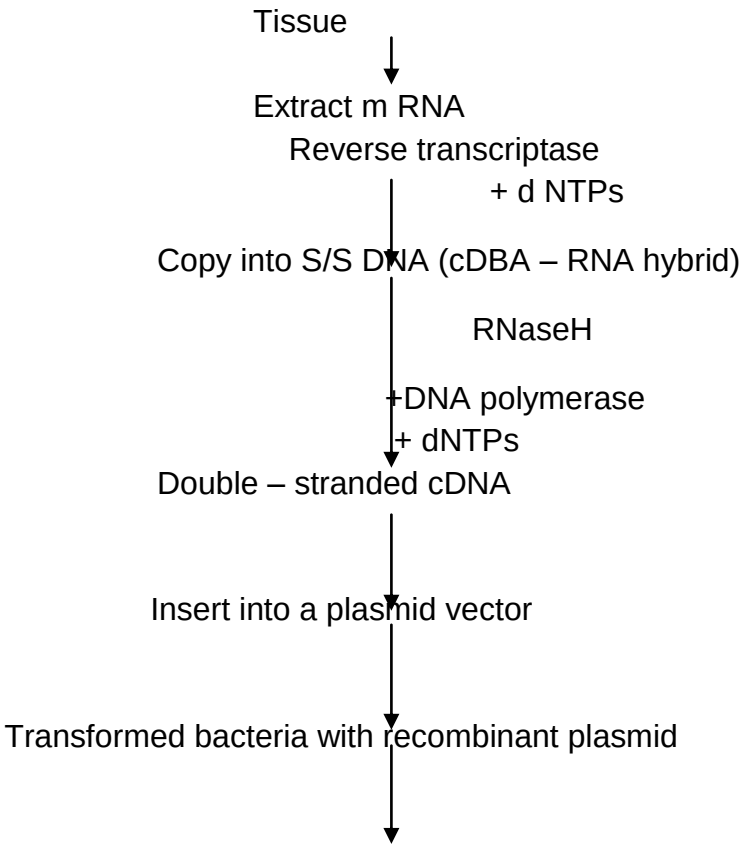
cDNA library :

cDNA is a single stranded DNA complementary to an RNA. cDNA is synthesized from RNA by invitro reverse transcription. A cDNA library is defined as a collection of recombinant molecules which contain all of the cDNA sequences of an individual genome.

The synthesis of DNA from its mRNA and cloning this copy DNA in cloning vectors such as E.coli is called cDNA cloning. This technique was proposed by Okayama and Berg.

Complementary DNA (cDNA) libraries can be prepared by isolating mRNAs from tissues which are actively synthesizing proteins, like young roots and leaves in plants, ovaries or reticulocytes in mammals etc. When a pure sample of mRNA coding for one specific protein is available, the mRNA is used instead of the gene, coding for it. Using this mRNA as a template, a copy DNA which is complementary to the mRNA is synthesized through the use of reverse transcriptase.

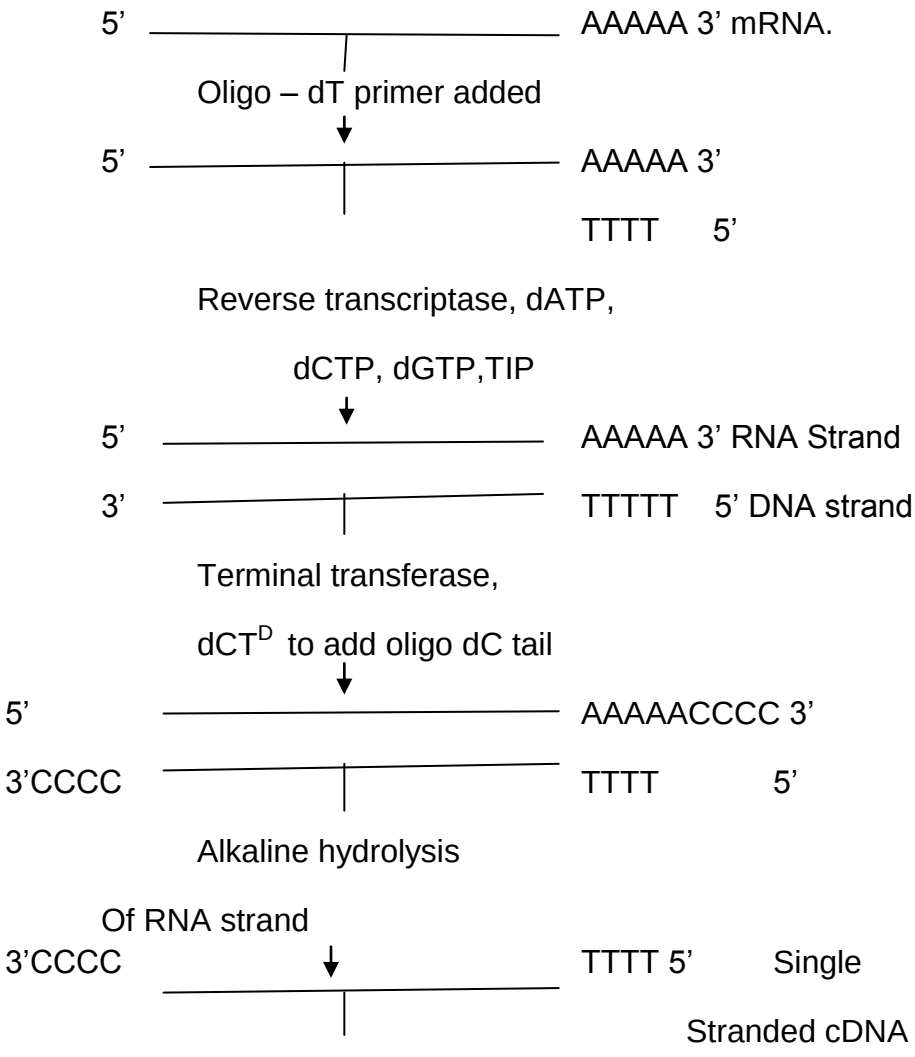
cDNA cloning is used in cloning a gene from hi8gher organisms in E.coli cells. For example, cloning the gene coding for human proinsulin is as follows. The B cells of islets of langerhans of human pancreas are lysed and mRNA are collected. Generally eukaryotic mRNA contains poly (A) at their 3' end. A primer sequence is added to the 3' OH end of each mRNA. It provides a 3'-OH group for the elongation of the polynucleotide chain. Usually an oligo d(T) primer is added to the mRNA because the mRBA contains copy poly A which is complementary to oT. Then the mRNA are treated with an enzyme reverse transcriptase which adds complementary nucleotides to the 3' OH group of the primer sequence. As a result of polymerization, RNA-DNA hybrid will be formed. The RNA –DNA hybrid is then mixed with the nucleotides dCTP and the enzyme terminal transferase. This enzyme helps to add an oligo dc tail to the 5' end. The RNA present in the RNA-DNA hybrid is denatured by treating it with alkali solution which removes the mRNA from the DNA. The DNA thus formed is a complementary DNA or cDNA.



Selection of transformed bacterial clone
Containing gene of interest

Fig. General scheme for cDNA cloning

After the formation of a cDNA stsrand, the enzyme DNA polymerase I and the nucleotides dATP. dCTP. dGTP. And dTTP are added. The single cDNA strand acts as a template for the synthesis of the second strand. As a result of polymerization a double stranded DNA is formed. A collection of cDNA synthesized from mRNAs constitute a cDNA library. The cDNA library represents the genes that are expressed in the cell or tissue. The cDNA is then cloned in E.coli.



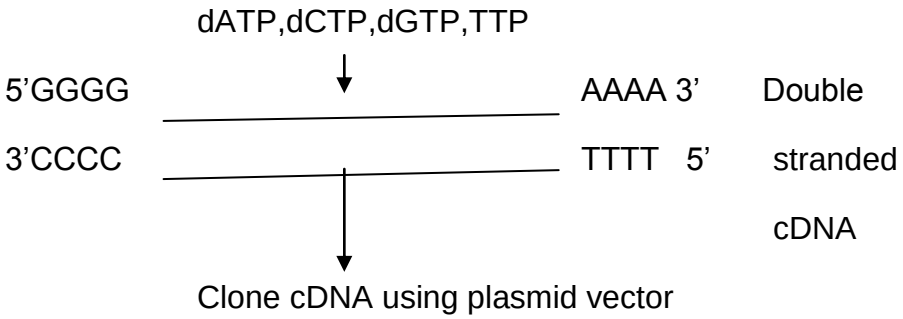


Fig : Synthesis of cDNA and construction of cDNA library

(2) Cloning by Gene synthesis :

This tecninque involves the artificial synthesis of the desired gene or DNA sequence in vitro and cloning it. For example, a gene coding for proinsujlin can be synthesized in a test tube and used in cloning. Various oligonucleotides can be synthesized and joined together to produce the desired gene. But, by using this procedure, genes coding for proteins up to 500 amino acids long can only be synthesized. Moreover, one should know.

Lecture 23

Biomass production- recycling of waste - Sewage - Domestic - Agricultural - Industrial -Treated sewage for single cell protein production - Single cell protein advantages – Source of SCP - Production of bacterial biomass - Production using waste - Starchy waste – from Algae - Nutritive value of SCP - Consumption of SCP - uses of SCP

Commercial production of single cell protein

Proteins are the source material needed to build and repair out body. Proteins occupied second position in our diet after carbohydrates. The conventional sources of protein are pulses and cereals of plants and meat, milk, eggs, fish and animals. Due to growing population the conventional proteins are insufficient hence there is a growing need for more and more protein for human nutrition. In some parts of the world protein deficiency is more prevalent. Hence search for non conventional protein had began long back. In recent decades single – cell protein has been found as an alternative protein supplement for human and animal feed.

The term single – cell protein (SCP) was coined by Prof. C.L. Wilson in 1966. According to Prof. C.L. Wilson single – cell protein is the cells of various microorganisms grown for their protein content. The dried biomass of the end product of microbial fermentation is referred to as single cell – protein (SCP). Microorganisms when grown on suitable substrate accumulate large quantity of proteins. The purified, dried cell biomass of these microorganisms can be utilized as protein diet.

Significance of SCP as a source of food and feed:

1. SCP can be produced in large amounts in short time because microorganisms are having very fast growth rate.
2. The protein content of SCP is quiet high. (35% to 60%)
3. They can be easily grown on a variety of substrata like waste materials of agriculture and industries.

When SCP has to be used as human food and animal feed it should show certain characters.

1. SCP should have satisfactory amount of protein, lipid, minerals and vitamins.
2. SCP products must be free from toxic substance.

3. The SCP should have satisfactory flavor and aroma.
4. It should not show adverse effects on human and animals.

SCP Microorganisms :

Organisms belonging to fungi, yeasts, algae, actinomycetes and bacteria are found to be most efficient SCP producers.

Group	Organism
Fungi	Fusarium gramininarum, Penicillium cyclopium, Aspergillus terreus, Cephalosporium etc.
Yeast's	Saccharomyces, Candida, Torulopsis.
Actinomycetes	Streptomyces, Mycobacterium, Nocardia, Actinomyces.
Algae	Chlorella, Spirulina, Senedesmus
Bacteria	Pseudomonas, Klebsiella, Methanomo nas, Rhodospirillum, Corynebacterium, Cellulo-Monos, Hydrogenomonas, Methy lomonas

A variety of substances are used as raw materials for the production of SCP. These include waste formed in agriculture, industrial and urban. Agricultural waste includes straw of rice, wheat, barley and grass, Cotton gin, husks from grain, vegetable and fruit peelings. Industrial waste includes paper mill sludge. Hydrocarbons includes methane, Propane, Petroleum, Kerosent, Methanol and Ethanol et. Basing on the waste used as raw material the microorganism should be selected. If Agriculture waste are used as raw material filamentous fungi should be selected for the production of SCP. Mostly Algae and photosynthetic bacteria are best microorganisms for producing SCP. It is evident from observations that nutritional values of SCP is equivalent to conventional sources of proteins and superior in some cases.

Commercial production of of SCP :

The worldwide food protein deficiency was about 12 million tones in 1985, and is likely to rise to about 25 million tones by 2005. In this context, SCP provides a solution by which edible proteins may be produced on a large scale by means of natural microorganisms.

A variety of raw materials are used as carbon and energy sources for SCP production. Single-cell proteins contain very high percentage of protein by weight (sometimes upto 70%). Some important raw materials used as carbon and energy sources, microorganisms concerned, and the protein contents (in %) are given.

(1) **SCP production using carbohydrates as raw materials** : Filamentous Fungi are used in producing SCP protein on raw material wheat, potato containing carbohydrates. Species of Fusarium mold are used as microorganisms in producing SCP protein. This protein produced is marketed under the trade name microprotein for human consumption.

(2) **SCP production using paper industry waste** : Waste products like sulphide liquor which is formed in industry become a serious problem in its disposal. In England using this waste SCP produced commercially. The sulphide liquor is fed to microorganisms like paecilomyces moldes. It give sprotein rich end products. This not only purifies the waste but also supplies rich protein feed for animals. This process is called “Pekilo” process. Through out the world the waste material from forestry, fruit industry, milk industry are utilized for the SCP production.

SCP production using cellulosic raw materials : A number of microorganisms are able to break cellulose into simple sugars. The sugars can be utilized for the growth of micro organisms producing SCP. In U.S.A. the bacterium Cellulomonas flavigena contain enzymes for the hydrolysis of cellulose. In India Cotton technological research laboratory, Bombay, by utilizing microorganism Penicillium funiculosum is producing SCP protein on large scale. In Calcutta yeasts are used as microorganisms for producing SCP on alcohol.

Commercial Production of SCP : In commercial production of SCP various raw material like methanol, ethanol, paraffins, sulphide liquor and carbohydrates are utilized. Recently starch is used as attractive substrate in the SCP production. Yeasts are used as microorganisms producing SCP using various starch materials.

Process of commercial production of SCP : Production of SCP on commercial basis is based on the continuous fermentation of microorganisms under carefully control conditions. This process requires a fermentation – broth which acts as substratum on which the microorganism grows under aerobic conditions. The nutrients is supplied in a sterilized state to prevent contamination. This fermentation process is usually carried continuously.

SCP Production using Chlorella, Spirulina (Algae) : Some algal members like Chlorella, Spirulina, Senedesmes are used in the commercial production of SCP SCP manufactured from Algae are used as food for animals and human beings. In Mysore Central Food Research station is actively producing SCP protein commercially by using Spirulina. The protein manufactured from Spirulina cultivation is commercially used as food in various parts of India.

Sewage is taken in large tanks and Spirulina grown. The Spirulina separated, cleaned and dried. This dired Spirulina is rich in proteins and Carbohydrates. This SCP protein contain very less percentage nucleic acids. Hence this is very useful protein food for humans also.

Growing Spirulina an Algae :

Commercial Production on large scale : The economics of SCP production depend critically on the cost of raw material and expenditure incurred in the process. Biotechnology helps in producing low cost production of SCP using cheaper raw material.

Spirulina is a cyanobacterium offers exllent prospects for low cost technology of SCP production. The SCP produces using Spirulina is quiet rich in aminoacids and B vitamin. In India NBRI Lucknow, CFTRI Mysore, started work on mass culture utilizing Spirulina as a source of SCP. The spirulina was grown on sewage waste. It gave high amount of SCP. It can be harvested in 15 days.

In Madras the AMM Murugappa Chettair Research Center grown Spirulina fusiformis in outdoor ponds. The mass cultivatin of species is done in ponds. After harvesting it is filtered dried and used as SCP food. It was proved that SCP is a good feed for animals.

Commercial Production of SCP using Spirullina : Spirulina is a member of blue – green Algae. Large rectangular cement tubs are taken having a height of 15 to 20 cm. These tubs are filled with filtered sewage water up to 15 cm height. In this water Spirulina filaments are cultivated. Sodium bicarbonate is added to the water. The growth of the Algae is maximum at pH 8 to 10 and the opoti8mum temperature is 35 to 40^0C. The growth of Algae will be maximum in 15 to 20 days. The grown Algal filaments are separated with the help of nylon net and dried in the direct sun light. The dried algal filaments made into the powder and used as animal feed.

This commercial production SCP protein using Spirulina is very cheep. These proteins have no harmful effects on animals or human beings. This SCP protein is very easily digestible and contain high percentage of protein, vitamins and minerals.

Uses of SCP protein manufactured by using Spirulina :

(1) The SCP protein produces from Spirulina contain high percentage of nutritive values. Spirulina cells contain 60 to 70 percent of vitamins and minerals.
(2) This SCP protein can be produced very cheaply and large amounts in a short time. Hence this protein will be better supplement in developing countries.
(3) In some countries the SCP produced by Spirulina is used as multivitamin tablets.
(4) This SCP contain less amount of fat and low calary value. Hence this can be used for diabetic patients. This is also useful in humans who want to reduce cholesterol and body weight.
(5) As this SCP contain A and B vitamins in large proportion, these are used in hair tonics.
(6) The cattle which feed on SCP protein produced from Spirulina gave 40% more milk yield.

Edible Mushrooms

Mushrooms literally means fungi. Generally fruiting bodies of basidiomycetous fungi are called Mushrooms. Mushrooms contained high quality proteins and other minerals hence they attract people for food. There are about four thousand species of mushrooms. Out of this nearly two thousands know to be edible. In practice a dozen or two are naturally used as food. The edible mushrooms are brightly colored or white. Some of them are poisonous and are known as toadstools.

The simple Mushrooms has fruiting body looking like umbrella. The fruiting bodies contained microscopic spores. The spores fall on substratum and germinate giving rise umbrella like fruiting body. From each fruiting body enormous number of spores are released but only some are able to produce fruiting bodies due to lake of favorable conditions.

Mushrooms are becoming popular for their nutritive value, flavor and taste. Mushrooms contain large amount of water and other food. Usually about 50 to 60% of proteins are present. They are the best plant sources of B-vitamin. Biotin, thiamine and vitamins C and K are also present in sufficient amounts. During the process of cooking the vitamins are well retained. In addition to proteins they also contain carbohydrates and lipids.

Nutritive Value of Mushrooms:

Mushrooms are best food having better taste and high nutritive value. These nutritionally equal with animal and plant proteins. These proteins have all 22 types of amino acids. They also contain minerals like Iron, Copper, Phosphorous etc in high percentage. ABC vitamins also present in large am. The proteins of Mushroom are easily digestible.

Lecture 24

Energy production methods - methanogens - incarnation - biofuel - Bioremediation -Bioventing - Land farming - Bio augmentation - use of spent mushroom compost

COMMERICIAL PRODUCTION OF BIOGAS

India is one of the pioneer country in Biogas technology. There are many institutions where reasearches and development programmes are carried out. These are Khadi and village Industries Commission (KVIC) Bombay, National Environmental Engineering Research Institute (NEERI) Nagapur, and NEDA of U.P.

Biogas can be defined as a composition of different gases which are produced as a result of action of anaerobic microorganisms on raw materials such as domestic and agricultural wastes in biogas plant. Biogas contains methane in bulk, and other gases like CO_2, H_2, N_2, O_2 etc.

Proportion of gases in biogas.

S.L	Name of Gas	Chemical Formula	Proportion (In %)
1.	Methane	CH_4	50 – 68
2.	Carbon dioxide	CO_2	25 – 35
3.	Hydrogen	H_2	1 – 5
4.	Nitrogen	N_2	2 – 7
5.	Oxygen	O_2	0 – 0.1
6.	Hydrogen sulphide H_2S		

Raw materials used in production of biogas : Biomass generated from animals include cattle dung, manure from poultry, goat, sheep, fisheries waste etc. Cattle dung is the most potential biomass for biogas production. Plant biomass includes agricultural residue, deteriorated wheat grain etc.

Biogas is the main product of anaerobic digestion (municipal treatment). The process of anaerobic digestion consist of three steps.

I. Hydrolysin or Solubilisation : In this stage the feed stock (Municipal waste, surg) is solubilised by water and enzymes. The complex organic polymers like carbohydrates, fats, proteins are hydroglysed into simple monomers by the action of enzymes of hydrolytic fermentative methanogenic bacteria.

Carbohydrates → Glucose → alcohol →
Fats → Fatty acids →

II. Acedogenesiss (Acid formation) : In this step hydrolysed compounds are converted intracellularly into simple compounds by Acedogenic bacteria.

Fatty acids / Alcohols — Acetogenci bacteria: $CH_3COOH \longrightarrow$ acetic acid $+ HCOOH + CO_2$

III. Methanogenesis (Methane formation) : This is final stage where methane gas is formed. In this stage simple compounds are mainly converted into methane and CO_2 by anaerobic bacteria like Methanosarcina, Methanothrix, Methanobacterium.

Methenogenic $12CH_4$

(1) $CH_3COOH + CO_2 \longrightarrow + 12CO_2$

(2) $H COOH + {}_cO_2 \longrightarrow 2H_2O + 3CO_2$

Methenogenic Bacteria

COMMERCIAL PRODUCTION : Biogas is produced on commercial scale from dung human waste and other waste with help of microorganisms by using biogas plant.

The dung consist nearly 20% of inorganic particles. The percentage of inorganic salts can be reduced by adding water. When dung and water is mixed in 1 : 1 ration 10% of inorganic salts are maintained. This ratio of inorganic salts is sufficient for optimum production of biogas. The required dung feeding rate for optimum production of biogas is estimated as 3500 kg per day.

Apart from cattle waste (dung) many other inputs are useful in generating more biogas through increasing the growth of bacteria. Dung + 2 – 3% of fresh slury and calcium ammonium nitrate which is equal to 50% of fresh slurry is an optimum combination for maximum production of biogas. Another 2% of biogas production can be increased by mixing human waste to the dung. 35^0C – 38^0C is optimum temperature required for maximum production. The optimum pH is 7 (neutral) required.

Use of culture : Some bacteria and archebacteria are more active than other bacteria. These types are isolated and mixed with inputs in the digester which can increase biogas production substantially.

Mechanism of Methane formation : Several coenzymes, CO_2 reducing factors, activating factors are necessary for methane formation. Coenzymes include methyl coenzymes. Coenzyme – F420, coenzyme F430 etc.

I. $CO + H_2O \longrightarrow CO_2 + H_2$
II. $CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O$

Methani is also formed form other substrates.

$$4CH_3OH \longrightarrow 3CH_4 + 2H_2O + CO_2$$

Factors affecting Methanogenesis :

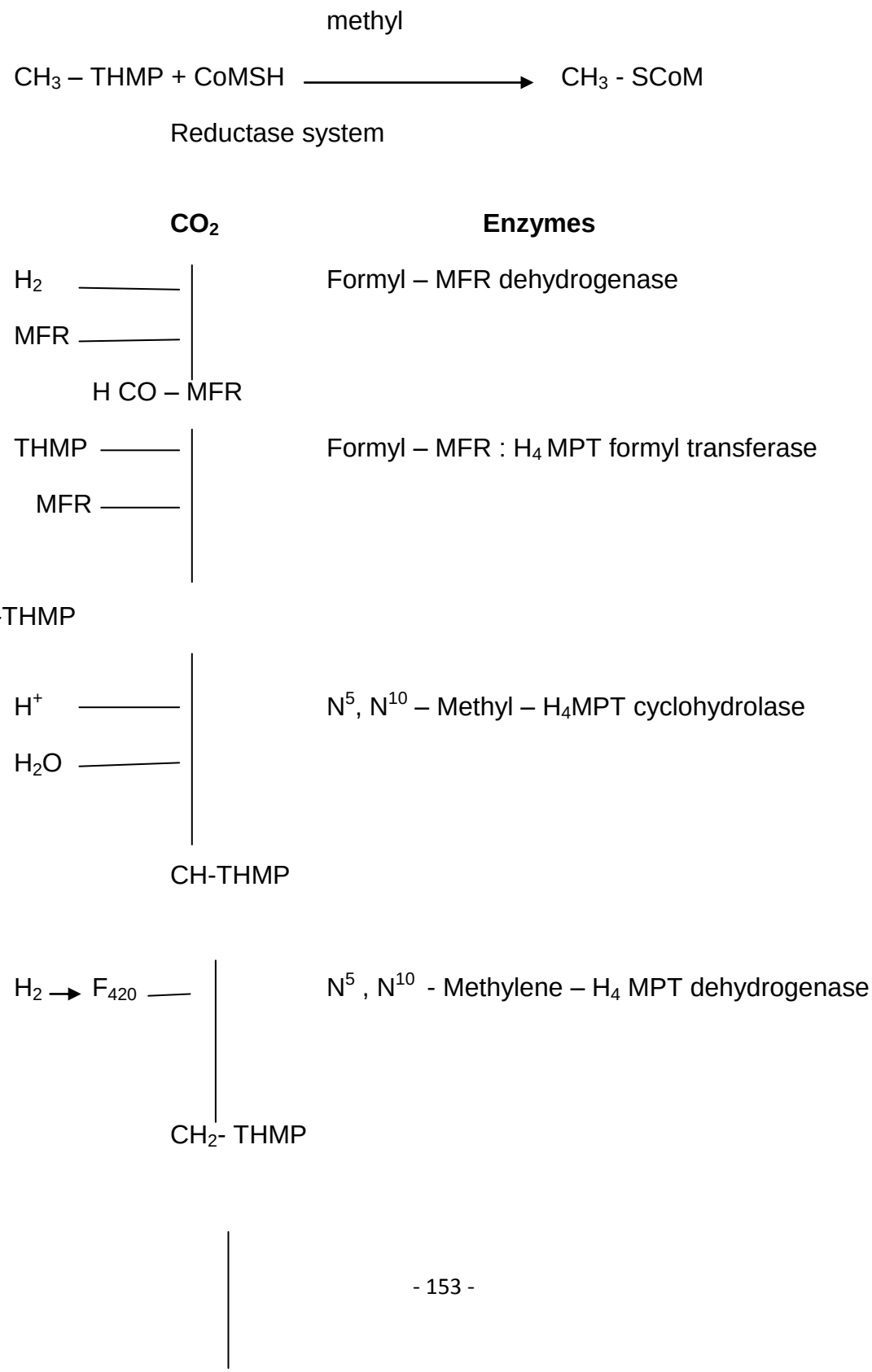
1. Small amount of sludge of another digester activate methane production.
2. Temperature between 30^0 – 60^0 C and pH at 6-8 favours the methane formation.
3. Proper C : N ratio i.e., 30 : 1 favours methane formation.
4. Methanogenesis takes place only in anaerobic conditions. Hence in India digesters are buried in the soil.

Advantages of Biogas :

(1) It is a substitute for fire wood, electricity, kerosene and LPG. Hence it can reduce environmental pollution.
(2) It can be used as a better fuel for cooking, heating lighting, power for irrigation etc.

Biochemistry : The biochemical conversion of H_2 and CO_2 to methane and of acetate of methane and CO_2 involve a number of coenzymes and prosthatic groups like “Deazari boflavin derivative EUR, Methanopterin, Methanofuran, the nickel tetrapyrol factor FU30 and coenzymke M-Merceaptoethane sulphate.

The production of methane yields ATP and is the only means of ATP formation for the methanogenic bacteria. For methane synthesis the methyl group is transferred from CH_3 – THMP to CoMSH to form CH_3 – SCOM. The terminal reduction is catalyzed by the "methyl reductase system".



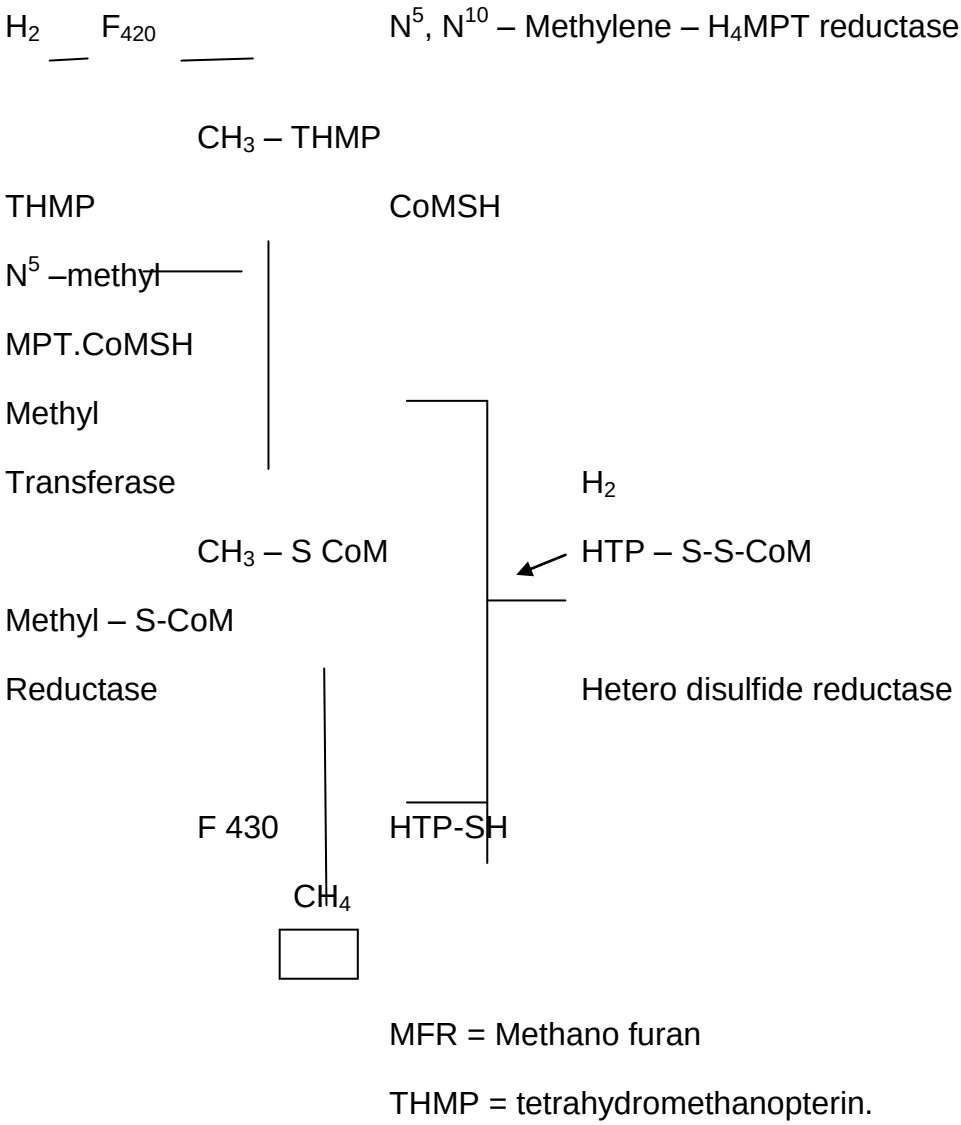


Fig : **The Methane production pathway**.

Solid state fermentation

Solid State Fermentation : In such fermentations, microbial growth and product formation occur at the surface of solid substrates. Examples of such fermentations are mushroom cultivation, mold – ripened cheeses, starter cultures etc.

More recently, this approach has been used for the production of extracellular enzymes, certain valuable chemicals, fungal toxins, and fungi spores (used for biotransformation). Traditional substrates are several agricultural products. Rice, wheat, maize, soybean etc. The substrate provides a rich and complex source of nutrients which may or may not need to be supplemented. Such substrates selectively

support mycelia organisms which can grow at high nutrient concentrations and produce a variety of extracellular enzymes, e.g., a large number of filamentous fungi, and a few bacteria (actinomycetes and one strain of Bacillus). According to the physical state, solid state fermentations are divided into two groups.

(i) Low moisture solids fermented without or with occasional/continuous agitation, and
(ii) Suspended solids fermented in packed columns through which liquid is circulated.

The fungi used for solid state fermentations are usually obligate aerobes

Patent concept :

Important part of the biotechnology industry concerns patenting or intellectual property rights. Intellectual property rights (IPRs) have been created to protect the right of individuals to enjoy their creations and discoveries.

Lecture 25

Biosensors - classification - Field of application - Transducers - Electrochemical biosensors- Voltammetric - Potentiometric - Conductometric - chemical sensitive field effect transistors(CHEMFET)

Biosensors

The marriage of electronics with the biotechnology has resulted an extremely significant device termed as biosensor. A biosensor is an analytical device consisting of a biocatalyst (enzyme, cell or tissue) and a transducer, which can convert a biological or biochemical signal or response into a quantifiable electrical signal. The biocatalyst component of most biosensors is immobilized onto a membrane or with in a gel, such that the biocatalyst is held in intimate contact with the transducers and may be reused. The biological recognition system in biosensors is microbial cell, an enzyme, antibody, hormone, nucleic acid that are immobilized in microchip devices for quantitative estimation of a substance.

Biosensors may be categorized as first, second and third generation instruments according to the degree of intimacy between the biocatalyst and transducers.

In **first generation** instruments, the two components (biocatalyst and transducer) may be easily separated and both may remain functional in the absence of the other.

In **second-generation** instruments, the two components interact in a more intimate fashion and removal of one of the components affects the usual functioning of the other.

In **third generation** instruments, the biochemistry and electrochemistry are even more closely linked and where the electrochemistry occurs at a semi-conductor, the term biochip may be applied to describe such instruments.

The biosensors may be of different kinds like electrochemical biosensors, amperometric biosensors, thermistor biosensors, bioaffinity biosensors, whole cells biosensors (microbial biosensors), optoelectronic biosensors etc. The sensor, which may be a carbon electrode, an ion-sensitive electrode, oxygen electrode, photocell or thermistor, analyzes the biological signals and converts them into electrical signals in the readable form, which is recorded on a meter. The substance to be analyzed passes through the membrane and interacts with the immobilized material to yield the biological signals

(electrons, heat, ions, gases, etc.). These biological signals are then converted int electrical signals by the sensors.

Biosensors are, in fact, biocatalysts. They are used as purified enzyme, antibody or as whole microbial cell or as an organelle. A biosensor used as an **immobilized** biological molecule (usually an enzyme or an antibody) or a whole microbial cell to detect or sense a particular substance. The biosensor does this by reacting specifically with the substance to be detected (hence the use of enzymes or antibodies) to give a product which is used to generate an electrical **signal** by means of a device called **transducer**.

Biosensor technology couples our knowledge of biology with advances in microelectronics. A biosensor is composed of a biological component, such as a cell or antibody, linked to a tiny transducer. Biosensors are detecting devices that rely on the specificity of cells and molecules to identify and measure substances at extremely low concentrations. When the substance of interest collides with the biological component, the transducer produces a digital electronic signal proportional to the concentration of the substance. Biosensors are used for following purposes :

1. Measure the nutritional value, freshness and safety of food
2. Provide emergency room physicians with bedside measures of vital blood components
3. Locate and measure environmental pollutants

Biosensor technology is progressing very fast on the front of techniques, their applications specially in the fields of analytic medicine, industry and environment. It is sometimes also useful in monitoring the presence of specific chemicals both accurately and rapidly.

Conventional Biosensor

Biosensors are composed of a bifunctional material and a transducer and have been developed and applied to analytical fields, clinical analysis, food industry and environmental measurements. Biosensors have their roots in military research, as means of detecting nerve gases and other chemical warfare toxins. Their applications have branched out to include simple to use alternate site diagnostic devices for home, doctor’s office, or drug use screening; medical and surgical monitors (small enough to fit inside a blood vessel) and environmental quality monitors.

Immobilized enzymes, microorganisms and antibodies are used as molecular recongnition materials. Electrochemical devices have often been used for transducers. Various enzymes have been used as molecular recognition elements. An enzyme electrode is composed of an enzyme immobilized membrane and an electrode. The

principle of an enzyme electrode is based on the detection of electro active species produced or consumed by the enzyme reaction. For example, a conventional glucose sensor is composed of glucose oxidase (GOD) and an electrode. GOD oxidizes glucose with the consumption of oxygen and produces gluconolactone and hydrogen peroxide. Measuring the consumption of oxygen with an oxygen electrode or production of hydrogen peroxide with hydrogen peroxide electrode, the concentration of glucose can be determined. This type of glucose sensor is in commercial use for the diagnosis of diabetes. There are many kinds of biosensors using the same principle and devices, and which are developed and used in the fields of clinical analysis and measurement of foodstuffs.

Microbial Biosensor

Microorganisms have also been utilized as molecular recognition elements. A microbial sensor consists of a microorganism immobilized membrane and an electrode. Various kinds of microbial sensors have been developed and applied to the measurement of biological compounds. The principle of a microbial sensor is based on either the change of respiration or the amount of produced metabolites as the result of assimilation of substrates by microorganisms. Furthermore, the use of auxotrophic mutants can selectively determine many kinds of substances. For example, the vitamin B_{12} sensor was constructed by using immobilized Escherichia coli 215. The E.coli 215 strain requires vitamin B12 for its growth. The linear relationship was obtained in the range between 5 × 109 and 25 × 109 g/ml. Within 25 days, the decrease in the response was approximately only 8 per cent.

Recently, microbial sensors using thermophilic bacteria have been developed. The use of thermophilic bacteria can possibly reduce contamination of other microorganisms by the use of high temperatures to obtain long term stability. For example, BOD and carbon dioxide sensors are constructed by using thermophiolic bacteria isolated from a hot spring, Good linear correlation was observed between the BOD sensor response and BOD value in the range 1 to 10 mg/1 BOD (JIS) at 50^0C. The Sensor signal was stable and reproducible for more than 40 days. For the carbon dioxide sensor, a linear relationship was obtained in $NaHCO_3$ concentration between 1 and 8 m M at 50^0C and the response time was 5 to 10 min. The linear relationship was also observed in the CO_2 concentration range 3 to 8 per cent.

Micro biosensors have many following advantages

1. Implantation in the human body and are suitable to in vivo measurement.
2. Can be integrated on one chip and are useful for measuring various substrates in a small amount of sample solution simultaneously.

3. Since semiconductor fabrication technology is applied to micro biosensors, it is possible to develop disposable transducers for biosensors through mass production.

Lecture 26

Optical biosensors- Biological components - Method of immobilization - Pressure biosensors- Thermonmetric biosensors- Glucose biosensors - Glycerol biosensors - Ethanol biosensors

Development of Micro Biosensor

Micro biosensors are based on ion sensitive field effect transistor (ISFET) and were first reported by Bergveld (1970). Matsuo et al. (1974) improved the ISFET using silicon nitride as the gate insulator to construct micro pH sensitive devices. They show rapid response, low power consumption, low noise and no need of a high impedance amplifier.

Some Common Examples of Application of Biosensors

Biosensor technology can be used to determine, how fresh food such as meat and fish are, as well as their quality. The biosensors are inserted in food, and react in the presence of certain chemical elements which develop during the process of decomposition or spoilage.

An alcohol sensor has been developed to make online measurements of alcohol concentration in culture broth, as required in brewing and fermentation industries. A microbial electrode consisting of immobilized yeast cells, a gas permeable Teflon membrane, and an oxygen electrode has been developed for determining ethyl alcohol.

It is possible to detect toxins employ glow-in-dark biosensors based on bioluminescence. Certain bacteria can be genetically engineered to glow in dark. These bacteria are used as living biosensors for detecting a variety of toxin agents. A convenient indicator gene is linked to a structural gene that is turned on or off by the some toxins such as polychlorinated biphenyls. The level of gene expression of the marker will reflect the expression of the target gene. The idea is to immobilize the engineered strains on dipsticks that could be used to assay for pollutants in a river or lake.

Glucose biosensor is used to detect blood glucose level. This biosensor is based on glucose – oxidase enzyme. The enzyme is immobilized on an electrode surface acting a san electrocatalyst for oxidation of glucose. The biosensor gives a reproducible electrical signal for glucose concentration as low as 0.15 mM and works for several weeks with no appreciable degradation of enzyme activity.

A biosensor containing immobilized Trichosporon cutaneum is used to test the extent of water contamination by measuring the biological oxygen demand (BOD).Biosensors have also been used for measuring pesticides and herbicides. One such sensor responds to carbamate and organophoporus pesticides. Immobilized acetylcholinestrerase activity is measured, as the enzyme reacts with acetylcholine using a potentiometric electrode. When above pesticides are present the enzyme is inhibited and the potentiometric response decreases. The acetylcholine based sensor is commercially available in U.K. as a pesticide/nerve gas detector.

A biosensor specific for the herbicide atrazine (weedicide) uses a piezoelectric device. The sensor consists of piezoelectric crystal coated with anti-atrazine antibodies, which changes its resonant frequency on the binding atrazine.

Rawson et al. (1989) developed a device sensitive to a broad range of toxins based ont eh inhibition of photosynthetic cyanobacteria. Synechococcus cells immobilized in the calcium alginate beads on a graphite electrode, and mediator potassium ferricyanide is supplied which shuttles electrons between the photosynthetic electrons transport chain and the electrode. In the presence of herbicides such as linuron, atrazine or metoxuran, photosynthesis is inhibited and the mediation current measured at the electrode decreases.

The other application areas of biosensor include agriculture, horticulture, vet nary analysis, detection of pollution and microbial contamination of water, direct biological measurement of flavours, essence and pheromones. Biosensors are already of major commercial importance and their significance is likely to increase as the technology develops.

TABLE 27.2 : Common biosensors

Analyte	Biocatalyst	Transducers	Immobilization	Stability	Response time
Alcohol	Alcohol-oxidase	O_2	Glutaraldehyde	>2 weeks	1-2 min
Arginine	Streptococcus Faecium	NH_3	Physically entrapped	20 days	20 min.
Cholesterol	Nocardia Erythyropois	O_2	Physically entrapped	4 weeks	35-70sec

D-Glucose	Glucose –oxidase	O_2	Chemical	3 weeks	1 min
Glutamate	Glutamate Decarboxylase	CO_2	Glutaraldehyde	1 weeks	10 min
NAD +	NADase and E.coli	NH_3	Dialysis membrane	1 weeks	5-10 min
Nitrate	Azatobacter Vinelandii	NH_3	Physically entrapped	2 weeks	7-8 min
Penicillium	Penicillinase	H+	Polyacralamide	>2 weeks	15-30sec
Urea	Urease	NH_4	Polyacralamide	>19days	20-40sec

Lecture 27

Enzyme technology - Microbial enzymes - production of enzymes - Solid state fermentation- Fermentors - Medium - Advantages and disadvantages -Submerged fermentation- Steps of enzyme production - Factors affecting Submerged culture

Microorganisms, plants and animals are the major sources of natural enzymes, but much focus has been diverted on microorganisms as a source of enzymes. Scientists and industrialists rely on enzymes of microbial source. Recombinant DNA technology has greatly influenced the possibilities for optimizing enzyme yield and type through strain selection, mutation, induction and selection of growth condition etc. Enzymes differ greatly in their stability to temperature and to extremes of pH. For example, bacterial proteases are relatively heat stable and they remain active under alkaline conditions and is being used as soap powder additives. On the other hand, fungal amylases have their greater sensitivity to heat and are being used in the baking industry. Microbial enzymes are used in fermentation processes at commercial level and therefore; substrate is required in large quantities. These substrates are starch hydrolysate, molasses, corn steep liquor, whey and cereals. For industrial production of enzymes suitable and low-prices substrates are required. Enzymes at industrial level are produced by solid substrate process using fungal sources for commercial extracellular enzymes production. Other technique is conventional batch submerged culture for bacterial source and is commonly used.

Solid substrate fermentation

Solid substrate methods for production of fungal enzymes include growth of filamentous fungus on moist wheat or rice bran with added nutrient salts as substrates to produce amylases, proteases, pectinases and cellulases. During the process, the control of pH, temperature and humidity is very difficult.

The fermenters are rectangular or circular trays of a few centimetre depths, mounted on racks and air is blown over the surface and beneath the mesh bottom. Another type is of two stage rotary system in which partially fermented bran from an upper chamber is conveyed to a lower rotary tray where fermentation process is completed.

Substrates composed of longer beds can be used. These can also fixed bed types in which air is forced though a bed of inoculated substrate which is periodically agitated by some mechanical device, or they can be of conveyed type. The other type of fermenter has been developed in which the rotating drum used horizontally, disposed cylinders, mounted on rollers and provides both support. It acts as rotation device.

The media comprise of cereal, which provide nutritional requirements to the microorganisms. The cereals are cracked and grinded to make them in utilizable form. They are sterilized using steaming technique at low pH or by chemical methods, or a combination of both. The mass of the moistened bran is inoculated with a suspension of spores or vegetative inoculum of the selected strain. Incubation is done for 1-10 days at temperature 40° to 84°C. Moisture content and temperature of the fermentil1g media should be controlled properly, though it is a difficult task. During the process, several metabolic reactions are under operation. Such reactions generate metabolic heat, which causes loss of water from cultures.

Advantages

The volume of fermenter is needed in less quantity as compared to submerged fermenter system. Inoculum stages are generally unnecessary. Spore seeding can be done directly. Recovery by extraction of the harvest with small volume of water yields relatively highly concentrated solutions of enzymes. The sterilization process is not expensive.

Disadvantages

Solid substrate fermentation process has several disadvantages and limitations. The process is limited to fungal organisms. The important one to maintain and control the environmental conditions of the fermenter, this includes temperature, pH, moisture content and oxygen transfer. It is a difficult task. Pretreatment of moist media except in

rice, is usually required. Control of the progress of the fermentation is difficult because of in-homogenous condition of the culture.

Submerged Fermentation

In submerged fermentation process bioreactor are used similar in design and function to those used in antibiotic production processes. It is constructed from stainless steel and has a capacity of 10-50 m^3 and includes systems for mixing by mechanical internal agitator, external pumps, bubble columns, and airlift loop. Cultivation involves the suspension growth in liquid medium. Sterilization and process control of submerged fermentation are easy.

The medium is prepared after adjusting optimum or required pH value. The medium is pumped to previously stern sterilized fermenter vessel through HTST sterilization units. Thermolabile additives are added separately to the sterile medium. Inoculum build up should be before transfer to the production stage vessels. Sterile compressed air is introduced into the fermentation broth through a sparger system of a ring type or a single or multiple inlet type. Agitation is done using multiple, flat bladed disc turbines mounted on a vertical shaft. Often types include axial flow propellers or counter current stirrers. Temperature of the fermenters is maintained by using internal cooling coils or external heat exchange surface.

Temperature

Optimum temperature is required for the production of a particular enzyme and growth of the microorganism and it varies. The activity of an enzyme towards a used substrate is also influenced by temperature. Therefore temperature during fermentation should range in such a way that cell growth is properly encouraged and subsequently changes with enzyme accumulation.

pH

The pH optima are required for stability of an enzyme and it varies. For getting maximum growth, rate of the organism it is essential to maintain pH optima. Therefore, pH profile during fermentation and enzyme production phase should be imposed in such a way that the maximum growth and enzyme production could be obtained.

Dissolved Oxygen Tension (DOT)

Proper aeration rate, agitation ratio and gas phase pressure are required to maintain dissolved oxygen tension. The oxygen below its critical concentration affects the growth of organisms. Adequate aerobic condition in growth culture is an essential requirement. The level of oxygen in submerged microbial cultures can be achieved by either increasing the mass of oxygen supplied to the fermenter/unit time by increasing the overpressure in the fermenter heads pace or by increasing the rate of air supply to the fermenter. Oxygen enriched air may effect higher oxygen transfer rates.

Solid-substrate fermentations have been used for producing various fermented foods in Asia for thousands of years, but this method is rarely used in Europe and North America. It involves the growth of microorganisms on solid, normally organic, materials in the absence or near absence of free water. The substrates used are cereal grains, bran, legumes and lignocelllulosic materials, such as straw, wood chippings, etc. Traditional processes are largely food fermentations producing oriental tempeh and sufu, cheeses and mushrooms along with compost and silage making. In addition, enzymes, organic acids and ethanol are now produced by solid substrate fermentations, particularly in areas where modern fermentation equipment is unavailable.

Solid-substrate fermentations lack the sophisticated control mechanisms that are usually associated with submerged fermentations. Their use is often hampered by lack of knowledge of the intrinsic kinetics of microbial growth under these operating conditions. Control of the environment within the bioreactors is also difficult to achieve, particularly the simultaneous maintenance of optimal temperature and moisture. However, in some instances, solid-substrate fermentations are the most suitable

methods for the production of certain products (see Table 6.4). For example, most fungi do not form spores in submerged fermentations, but

6.4 Advantages and disadvantages of solid-substrate fermentations

Advantages	Disadavantages
Potentially provide superior productivity	Slower microbial growth
Low-cost media	Problems with heat build-up
Simple technology	Bacterial contamination can be problematic
Low capital costs	Difficulties often encountered on scale-up
Reduced energy requirements	Substrate moisture level difficult to control
Low waste-water output	No problems with foaming

sporulation is often accomplished in solid-substrate fermentations. This method is successfully employed in the production of Coniothyrium minitans spores for the biocontrol of the fungal plant pathogen, Sclerotinia sclerotiorum. Solid-substrate fermentations are normally multistep processes, involving:

1 pretreatment of a substrate that often requires mechanical, chemical or biological processing;

2 hydrolysis of primarily polymeric substrates, e.g. polysaccharides and proteins;

3 utilization of hydrolysis products; and

4 separation and purification of end-products.

The microorganisms associated with solid-substrate fermentations are those that tolerate relatively low water activity down to Aw values of around 0.7. They may be employed in the form of:

1 monocultures, as in mushroom production, e.g. Agaricus bisporus;

2 dual cultures, e.g. straw bioconversion using Chaetomium celulilyticum and Candida tropicalis; and 3 mixed cultures, as used in composting and the preparation of silage, where the microorganisms may be indigenous or added mixed starter cultures (inoculants).

Environmental parameters that influence solid-substrate fermentations

WATER ACTIVITY, Aw

Water is lost during fermentation through evaporation and metabolic activity. This is normally replaced by humidification or periodic additions of water. If moisture levels are too low, the substrate is less accessible, as i does not swell and microbial growth is reduced. How ever, if the moisture levels are too high there is a reduction in the porosity of the substrate, lowering the oxygen diffusion rates and generally decreasing gaseous exchange. Consequently, the rate of substrate degradation is reduced and there is also an increased risk of microbial, contamination.

Heat Generation:

Heat generation can be more problematic than in Liquid . fermentations and has a major influence on re . humidity within a fermentation. The temperature if largely controlled by aeration and/or agitation of ' substrate.

Bioreactors for solid substrate fermentation:

Most solid-substrate fermentations are aerobic, but particular requirements for oxygen depend upon microorganism(s) used and the specific process. Rates aeration provided are also closely related to the need, dissipate heat, CO2 and other volatile compounds may be inhibitory. The kinetics of oxygen transfer' solid-substrate fermentations are poorly understand However, the rate of oxygen transfer is greatly influenced by the size

of the substrate particles, which de mines the void space. Oxygen transfer within this vo', space is closely related to the moisture level, as oxygen dissolves in the water film'around ,the substrate particles.. However, as mentioned above, if ex ' water fills the void spaces, it has a detrimental effect oxygen transfer.

Most solid-substrate fermentations are batch process although attempts are being made to develop semi continuous and continuous systems. Some processes not require bioreactors, they simply involve spreading the substrate onto a suitable floor. Those process employing vessels exhibit considerable variations. few anaerobic processes, such as silage production require no mechanisms for agitation or aeration .The majority are aerobic fermentation requiring aeration and occasional or continuo agitation. Bioreactors commonly used include following

Rotating drum fermenters, comprising a cylindrical I of around 100 L capacity mounted on its side onto that both support and rotate the vessel .These fermenters are used in enzyme and micro-biomass production. Their main disadvantage is the drum is filled to only 30% capacity, otherwise in is inefficient.

Tray fermenters, which are used extensively for production of fermented oriental foods and IDCS. Their substrates are spread onto each tray to depth of only a few centimetres and then stacked in chamber through which humidified air is circulated. these systems require numerous trays and large volume combustion chambers of up to 150m3 capacity .

3 Bed systems, as used in commercial koji production consisting of a bed of substrate up to 1 m deep, through which humidified air is continuously forced from below

4 Column bioreactors, consisting of a glass or plastic column, into which the solid substrate is loosely packed, surrounded by a jacket that provides a means of temperature control. These vessels are used to produce organic acids, ethanol and biomass.

5 Fluidized bed reactors, which provide continuous agitation with forced air to prevent adhesion and aggregation of substrate particles. These systems have been particularly useful for biomass production for animal feed.

Batch Culture

In this culture growth proceeds, nutrients are consumed cell mass increases, and the enzyme yield rises to a maximum level before conditions become deleterious. The

deleterious conditions develop due to exhaustion of essential nutrients or toxic/inhibitory end product accumulation. At the termination stage of the fermentation may be judged by various parameters like changes in dissolved oxygen, pH. $C0_2$ output, etc. At final stage the broth the transferred from the fermenter for harvesting of the cells followed by extraction of intracellular enzymes from biomass or extracellular enzymes from culture filtrate.

Fed Batch Culture

This fermentation technology is used when the substrate causes catabolite repression. The process needs feeding of additional nutrients, to extend the production phase at particular times and in response to specific physiological state of culture.

Continuous Culture

It needs continuous feeding of fresh sterile medium to the culture, while at the same time removing the same volume of broth. It maintains constant volume of culture for indefinite time in a steady state. The fermentation is carried in a chemostat.

Dual or multiple fermentations

Dual or multiple fermentations are those fermentations in which more than (me microorganism is employed. The organisms may be inoculated simultaneously into the growth medium, or one organism may be grown first in the medium, followed by the inoculation and growth of a second microorganism. Alternatively, after growth has occurred in the original media, two separate fermentations may be combined for further fermentation activity. The basic concept is that two or more microooorganisms accomplish something that neither organism can do' alone. Admittedly, in the state of present-day fermentation technology, this concept is more of a dream than a reality. The most obvious use of dual or multiple fermentations is to utilize one microorganism to_produce a fermentation product that is then converted or changed by a second microorganism or further microorganisms into a different fermentation product possessing greater economic value. Thus, a yeast first produces ethyl alcohol, and then an Acetobacter species converts the alcohol to vinegar. Another approach is to use one microorganism to change or prepare the medium so that it becomes suitable for the growth of a second microorganism. For example, the first microorganism may provide amylase or protease activity for the second microorganism, which lacks these abilities. Further uses of dual or multiple fermentations are the use of an organism to remove the

toxic metabolic by-products of another organism, an organism to provide growth factors for another organism, an organism to remove oxygen or depress oxidation-reduction potential for an anaerobic organism, or an organism to maintain a pH range critical for a second organism. In addition, one organism may produce a metabolic product, such as lactic acid, which both is beneficial to the growth of a second organism, such as a yeast, and at the same time helps to control contamination.

Simultaneous growth of two fermentation microorganisms in a single medium presents a problem in microbial ecology. Each organism must contend with the physiological, growth, and nutrient utilization activities of the other, and it is likely that their growth rates will differ so that one organism will outgrow the other. Thus, extensive studies of media and other fermentation conditions are required to balance the growth of the two or more organisms. This problem becomes either simplified or magnified if some form of symbiosis exists between the organisms, so that they are dependent on each other for growth.

Dual or multiple fermentations in which one organism is grown in a medium followed by the inoculation and growth of a second organism are easier to control from a fermentation standpoint. This is particularly true if it is feasible to kill the first organism by heat or other form of sterilization before inoculation with the second organism. The basic achievements of this approach are similar, however, to those of simulltaneous-inoculation dual fermentations. Examples of this approach are the initial growth of a proteolytic or amylolytic organism \n the medium to prepare it for succeeding growth of an organism not possessing these activities, the growth of lactic acid bacteria for succeeding yeasts, and the production of ethanol by a yeast followed by oxidation of the ethanol to acetic acid by an Acetobacter species.

Dual or multiple fermentations, as described above, present intriguing possibilities for the industrial utilization of microorganisms. However, much study still is needed in this area, particularly in those instances in which microorganisms are to be grown simultaneously in the same medium. Thus, a greater understanding of microbial ecology would contribute greatly to the industrial potential for various of these fermentations.

Lecture 28

Immobilization of enzymes - Arresting of cell in insoluble matrix - Immobilized cell systems- cell attachment to a surface - Aggregation - Entrapment - Containment - Physical adsorption- Covalent binding - Cross linking - Entrapment into polymeric films - Microencapsulation -Large scale cell immobilization - uses and applications in industries

Immobilization of enzymes

Enzymes are the nature's supreme catalysts, which exhibit great specificity and enormous catalytic power. Their use in food processing for many centuries have been extended in recent years to use in chemical production, analytical and diagnostic systems and in treatment of diseases. The prospects of enzyme application in various industries has been revolutionized by the introduction of technique namely "enzyme immobilization".

The process of immobilization is defined as "the imprisonment of a biocatalyst in a distinct phase that allows exchange with, but is separated from, bulk phase in which substrate, effector or inhibitor molecules are dispersed and monitored". In simple words, it is a technique in which the enzyme is arrested in an inert and usually insoluble matrix belongs to a high molecular weight polymer such as polyacrylamide, cellulose, starch, glass, beads etc. For the first time, J,M Nelson and E.G. Griffin have reported the phenomenon of enzyme immobilization. Some of the important immobilized enzymes having wider usage are amino acid acylase, penicillin Gacylase, glucose isomerase, aspartase, esterase and nitrilase.

Immobilized enzymes are preferred over immobilized cells or tissues, as the enzymes show high specificity and ability to yield pure products. The enzyme immobilization process under mild and controlled conditions allows the enzymes to retain their tertiary and quaternary structures which are necessary for their enzymatic activity. The process

of enzyme immobilization can become effective when follow some important points such as

(1) Selection of appropriate enzyme.
(2) Selection of appropriate immobilization procedure.
(3) There should be no leakage of enzymes.
(4) There should be no diffusion restrictions for substrate.
(5) There should be no compression or deterioration in reactor.
(6) Selection of conditions to maximize activity and stability.

The advantages of using immobilized enzymes are :

(1) Reuse or continuous use is possible.
(2) The enzyme (biocatalyst) does not contaminate the product.
(3) Saving in capital cost.
(4) Stability of enzyme is increased.
(5) Smaller size reactor can be used to achieve the same productivity because of increased concentration of biocatalyst.
(6) Even distribution of enzymes throughout the reactor is possible in immobilized system which ensures the proper supply of the enzyme.
(7) Various types of available reactors can be advantageously exploited.
(8) Reaction can be achieved in minimum reaction time.

METHODS OF IMMOBILIZATION

Basing on the physical relationship of the catalyst to the polymer matrix, the enzyme immobilization methods may be subdivided into various groups viz.,

(1) Adsorption method
(2) Covalent bonding method
(3) Cross binding method
(4) Entrapping method and
(5) Encapsulation method

(1) Adsorption method : This is this mildest and probably the earliest method of immobilization of enzymes. Adsorption of an enzyme to a polymer material is mediated by a range of specific or non-specific bonding forces such as electrostatic, hydrophpobic or affinity bonding to specific ligands. The cellulose based ionexchange resin composed of substances such as DEAE-cellulose, DEAE-sephadex. Carboxymethyl cellulose, Carboxy-methyl Sephades, Collagen, Silica gel etc., are used as adsorbents.

This method involves simple stirring of biocatalyst with ion exchange resin. During immobilization by this method, the p^H and ionic conditions of enzyme and adsorbent solution should be carefully controlled.

Advantages : The potential advantages of this method are (1) The ease and simplicity, (2) Mildest treatment during preparation of immobilized material, (3) No limitation or little limitation access of substrate to the bound enzyme. (4) Availability of readily prepared polymer material suitable for use in column reactors and (5) Potential for regeneration of the immobilized catalyst.

Disadvantages : However, this method also possess two main disadvantages viz., (1) Occurrence of relatively easy leaching of biocatalyst with changes in p^H or ionic strength and (ii) Some problems may across if the substrate carry the same charge as that polymer used as support material.

(2) Covalent bonding method : It is the most widely used technique. During the process, the enzyme covalently attach to the supporting polymer material. The supporting materials used for the purpose are Cellulose, Agar, Agarose, Sephades, Acrylate, Ureathane and Methancrylate polymers. The covalent bonding of an enzyme to a polymer can be brought out either (i) by activating the polymer with reactive group or (ii) by the use of bifunctional reagent to bridge between enzyme and polymer. The principal groups of an enzyme through which it is coupled are hydroxyl and amino groups and to a lesser extent, sulphydryl groups.

Advantages : The advantages of this method are (i) specific binding (ii) little or no clution, (iii) wide choice of carriers ad methods etc.

Disadvantages : The disadvantages are (i) expensiveness, (ii) procedural complication and (iii) difficulty in regeneration of immobilized catalyst.

Covalent bonding, (b) Ionic bonding

(3) Cross – Binding method : This method is an extension of covalent bonding technique. In this method, the enzyme can be immobilized by cross linking to a bifunctional or multifunctional reagent without involving a solid support. The most oftenly used reagent is glutaraldehyde. Some other examples of reagents used to immobilize enzymes by cross linking are diazobenzidine, Hexamethylene diisocyanate, Toulene 2, 4-diisothiocyanate etc. The reagent or the bridge chemical reacts with the enzyme and forms a linkage. This technique is cheap and simple but it is not often used with pure proteins because it yields very little bulk of immobilized enzyme with a very high intrinsic activity. Best because of its low cost. This method is widely used in commercial preparations of immobilized enzymes derived from non-viable cells or crude cell extracts.

(4) Entrapping method : Immobilization process by this method involves the entrapping of enzyme, instead of direct attachment to support surface, within a cross linked matrix of a water soluble polymer. Entrapment is carried out by mixing biocatalyst in monomer solution, followed by polymerization initiated by chemical reaction or change in temperature. The polymer matrix can be found in particulate form or block. The important polymer matrices widely used for entrapping the enzymes are polyacrylamide, cellulose triacetate, agar, collagen, gelatin, carrageenan, alginate etc.

The advantages of this method are simplicity, milder conditions of preparation, minimum constraints on enzymes. The disadvantages are elution of biocatalyst from some tells; abrasion; compaction of some gels and diffusion restrictions for larger substrates.

The recent modification of this technique is spinning fibers of cellulose triacetate or calcium alginate within which enzyme is trapped. The particular advantage of these fibre preparations is their relatively high surface area to volume ratio and thus reduced diffusion limitation.

(5) Encapsulation method : This technique involves the encapsulation of enzyme in a semipermeable membrane capsule which allows the passage of substrate molecules and product molecules. But the enzyme is not allowed to pass through membrane. Materials used to propare membrane capsules are nylon, polylactic acid, phospholipid liposomes, cellulose nitrate phospholipids, silastic resin etc. The capsules of defined size can be easily formed by alteration of the preparation conditions. To be effective, the biocatalyst must be stable in solution. The advantages of this technique are simplicity, less expensive and very high surface area to volume ratio. The disadvantages are less protection and less stability of the encapsulatd enzyme.

The choice of immobilization process is usually based on several factors. The choice of catalyst for use in a reactor should consider some points such as the nature of the biocatalyst the need for immobilization, the nature of reaction, polymer support material etc. The selecting polymer matrix for immobilization should be in expensive, good grade material, stable, incompressible, non-brittle, retainable with the reactor resist microbial attack, show no leaching, have a high surface area to volume ratio, allow easy coupling of enzyme etc. The various immobilization methods can be employed singly or in combination of one or two as per the necessity to obtain good results.

Lecture 29

Application of Biotechnology in food - building up of high biological value protein - Nucleicacid sequences as diagnostic tools - Protein engineering - Vitamin production - Amino acid production - Antibiotic production – Biopolymers

Increased nutritional qualities

Proteins in foods may be modified to increase their nutritional qualities. Proteins in legumes and cereals may be transformed to provide the amino acids needed by human beings for a balanced diet. A good example is the work of Professors Ingo Potrykus and Peter Beyer in creating Golden rice.

Improved taste, texture or appearance of food

Modern biotechnology can be used to slow down the process of spoilage so that fruit can ripen longer on the plant and then be transported to the consumer with a still reasonable shelf life. This alters the taste, texture and appearance of the fruit. More importantly, it could expand the market for farmers in developing countries due to the reduction in spoilage. However, there is sometimes a lack of understanding by researchers in developed countries about the actual needs of prospective beneficiaries in developing countries. For example, engineering soybeans to resist spoilage makes them less suitable for producing tempeh which is a significant source of protein that depends on fermentation. The use of modified soybeans results in a lumpy texture that is less palatable and less convenient when cooking.

The first genetically modified food product was a tomato which was transformed to delay its ripening. Researchers in Indonesia, Malaysia, Thailand, Philippines and Vietnam are currently working on delayed – ripending papaya in collaboration with the University of Nottingham and Zeneca. Biotechnology in cheese production: enzymes

produced by micro-organisms provide and alternative to animal rennet—a cheese coagulant – and an alternative supply for cheese makers. This also eliminates possible public concerns with animal-derived material, although there are currently no plans to develop synthetic milk, thus making this argument less compelling. Enzymes offer an animal-friendly alternative to animal rennet. While providing comparable quality, they are theoretically also less expensive.

About 85 million tons of wheat flour is used every year to bake bread. By adding an enzyme called maltogenic amylase to the flour, bread stays fresher longer. Assuming that 10 -15% of bread is thrown away as slate, if it could be made to stay fresh another 5-7 days then perhaps 2 million tones of flour per year would be saved. Other enzymes can cause bread to expand to make a lighter loaf, or alter the loaf in a range of ways.

Reduced dependence on fertilizers, pesticides and other agrochemicals

Most of the current commercial applications of modern biotechnology in agriculture are on reducing the dependence of farmers on agrochemicals. For example, Bacillus thuringiensis (Bt) is a soil bacterium that produces a protein with insecticidal qualities. Traditionally, a fermentation process has been used to produce an insecticidal spray from these bacteria. In this form, the Bt toxin occurs as an inactive protoxin, which requires digestion by an insect to be effective. There are several Bt toxins and each one is specific to certain target insects. Crop plants have now been engineered to contain and express the genes for Bt toxin, which they produce in its active form. When a susceptible insect ingests the transgenic crop cultivar expressing the Bt protein, it stops feeding and soon thereafter dies as a result of the Bt toxin binding to its gut wall. Bt corn is now commercially available in a number of countries to control corn borer (a lepidopteran insect), which is otherwise controlled by spraying (a more difficult process).

Crops have also been genetically engineered to acquire tolerance to broad-spectrum herbicide. The lack of herbicides with broad-spectrum activity and no crop injury was a consistent limitation in crop weed management. Multiple applications of numerous herbicides were routinely used to control a wi9de range of weed species detrimental to agronomic crops. Weed management tended to rely on pre emergence – that is, herbicide applications were sprayed in response to expected weed infestations rather than in response to actual weeds present. Mechanical cultivation and hand weeding were often necessary to control weeds not controlled by herbicide applications. The introduction of herbicide-tolerant crops has the potential of reducing the number of herbicide active ingredients used for weed management, reducing the number of herbicide applications made during a season, and increasing yield due to improved weed management and less crop injury. Transgenic crops that express tolerance to

glyphosate, glufosinate and bromoxynil have been developed. These herbicides can now be sprayed on transgenic crops without inflicting damage on the crops while killing nearby weeds.

From 1996 to 2001, herbicide tolerance was the most dominant trait introduced to commercially available transgenic crops, followed by insect resistance. In 2001, herbicide tolerance deployed in soybean, corn and cotton accounted for 77% of the 626,000 square kilometers planted to transgenic crops; Bt crops accounted for 15%; and “stacked genes” for herbicide tolerance and insect resistance used in both cotton and corn accounted for 8%.

Production of novel substances in crop plants

Biotechnology is being applied for novel uses other than food. For example, oilseed can be modified to produce fatty acids for detergents, substitute fuels and petrochemicals. Potatoes, tomatoes, rice tobacco, lettuce, safflowers, and other plants have been genetically engineered to produce insulin and certain vaccines. If future clinical trials prove successful, the advantages of edible vaccines would be enormous, especially for developing countries. The transgenic plants may be grown locally and cheaply. Homegrown vaccines would also avoid logistical and economic problems posed by having to transport traditional preparations over long distances and keeping them cold while in transit. And since they are edible, they will not need syringes, which are not only an additional expense in the traditional vaccine preparations but also a source of infections if contaminated. In the case of insulin grown in transgenic plants, it is well-established that the gastrointestinal system breaks the protein down therefore this could not currently be administered as an edible protein. However, it might be produced at significantly lower cost than insulin produced in costly bioreactors. for example, Calgary, Canada-based SemBioSys Genetics, Inc.reports that its safflower-produced insulin will reduce unit costs by over 25% or more and approximates a reduction in the capital costs associated with building a commercial scale insulin manufacturing facility of over $100 million, compared to traditional biomanufacturing facilities.

Herbicide

There is another side to the agricultural biotechnology issue. It includes increased herbicide usage and resultant herbicide resistance, “ super weeds,” residues on and in food crops, genetic contamination of non-GM crops which hurt organic and conventional farmers ,etc.

Bioremediation and biodegradation

Biotechnology is being used to engineer and adapt organisms especially microorganisms in an effort to find sustainable ways to clean up contaminated environments. The elimination of a wide range of pollutants and wastes from the environment is an absolute requirement to promote a sustainable development of our society with low environmental impact. Biological processes play a major role in the removal of contaminants and biotechnology is taking advantage of the astonishing catabolic versatility of microorganisms to degrade/convert such compounds. New methodological breakthroughs in sequencing, genomics, proteomics, bioinformatics and imaging are producing vast amounts of information. In the field of Environmental Microbiology, genome based global studies open a new era providing unprecedented in silico views of metabolic and regulatory networks, as well as clues to the evolution of degradation pathways and to the molecular adaptation strategies to changing environmental conditions. Functional genomic and metagenomic approaches are increasing our understanding of the relative importance of different pathways and regulatory networks to carbon flux in particular environments and for particular compounds and they will certainly accelerate the development of bioremediation technologies and biotransformation processes.

Marine environments are especially vulnerable since oil spills of coastal regions and the open sea are poorly containable and mitigation is difficult. In addition to pollution through human activities, millions of tons of petroleum enter the marine environment every year from natural seepages. Despite its toxicity, a considerable fraction of petroleum oil entering marine systems is eliminated by the hydrocarbon-degrading activities of microbial communities, in particular by a remarkable recently discovered group of specialists, the so-called hydrocarbonoclastic bacteria (HCCB).

Lecture 30

Application of Biotechnology in Pharmaceuticals - Identification of disease causing genes- Production of Hormones - Vaccines - Interferon - Regulatory proteins – antibiotics

Applications

Biotechnology has applications in four major industrial areas, including health care (medical), crop production and agriculture, non food (industrial) uses of crops and other products (e.g. biodegradable plastics, vegetable oil, biofuels), and environmental uses.

For example, one application of biotechnology is the directed use of organisms for the manufacture of organic products (examples include beer and milk products). Another example is using naturally present bacteria by the mining industry in bioleaching. Biotechnology is also used to recycle, treat waste, clean up sites contaminated by industrial activities (bioremediation), and also to produce biological weapons.

Pharmaceuticals

In medicine, modern biotechnology finds promising applications in such areas as

- Drug production
- Pharmacogenomics

Pharmacogenomics

Pharmacogenomics is the study of how the genetic inheritance of an individual affects his /her body's response to drugs. It is derived from the words "pharmacology" and "genomics". It is hence the study of the relationship between pharmaceuticals and

genetics. The vision of pharmacogenomics is to be able to design and produce drugs that are adapted to each person's genetic makeup.

Pharmacogenomics results in the following benefits:

1. Development of tailor-made medicines. Using pharmacogenomics, pharmaceutical companies can create drugs based on the proteins, enzymes and RNA molecules that are associated with specific genes and diseases. These tailor-made drugs promise not only to maximize therapeutic effects but also t decrease damage to nearby healthy cells.
2. More accurate methods of determining appropriate drug dosages. Knowing a patient's genetics will enable doctors to determine how well his/her body can process and metabolize a medicine. This will mazimize the value of the medicine and decrease the likelihood of overdose.
3. Improvements in the drug discovery and approval process. The discovery of potential therapies will be made easier using genome targets. Genes have been associated with numerous diseases and disorders. With modern biotechnology, these genes can be used as targets for the development of effective new therapies, which could significantly shorten the drug discovery process.
4. Better vaccines. Safer vaccines can be designed and produced yb organisms transformed by means of genetic engineering. These vaccines will elicit the immune response without the attendant risks of infection. They will be inexpensive, stable, easy to store, and capable of being engineered to carry several strains of pathogen at once.

Pharmaceutical products

Most traditional pharmaceutical drugs are relatively simple molecules that have been found primarily through trial and error to treat the symptoms of a disease or illness.

Biopharmaceuticals are large biological molecules such as proteins and these usually target the underlying mechanisms and pathways of a malady (but not always, as is the case with using insulin to treat type I diabetes mellitus, as that treatment merely addresses the symptoms of the disease, not the underlying cause which is autoimmunity); it is a relatively young industry. They can deal with targets in humans that may not be accessible with traditional medicines. A patient typically is dosed with a small molecule via a tablet while a large molecule is typically injected.

Small molecules are manufactured by chemistry but larger molecules are created by living cells such as those found in the human body: for example, bacteria cells, yeast cells, animal or plant cells.

Modern biotechnology is often associated with the use of genetically altered microorganisms such as E.coli or yeast for the production of substances like synthetic insulin or antibiotics. It can also refer to transgenic animals or transgenic plants, such as Bt corn. Genetically altered mammalian cells, such as Chinese Hamster Ovary cells (CHO), are also used to manufacture certain pharmaceuticals. Another promising new biotechnology application is the development of plant-made pharmaceuticals.

Biotechnology is also commonly associated with landmark breakthroughs in new medical tehreapies to treat hepatitis B, hepatitis C, cancers, arthritis, haemophilia, bone fractures, multiple aclerosis, and cardiovascualar disorders. The biotechnology industry has also been instrumental in developing molecular diagnostic devices that can be used to define the target patient population for a given biopharmaceutical. Herceptin, for example, was the first drug approved for use with a matching diagnostic test and is used to treat breast cancer in women whose cancer cells express the protein HER2.

Modern biotechnology can be used to manufacture existing medicines relatively easily and cheaply. The first genetically engineered products were medicines designed to treat human diseases. To cite one example, in 1978 Genentech developed synthetic humanized insulin by joining its gene with a plasmid vector inserted into the bacterium Escherichia coli. Insulin, widely used of the treatment of diabetes, was previously extracted from the pancreas of abattoir animals (cattle and /or pigs). The resulting genetically engineered bacterium enabled the production of vast quantities of synthetic human insulin at relatively low cost. According to a 2003 study undertaken by the International Diabetes Federation (IDF) on the access to and availability of insulin in its member countries, synthetic ‘human’ insulin is considerably more expensive in most countries where both synthetic ‘human’ and animal insulin are commercially available: e.g. within European countries the average price of synthetic ‘human’ insulin was twice as high as the price of pork insulin. Yet in its position statement, the IDF writes that “there is no overwhelming evidence to prefer one species of insulin over anther” and “(modern, highly purified) animal insulins remain a perfectly acceptable alternative.

Modern biotechnology has evolved, making it possible to produce more easily and relatively cheaply human growth hormone, clotting factors for hemophiliacs, fertility drugs, erythropoietin and other drugs. Most drugs today are based on about 500 molecular targets. Genomic knowledge of the genes involved in diseases, disease pathways, and drug-response sites are expected to lead to the discovery of thousands more new targets.

Lecture 31

Application of Biotechnology in Agriculture - Improvement of nutritional quality - post harvest technology - Changing plants at their genetic level - To develop nitrogen fixation – Production of disease resistant plants

Agriculture

- Crop yield
- Reduced vulnerability of crops to environmental stresses
- Increased nutritional qualities
- Improved taste, texture or appearance of food
- Reduced dependence on fertilizers, pesticides and other agrochemicals
- Production of novel substances in crop plants
- Herbicide
- Bioremediation and biodegradation

Crop yield

Using the techniques of modern biotechnology, one or two genes(Smartstax from Monsanto in collaboration with Dow AgroSciences will use 8, starting in 2010) may be transferred to a highly developed crop variety to impart a new character that would increase its yield. However, while increases in crop yield are the most obvious applications of modern biotechnology in agriculture, it is also the most difficult one. Current genetic engineering techniques work best for effects that are controlled by a single gene. Many of the genetic characteristics associated with yield (e.g., enhanced growth) are controlled by a large number of genes, each of which has a minimal effect on the overall yield. There is, therefore, much scientific work to be done in this area.

Reduced vulnerability of crops to environmental stresses

Crops containing genes that will enable them to withstand biotic and abiotic stresses may be developed. For example, drought and excessively salty soil are two important limiting factors in crop productivity. Biotechnologists ar3e studying plants that can cope with these extreme conditions in the hope of finding the genes that enable them to do so and eventually transferring these genes to the more desirable crops. One of the latest developments is the identification of a plant gene, At-DBF2, from Arabidopsis thaliana, a tiny weed that is often used for plant research because it is very easy to grow and its genetic code is well mapped out. When this gene was inserted into tomato and tobacco cells (see RNA interference), the cells were able to withstand environmental stresses like salt, drought, cold and heat, far more than ordinary cells. If these preliminary results prove successful in larger trials, then At-DBF2 genes can help in engineering crops that can better withstand harsh environments. Researchers have also created transgenic rice plants that are resistant to rice yellow mottle virus (RYMV). In Africa, this virus destroys majority of the rice crops and makes the surviving plants more susceptible to fungal infections.

Lecture 32

Bio gas plant - Anaerobic digestion - Methane formation - Methanogenic fermentations -Methane oxidation - Hydrocarbon degradation - Anaerobic digester designs - positive andnegative features of anaerobic process Energy production methods - methanogens - incarnation - biofuel - Bioremediation - Bioventing - Land farming - Bio augmentation - use of spent mushroom compost

COMMERICIAL PRODUCTION OF BIOGAS

India is one of the pioneer country in Biogas technology. There are many institutions where reasearches and development programmes are carried out. These are Khadi and village Industries Commission (KVIC) Bombay, National Environmental Engineering Research Institute (NEERI) Nagapur, and NEDA of U.P.

Biogas can be defined as a composition of different gases which are produced as a result of action of anaerobic microorganisms on raw materials such as domestic and agricultural wastes in biogas plant. Biogas contains methane in bulk, and other gases like CO_2, H_2, N_2, O_2 etc.

Raw materials used in production of biogas : Biomass generated from animals include cattle dung, manure from poultry, goat, sheep, fisheries waste etc. Cattle dung is the most potential biomass for biogas production. Plant biomass includes agricultural residuce, deteriorated wheat grain etc.

Biogas is the main product of anaerobic digestion (municipal treatment). The process of anaerobic digestion consist of three steps.

IV. Hydrolysin or Solubilisation : In this stage the feed stock (Municipal waste, surg) is solubilised by water and enzymes. The complex organic polymers like carbohydrates, fats, proteins are hydroglysed into simple monomers by the action of enzymes of hydrolytic fermentative methanogenic bacteria.

Carbohydrates ⟶ Glucose ⟶ alcohol

Fats ⟶ Fatty acids

V. Acedogenesiss (Acid formation) : In this step hydrolysed compounds are converted intracellularly into simple compounds by Acedogenic bacteria.

Fatty acids Acetogenci CH_3 COOH

⟶ + HCOOH + CO_2

Alcohols bacteria acetic acid

VI. Methanogenesis (Methane formation) : This is final stage where methane gas is formed. In this stage simple compounds are mainly converted into methane and CO_2 by anaerobic bacteria like Methanosarcina, Methanothrix, Methanobacterium.

Methenogenic $12CH_4$

(3) CH_3 COOH + CO_2 + $12CO_2$

(4) H COOH + $_cO_2$ ⟶ $2H_2O + 3CO_2$

Methenogenic

Bacteria

COMMERCIAL PRODUCTION : Biogas is produced on commercial scale from dung human waste and other waste with help of microorganisms by using biogas plant.

The dung consist nearly 20% of inorganic particles. The percentage of inorganic salts can be reduced by adding water. When dung and water is mixed in 1 : 1 ration 10% of inorganic salts are maintained. This ratio of inorganic salts is sufficient for optimum production of biogas. The required dung feeding rate for optimum production of biogas is estimated as 3500 kg per day.

Apart from cattle waste (dung) many other inputs are useful in generating more biogas through increasing the growth of bacteria. Dung + 2 – 3% of fresh slurry and calcium ammonium nitrate which is equal to 50% of fresh slurry is an optimum combination for maximum production of biogas. Another 2% of biogas production can be increased by mixing human waste to the dung. 35^0C – 38^0C is optimum temperature required for maximum production. The optimum pH is 7 (neutral) required.

Use of culture : Some bacteria and archebacteria are more active than other bacteria. These types are isolated and mixed with inputs in the digester which can increase biogas production substantially.

Mechanism of Methane formation : Several coenzymes, CO_2 reducing factors, activating factors are necessary for methane formation. Coenzymes include methyl coenzymes. Coenzyme – F420, coenzyme F430 etc.

III. $CO + H_2O \longrightarrow CO_2 + H_2$

IV. $CO_2 + 4H_2 \longrightarrow CH_4 + 2H_2O$

Methani is also formed form other substrates.

$$4CH_3OH \longrightarrow 3CH_4 + 2H_2O + CO_2$$

Factors affecting Methanogenesis :

5. Small amount of sludge of another digester activate methane production.
6. Temparature between 30^0 – 60^0 C and pH at 6-8 favours the methane formation.
7. Proper C : N ratio i.e., 30 : 1 favours methane formation.
8. Methanogenesis takes place only in anaerobic conditions. Hence in India digesters are buried in the soil.

Advantages of Biogas :

(3) It is a substitute for fire wood, electricity, kerosene and LPG. Hence it can reduce environmental pollution.

(4) It can be used as a better fuel for cooking, heating lighting, power for irrigation etc.

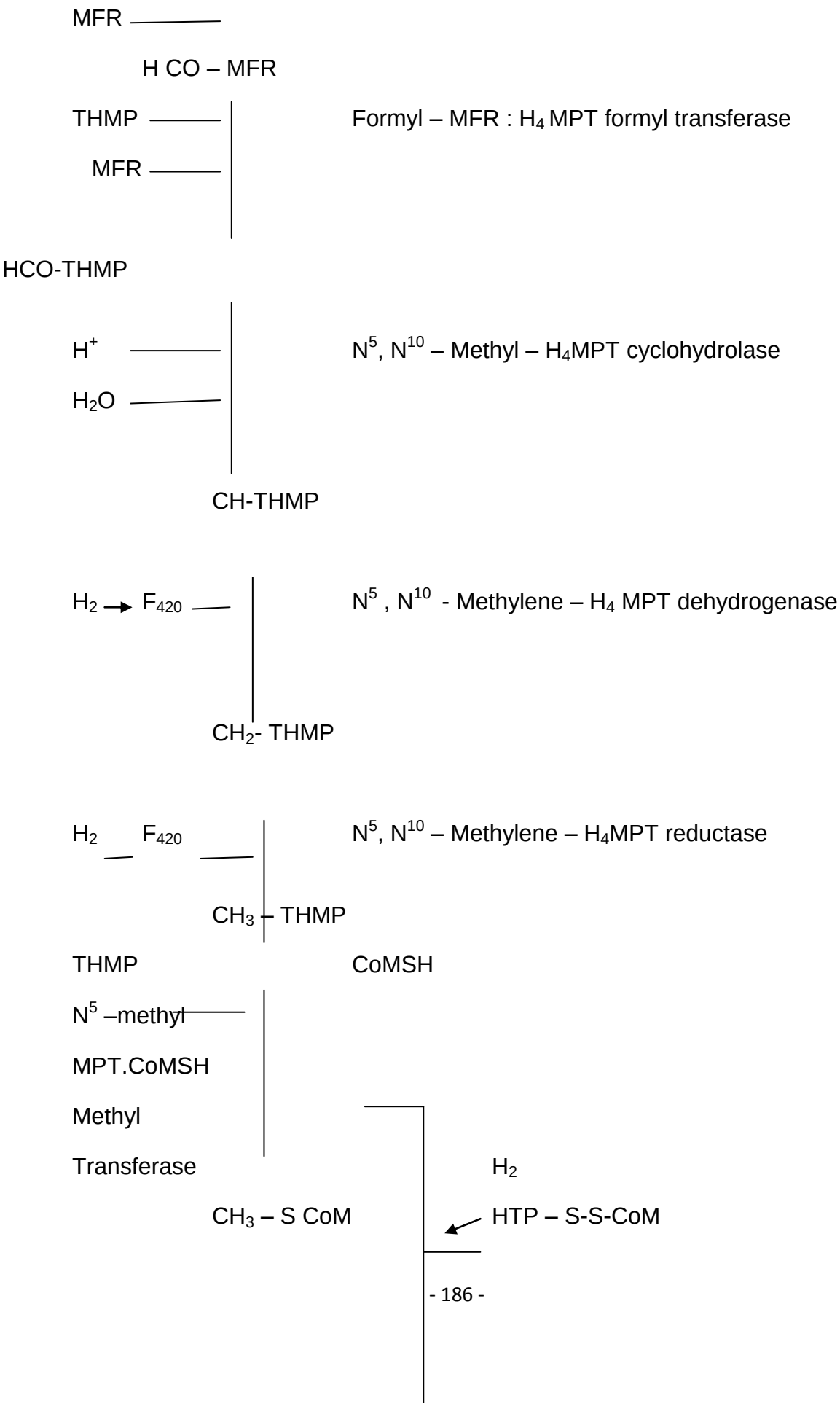
Biochemistry : The biochemical conversion of H_2 and CO_2 to methane and of acetate of methane and CO_2 involve a number of coenzymes and prosthatic groups like “Deazari boflavin derivative EUR, Methanopterin, Methanofuran, the nickel tetrapyrol factor FU30 and coenzymke M-Merceaptoethane sulphate.

The production of methane yields ATP and is the only means of ATP formation for the methanogenic bacteria. For methane synthesis the methyl group is transferred from CH_3 – THMP to CoMSH to form CH_3 – SCOM. The terminal reduction is catalyzed by the “methyl reductase system”.

$$CH_3 – THMP + CoMSH \xrightarrow{\text{methyl}} CH_3 - SCoM$$

Reductase system

CO_2	**Enzymes**
H_2	Formyl – MFR dehydrogenase


MFR
H CO – MFR
THMP
MFR
Formyl – MFR : H_4 MPT formyl transferase
HCO-THMP
H^+
H_2O
N^5, N^{10} – Methyl – H_4MPT cyclohydrolase
CH-THMP
H_2
F_{420}
N^5 , N^{10} - Methylene – H_4 MPT dehydrogenase
CH_2- THMP
H_2
F_{420}
N^5, N^{10} – Methylene – H_4MPT reductase
CH_3 – THMP
THMP
CoMSH
N^5 –methyl
MPT.CoMSH
Methyl
Transferase
CH_3 – S CoM
H_2
HTP – S-S-CoM

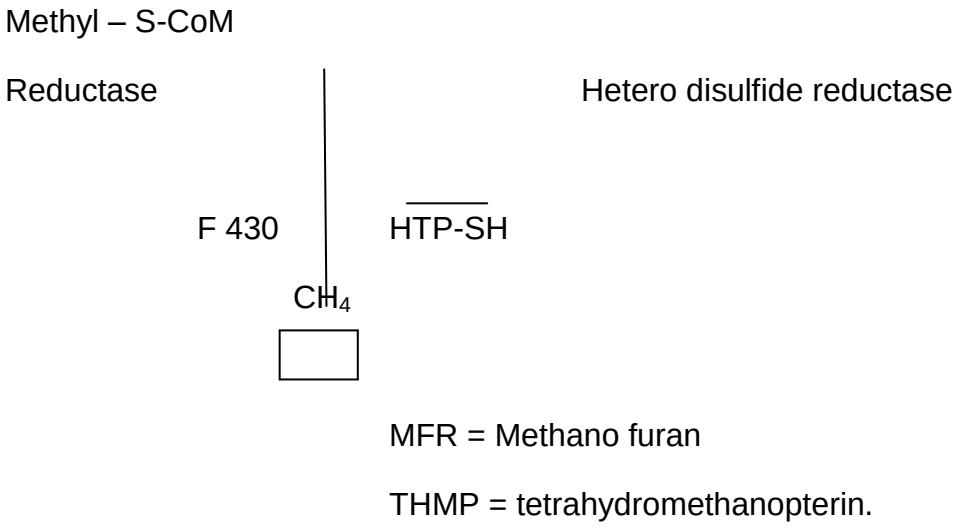


Fig : **The Methane production pathway**.

Solid state fermentation

Solid State Fermentation : In such fermentations, microbial growth and product formation occur at the surface of solid substrates. Examples of such fermentations are mushroom cultivation, mold – ripened cheeses, starter cultures etc.

More recently, this approach has been used for the production of extracellular enzymes, certain valuable chemicals, fungal toxins, and fungi spores (used for biotransformation). Traditional substrates are several agricultural products. Rice, wheat, maize, soybean etc. The substrate provides a rich and complex source of nutrients which may or may not need to be supplemented. Such substrates selectively support mycelia organisms which can grow at high nutrient concentrations and produce a variety of extracellular enzymes, e.g., a large number of filamentous fungi, and a few bacteria (actinomycetes and one strain of Bacillus). According to the physical state, solid state fermentations are divided into two groups.

(iii) Low moisture solids fermented without or with occasional/continuous agitation, and
(iv) Suspended solids fermented in packed columns through which liquid is circulated.

The fungi used for solid state fermentations are usually obligate aerobes

Patent concept :

Important part of the biotechnology industry concerns patenting or intellectual property rights. Intellectual property rights (IPRs) have been created to protect the right of individuals to enjoy their creations and discoveries.