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Lecture one

Apdvanced Microbial Technology Lec. 1

- Principles

Biotechnology- "Any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants or animals, or to develop microorganisms for specific use.

Microbial technology involve the use of microorganisms to achieve specific goals, whether creating new products or improving the environment. For example, one application is the directed use of organisms for the manufacture of products such as pharmaceutical and medical compounds (antibiotics, hormones, transformed steroids), solvents, organic acids, amino acids, and enzymes that have direct economic value. Another example is using naturally present bacteria by the mining industry in bioleaching. It is also used to recycle, treat waste, cleanup sites contaminated by industrial activities (bioremediation), and also to produce biological weapons.

biotechnology has developed rapidly in the last several decades, and is characterized by the modification of microorganisms through the use of molecular biology, including the use of recombinant DNA technology .

Although there several classifications of biotechnology, the one using color code is most popularly used. Most commonly four major colors are considered, namely red, green, white and purple.

The biggest number of colors is used by the code, which divides biotechnologies into *:*

· green one, which is devoted to the development of agriculture
· yellow one, which might be called nutritional biotechnology
· red one, which is devoted to medicine and human health
· white one, namely industrial biotechnology
· gray one, which is devoted to the problems of environmental protection
· blue biotechnology of marine (aquatic) regions
· brown biotechnology of dessert and dry regions

· gold one, which is connected with bioinformatics, computer science and chip technology
· violet one, which deals with law, ethical and philosophic issues
· dark biotechnology connected biological weapons.

Choosing Microorganisms for Microbial technology

Selection and use of microorganisms in microbial technology are challenging tasks that require a solid understanding of microorganism growth and manipulation, as well as microbial interactions with other organisms. The use of microorganisms in microbial technology follows a logical sequence. It is necessary first to identify or create a microorganism that carries out the desired process in the most efficient manner.

The major sources of microbial cultures for use were natural materials such as soil samples, waters, and spoiled bread and fruit. Cultures from all areas of the world were examined in an attempt to identify strains with desirable characteristics. A wide variety of alternative approaches are available, ranging from isolating microorganisms from the environment to using molecular techniques to modify an existing microorganism. This microorganism then is used, either in a controlled environment such as a fermenter or in complex systems such as in soils or waters to achieve specific goals.

***Properties of useful microorganisms**

•Able to produce substance of interest in high yield
•Capable of growth and product formation in large scale
•Able to grow in cheap substrate
•Should not be pathogenic
•Should be genetically stable and easy to manipulate
•Preferable produce spores/other reproductive cells that easily inoculated into large fermenters.

Preservation of Microorganisms

Once a microorganism has been selected or created to serve a specific purpose, it must be preserved in its original form for further use and study.

Periodic transfers of cultures have been used in the past, although this can lead to mutations and phenotypic changes in microorganisms. To avoid these problems, a variety of culture preservation techniques may be used to maintain desired culture characteristics . **Lyophilization,** or freeze-drying, and storage in liquid nitrogen are frequently employed with microorganisms. Although lyophilization and liquid nitrogen storage are complicated and require expensive equipment, they do allow microbial cultures to be stored for years without loss of viability or an accumulation of mutations.

Genetic Manipulation of Microorganisms

Genetic manipulations are used to produce microorganisms with new and desirable characteristics. The manipulation of the genetic material in organisms can now be achieved in three clearly definable ways – organismal, cellular and molecular.

Organismal manipulation

Genetic manipulation of whole organisms has been happening naturally by sexual reproduction. In more recent times it has been used with several industrial microorganisms, e.g. brewing yeasts.

It involves selection, mutation, sexual crosses, hybridization, etc. However, it is a very random process and can take a long time to achieve desired results – if at all in some cases. The benefits in agriculture, there have been much improved plants and animals, while in the biotechnological industries there have been greatly improved productivities, e.g. antibiotics and enzymes.

Cellular manipulation

Cellular manipulations of DNA have been used for over two decades, and involve either cell fusion or the culture of cells and the regeneration of whole plants from these cells. examples of these methods include monoclonal antibodies and the cloning of many important plant species.

Molecular manipulation

Molecular manipulations of DNA and RNA a new era of genetic manipulations enabling – for the first time in biological history – a directed control of the changes. use of *genetic engineering* or *recombinant DNA technology*, which is now bringing dramatic changes to biotechnology. In

these techniques it is able to know much more about the genetic changes being made.
The classical methods of microbial genetics play a vital role in the development of cultures for microbial technology.

Mutation

Once a promising culture is found, a variety of techniques can be used for culture improvement, including chemical mutagens and ultraviolet light . As an example, the first cultures of *Penicillium notatum*, which could be grown only under static conditions, yielded low concentrations of penicillin then further improved through mutation . Today most penicillin is produced with *Penicillium chrysogenum*, grown in aerobic stirred fermenters, which gives 55-fold higher penicillin yields than the original static cultures.

Protoplast Fusion

Protoplast fusion is now widely used with yeasts and molds. Most of these microorganisms are asexual or of a single mating type, which decreases the chance of random mutations. To carry out genetic studies with these microorganisms, protoplasts are prepared by growing the cells in an isotonic solution while treating them with enzymes, including cellulase and beta-galacturonidase. The protoplasts are then regenerated using osmotic stabilizers such as sucrose.. After regeneration of the cell wall, the new protoplasm fusion product can be used in further studies. A major advantage of the protoplast fusion technique is that protoplasts of different microbial species can be fused, For example, protoplasts of *Penicillium roquefortii* have been fused with those of *P. chrysogenum*

Insertion of Short DNA Sequences

Short lengths of chemically synthesized DNA sequences can be inserted into recipient microorganisms by the process of **site directed mutagenesis.** This can create small genetic alterations leading to a change of one or several amino acids in the target protein. Such minor amino acid changes have been found to lead, in many cases, to unexpected changes in protein characteristics, and have resulted in new products such as more

environmentally resistant enzymes and enzymes that can catalyze desired reactions.

These approaches are part of the field of **protein engineering.** Site-directed mutagenesis Enzymes and bioactive peptides with markedly different characteristics (stability, kinetics, activities) can be created. The molecular basis for the functioning of these modified products also can be better understood.

Transfer of Genetic Information between Different Organisms

New alternatives have arisen through the transfer of nucleic acids between different organisms, which is part of the rapidly developing field of combinatorial biology . This involves the transfer of genes for the synthesis of a specific product from one organism into another, giving the recipient varied capabilities such as an increased capacity to carry out hydrocarbon degradation. degradation. The genes for antibiotic production can be transferred to a microorganism that produces another antibiotic, or even to a non-antibiotic-producing microorganism. For example, the genes for synthesis of bialophos (an antibiotic herbicide) were transferred from *Streptomyces hygroscopicus* to *S. lividans.*

DNA expression in different organisms can improve production efficiency and minimize the purification steps required before the product is ready for use. For example, recombinant baculoviruses can be replicated in insect larvae to achieve rapid large scale production of a desired virus or protein. Transgenic plants may be used to manufacture large quantities of a variety of metabolic products..

Genetic information transfer allows the production of specific proteins and peptides without contamination by similar products that might be synthesized in the original organism. This approach can decrease the time and cost of recovering and purifying a product.

Modern biotechnology

Modern biotechnology offers opportunities to improve product quality, nutritional content, and economic benefits. Among many, the two core techniques that enabled birth of modern biotechnology are :

(i) Genetic engineering : Techniques to alter the chemistry of genetic material (DNA and RNA),to introduce these into host organisms and thus change the phenotype of the host organism.

(ii) Maintenance of sterile (microbial contamination-free) ambience in chemical engineering processes to enable growth of only the desired microbe/eukaryotic cell in large quantities for the manufacture of biotechnological products like antibiotics,vaccines, enzymes, etc.

In microbial technology these techniques will be widely used to improve existing microbial processes by improving stability of existing cultures and eliminating unwanted side products.Recombinant DNA techniques will form the basis of new strains of microorganisms with new and unusual metabolic properties. A full understanding of the working concepts of recombinant DNA technology requires a good knowledge of molecular biology.

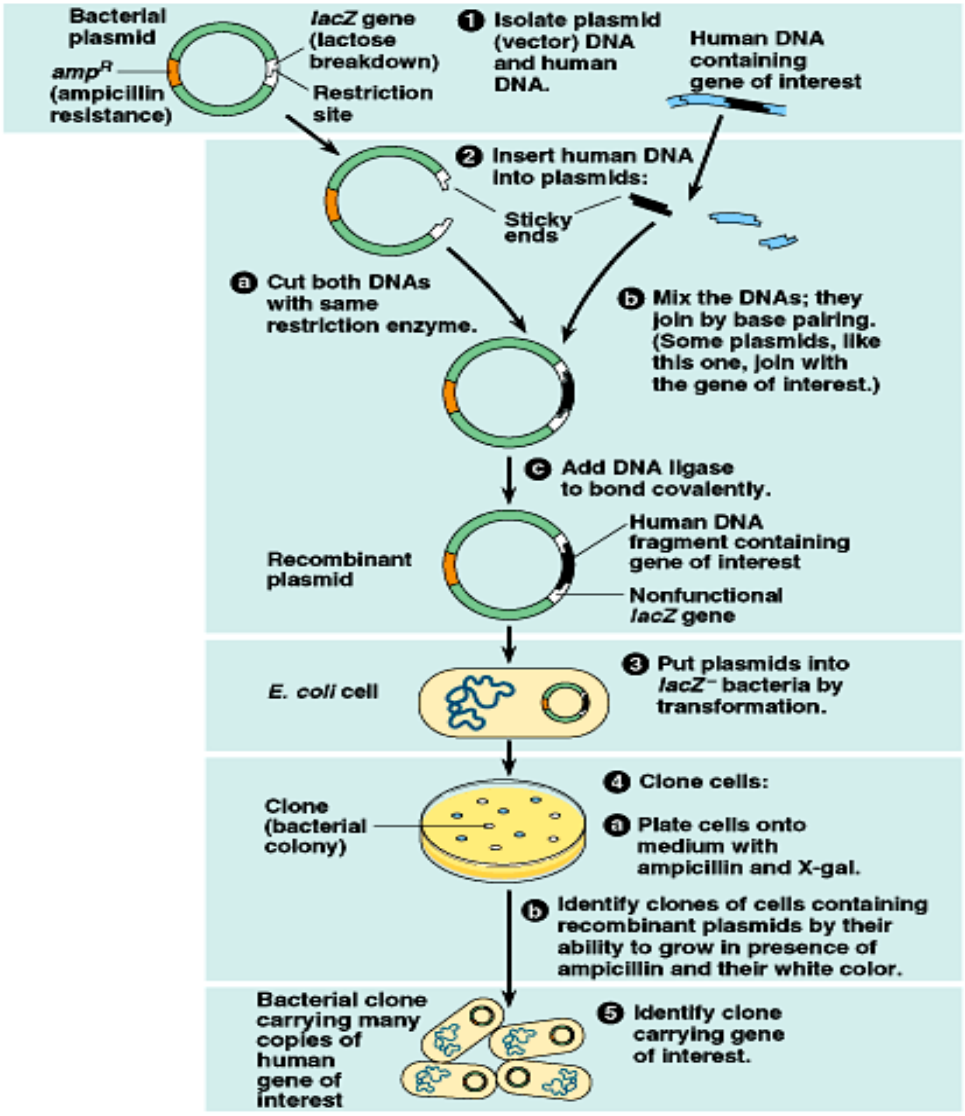
There are three basic steps in genetically modifying an organism :

(i) identification of DNA with desirable genes;
(ii) introduction of the identified DNA into the host;
(iii) maintenance of introduced DNA in the host and transfer of the DNA to its progeny.

TOOLS OF RECOMBINANT DNA TECHNOLOGY

Recombinant DNA technology involves several steps :

1. Isolation of donor DNA fragment or gene
2. Selection of suitable vector
3. Incorporation of donor DNA fragment into the vector
4. Transformation of recombinant vector into a suitable host cell
5. Isolation of recombinant host cell



1. Isolation of donor DNA fragment or gene

- At first a donor DNA fragment should be isolated. There are two method for isolation of desired gene or DNA fragment.
- Using restriction endonuclease enzyme: the enzyme restriction endonuclease is a key enzyme in molecular gene cloning. It has specific restriction site for its action. The enzyme RE generates a DNA fragment either with blunt end or with sticky end
- Using reverse transcriptase enzyme: reverse transcriptase enzyme Synthesizes complementary DNA strand of the desired gene using its mRNA.

2. Selection of suitable cloning vector:

- When donor DNA fragment is incorporated into a host cell, it will not replicates because the isolated gene do not have the capacity to replicated itself. So before introduction of donor fragment into host, a suitable vector should be selected.
- Cloning vector is the DNA molecule capable of self-replication inside the host cell. the main function of cloning vector is to replicates the inserted DNA fragment inside the host cell.
- Examples of cloning vectors: Plasmid, BAC, YAC, Λ-bacteriophase, expression vectors etc.

Some Distinct Features of Cloning Vectors

1. Origin of Replication – The specific sequence of nucleotide in a DNA segment recognized by the cellular DNA-replication enzymes. , which acts, as the origin of the replication process is known as ORI. Without replication origin, DNA cannot be replicated in the cell. The foreign DNA starts replicating along with the host cell when it is integrated or connected to this sequence.

2. Selectable marker gene – The cloning vector must possess a selectable marker gene as it allows the selection of the host cells, which carry the recombinant DNA, and separates them from those that do not

3. Presence of restriction sites – It should have restriction sites to enable breakup of certain sequences with respect to restriction endonuclease.

4. It must be not too big in size.
5. The insertion of donor DNA should not hamper the replication process and property of the cloning vector.
6. There must be multiple sites for cloning.
7. The vector and the sample DNA are both digested with the same restriction enzyme. It is then recombined so as to enable them to grow in a host. Vectors contain selectable markers that aid in determining which recombinant has to be inserted.

8. A vector need not necessarily contain elements suitable for the expressions of the target gene, but many do and hence may work as an expression vector.

Kinds of Cloning Vectors:

a. **Plasmid** – The size of a plasmid is 4361 bp, and the cloning limit is 0.1-10 kb. The marker gene is ampicillin and the tetracycline resistant gene whereas it remains isolated from the E.Coli. Example: PBR322
b. **Bacterial Artificial Chromosome** – The size of BAC is 11827 bp and the cloning limit is 35-300 kb. The marker gene is chloramphenicol and lactose metabolizing gene. It is a modification of f-plasmid and is artificially synthesized. Example: pUvBBAC
c. **Yeast Artificial Chromosome** – The size of YAC is 11400 bp and the cloning vector 100-1000 kb. The marker is similar to that of yeast. It is artificial and has yeast centromere, which is isolated from the Saccharomyces cerevisiae.
d. **Λ Bacteriophase** – Its size is 48502 bp and 1/3rd of this is not essential. It can only recombinant about 4-5 kbp of the donor DNA. An example is lambda genome.
e. **Cosmid** – The size of cosmid is 7900 bp and the cloning limit is 30-50 kb. It has features similar to both phase and plasmid and an example of it is super COS1
f. **Human Artificial Chromosome** – It is an artificial chromosome that is used to transfer human gene and has no limit on cloning as it can carry a large segment of the DNA.

3. Incorporation of donor DNA fragment with Plasmid vector:

- The plasmid vector is cut open by the same RE enzyme used for isolation of donor DNA fragment
- The mixture of donor DNA fragment and plasmid vector are mixed together.

- In the presence of DNA ligase, base pairing of donor DNA fragment and plasmid vector occurs forming recombinant vector in the mixture

4. Transformation of recombinant vector into suitable host:

- The recombinant vector is transformed into suitable host cell. ie bacterial cell
- Some bacteria are naturally transformable, they take up the recombinant vector automatically. For examples: *Bacillus*, *Haemophillus*, *Helicobacter pylori*, are naturally competent
- Some other bacteria are not naturally competent, in those bacteria recombinant vector are incorporated by artificial method such as Ca++ ion treatment, electroporation etc

5. Isolation of recombinant cell:

- The recombinant host cell is then grown in culture media but the culture may contains colonies both recombinant cell and non-recombinant cell.
- For isolation of recombinant cell from non-recombinant cell, marker gene of plasmid vector is employed.
- For examples, PBR322 plasmid vector contains different marker gene (Ampicillin resistant gene and Tetracycline resistant gene. When pst1 RE is used it knock out Ampicillin resistant gene from the plasmid, so that the recombinant cell become sensitive to Ampicillin.

Obtaining the Foreign Gene Product

When recombinant DNA is transferred into a bacterial, plant or animal cell, the foreign DNA is multiplied. Most of the recombinant technologies are aimed to produce a desirable protein. So there is a need for expression of recombinant DNA.

After the cloning of the gene of interest one has to maintain the optimum conditions to induce the expression of the target protein one should consider producing it on a large scale. If any protein encoding gene is expressed in a heterologous host it is known as a "recombinant protein".

The cells having cloned genes of interest can be grown on a small scale in the laboratory. The cultures may be used for extracting and purifying the desired protein.

The cells can also be multiplied in a continuous culture system where the used medium is passed out from one side and fresh medium is added from the other side to maintain the cells in their physiologically most active log/exponential phase— rapid multiplication of the cells. This type of culturing method produces a larger biomass to get higher yields of desired protein.

Bioreactors (Fermenters):

Bioreactors are considered as vessels in which raw materials are biologically converted into specific products by microbes, plant and animal cells and/or their enzymes. Small volume cultures cannot give large quantities of the products. Large scale production (100 – 1000 litres) of the products is carried out in bioreactors (Fig. 11.7). A bioreactor provides the optimal conditions for obtaining the desired product by providing optimum growth conditions such as temperature, substrate, vitamins, oxygen and salts.

Types of Bioreactors:

The most commonly used bioreactors are of stirring type. Stirring type bioreactors are:

(i) Simple stirred-tank bioreactor and

(ii) Sparged stirred-tank bioreactor as shown in Fig. 11.7. In the sparged bioreactor, sterile air bubbles are sparged. The surface area for oxygen transfer is increased.

Fermentation Process:

Fermentation is the process by which microorganisms turn raw material such as glucose into products such as alcohol. The term fermentations originally applied only to anaerobic processes but is now used more broadly to include all processes whether aerobic or anaerobic.

All operations are carried out under sterile conditions to avoid contamination of the culture. The product is either the cells themselves (biomass) or some useful cell product.

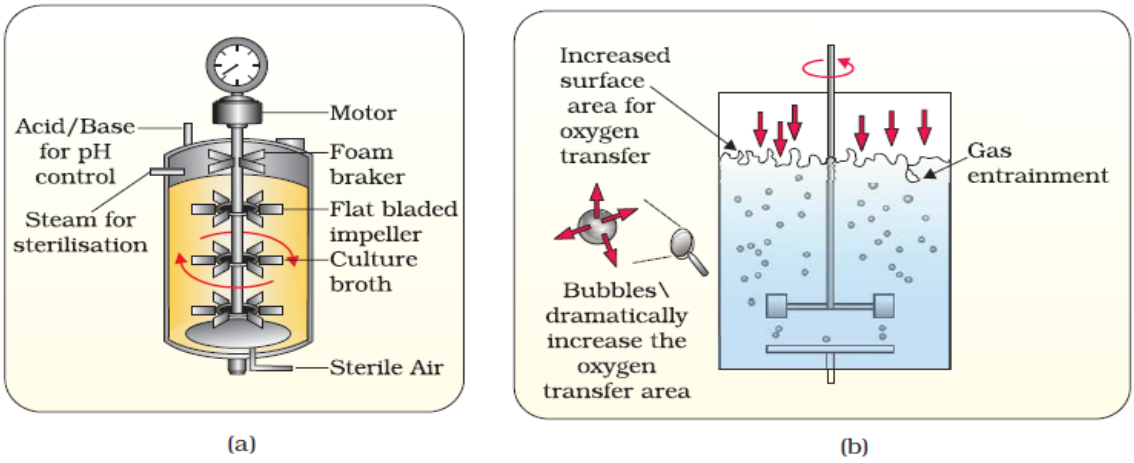


Figure 11.7 (a) Simple stirred-tank bioreactor; (b) Sparged stirred-tank bioreactor through which sterile air bubbles are sparged

Two basic types of fermentation are possible. These are batch fermentation and continuous fermentation. In batch fermentation, the nutrients and microorganisms are put in a closed reactor and not changed from outside once the fermentation starts, for example, no more nutrients are added.

When nutrients are utilized, the product is separated from microorganisms. In continuous fermentation nutrients are replaced as fast as they are used and products are removed as fast as they are made.

Lecture two

Application of Microbial technology Lec 2

Application of microbial metabolism

Living organisms have numerous biochemical reactions operating in them. These reactions allow the organisms to survive by processes such as generation of energy, production of fundamental building blocks required for structural organization and synthesis of biomolecules having specialized functions.

Microbial products often are classified as primary and secondary metabolites , primary metabolites consist of compounds related to the synthesis of microbial cells in the growth phase. They include amino acids, nucleotides, and fermentation end products such as ethanol and organic acids. In addition, industrially useful enzymes, either associated with the microbial cells or exoenzymes, often are synthesized by microorganisms during growth.
Secondary metabolites usually accumulate during the period of nutrient limitation or waste product accumulation that follows the active growth phase. These compounds have no direct relationship to the synthesis of cell materials and normal growth. Most antibiotics and the mycotoxins fall into this category.

Strategies For Overproduction Of Microbial Products

Improvement of the productivity of commercially viable microbial strains is an important field in microbiology, especially since wild type strains isolated from nature usually produce only a low level (1–100 g/ml) of antibiotics.
Microbial strain can be regarded as the heart of fermentation industry, so improvement of the production strains usually resides in increase yields of the desire metabolites thus it offer greatest opportunities for coast reduction.
Production of primary metabolites is regulated by feedback inhibition(by the end product of a particular pathway).
Overproduction of primary metabolites based on :
-mutation to cause accumulation of metabolite of interest

-mutants resistant to anti metabolites through modification of enzyme structure at allosteric site
-modification of operator or regulator gene to express the enzyme constitutively

Overproduction Of Secondary Metabolites based on genetic engineering of regulatory genes, antibiotic resistance gene(immunizing responsible for their own metabolites) and genes involved in primary metabolism affecting the biosynthesis of secondary metabolites , include :

Classical strain improvement (CSI)
Random mutagenesis to accumulate genomic alterations and screening for the phenotypes with desirable process characteristics

Rational metabolic engineering
The directed improvement of cellular properties through the modification of specific biochemical reactions or the introduction of new ones, with the use of recombinant DNA technology**.**

Improvement strain advantages include :
- increasing yields of the desired metabolite
- improving cell growth efficiency (energy efficiency)
- Eliminate (reduce) undesirable byproducts
-removal of unwanted co metabolites
- improving utilization of inexpensive carbon and nitrogen sources
- Helping media design and Extension of substrate range
- alteration of cellular morphology to a form better suited for separation of the mycelium from the product and /or for improved oxygen transfer in the fermenter .
- Production of novel compounds – polyketides
- Improving cell growth and fermentation kinetics

Modification of Gene Expression
In addition to inserting new genes in organisms, it also is possible to modify gene regulation by changing gene transcription, fusing proteins, creating hybrid promoters, and removing feedback regulation controls. These approaches make it possible to overproduce a wide variety of

products, as shown in table 1. As a further example, genes for the synthesis of the antibiotic actinorhodin have been transferred into strains producing another antibiotic, resulting in the production of two antibiotics by the same cell.

This approach of modifying gene expression also can be used to alter metabolic pathways by inactivation or deregulation of specific genes. Alternative routes can be used to add three functional groups to a molecule. Some of these pathways may be more efficient than the others. Understanding pathway architecture makes it possible to design a pathway that will be most efficient by avoiding slower or energetically more costly routes. This approach has been used to improve penicillin production by **metabolic pathway engineering** (MPE).

An interesting recent development in modifying gene expression, which illustrates metabolic control engineering, is that of altering controls for the synthesis of lycopene, an important antioxidant normally present at high levels in tomatoes and tomato products. In this case, an engineered regulatory circuit was designed to control lycopene synthesis in response to the internal metabolic state of *E. coli.* An artificially engineered region that controls two key lycopene synthesis enzymes is stimulated by excess glycolytic activity and influences acetyl phosphate levels, thus allowing a significant increase in lycopene production while reducing negative impacts of metabolic imbalances.

Table 1: Examples of Recombinant DNA Systems Used to Modify Gene Expression

Product	**Microorganism**	**Change**
Actinorhodin	*Streptomyces coelicolor*	Modification of gene transcription
Cellulase	*Clostridium* genes in *Bacillus*	Amplification of secretion through chromosomal DNA amplification
Amino acids	*Corynebacterium*	Isolation of biosynthetic genes that lead to enhanced enzyme activities or removal of feedback regulation

Procedures for using microorganisms in the production of chemical feedstocks also have been developed using this MPE approach. By turning on and off specific genes, feedstock chemicals such as 1,2-propanediol and 1,3-propanediol can be produced at high levels These particular chemicals are used in semi moist dog foods!
Other examples include the increased synthesis of antibiotics and cellulases, by modification of gene expression, DNA amplification and interactive enzyme overproduction or removal of feedback inhibition. Recombinant plasminogen, for example, may comprise 20 to 40% of the soluble protein in a modified strain, a tenfold increase in concentration over that in the original strain.

Metabolic engineering (ME **)**

In the past, to increase the productivity of a desired metabolite, a microorganism was genetically modified by chemically induced mutation, and the mutant strain that overexpressed the desired metabolite was then chosen. However, one of the main problems with this technique was that the metabolic pathway for the production of that metabolite was not analyzed, and as a result, the constraints to production and relevant pathway enzymes to be modified were unknown.

In 1990s, a new technique called metabolic engineering emerged. This technique analyzes the metabolic pathway of a microorganism, and determines the constraints and their effects on the production of desired compounds. It then uses genetic engineering to relieve these constraints.

Metabolic engineering (ME) is "the improvement of cellular activities by manipulating enzymatic, regulatory and transport functions of the cell with the use of recombinant DNA technology. Metabolic engineering considers metabolic and cellular system as an entirety and accordingly allows manipulation of the system with consideration of the efficiency of overall bioprocess, which distinguishes itself from simple genetic engineering .

Strategies for Metabolic Engineering

Most of the metabolic engineering approaches are based on genetic engineering techniques. Some of the fundamental requirements for metabolic engineering are knowledge about :
(1) the biosynthetic pathway of the chemical to be produced,
(2) genes encoding the related enzymes,
(3) regulation of such enzymes, with ability to (4) transfer and express or suppress the required genes in the host organism,
(5) mutate the gene *in vivo* and *in vitro* to be able to alter properties of the encoded enzyme, and
(6) assemble an array of genes for their expression inside the host cell.

Although bacteria and yeast are the pioneering hosts for metabolic engineering, other organisms such as fungi, animal as well as plant cells are also used nowadays for similar experiments.

There are different approaches of metabolic engineering for achieving the required production of the desired biochemicals. Some of them are described below along with examples.

(1) One of the most obvious approaches is overexpressing the gene encoding the rate-limiting enzyme of the biosynthetic pathway of the desired end-product. Using a similar strategy, the vitamin E content of Arabidopsis (a model plant system) has been increased by overexpression of a gene encoding the enzyme γ-tocopherolmethyltransferase . *Figure* 1.

(2) Another way of overproducing the product of a given pathway is to inhibit the competing metabolic reactions which involve the same substrate. In this way, the substrate is metabolically channeled specifically towards the desired chemical. An example of this strategy is increasing the production of 1,2-propanediol, which is mainly used for production of biodegradable polymers, by inhibiting the lactate dehydrogenase and glyoxylase genes which encode for the competing enzymes (*Figure* 1).
(3) In some cases, the production of the desired biochemical can be carried out in the non-native organism, i.e., heterologous host (*Figure* 2).

In other words, a gene can be isolated from the organism which naturally produces the desired biochemical and can be expressed in another organism which might be easier to cultivate than the host organism. In such a case, an important factor is the availability of the substrate of the desired pathway.

Thus, multiple genes encoding an array of the enzymes of a pathway can be expressed in the non-native host. Expressing the combination of genes encoding the most efficient enzymes from different organisms is another way to achieve the product which is otherwise not produced or produced at a very low level. One of the most successful examples of this strategy is the production of biofuel molecules – fatty acid ethyl esters – in *E. coli* by expressing the genes encoding the successive enzymes of the pathway which are obtained from various sources such as plants and bacteria .

(4) The most interesting approach for production of non-natural chemicals is to engineer an enzyme which is not found in Nature. This mode of metabolic engineering relies on creating mutations in the related gene so that the amino acid composition of the enzyme is altered. This might result in alteration in the substrate and product specificities of the enzymes, as they are dependent on the amino acid composition and sequence.

There are two ways for achieving this. One involves generation of the mutations in a random manner followed by selection of the desired mutant and in the other rational mutations are created at predefined sites in the enzymes based on the available knowledge of its reaction mechanism.

The latter way requires extensive computational modeling of the desired reaction in terms of the structure of the substrates and products, the active site and overall structure of the enzyme. Such a method has been used for the production of a non natural amino acid L-homoalanine, (which is an important precursor of the many drugs), by creating rational mutations in glutamate dehydrogenase enzyme in *E. coli* (*Figure* 1).

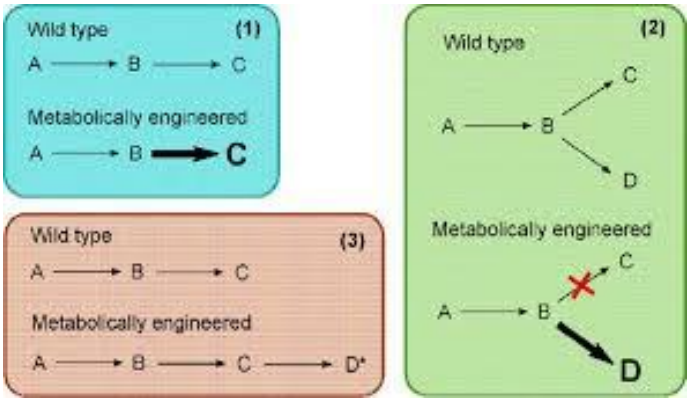


Figure 1. Strategies for metabolic engineering for the production of a desired chemical:
(1) overexpression of the rate-limiting enzyme, (2) inhibition of the competing pathway and (3) engineering a novel enzyme for the production of non-natural chemical

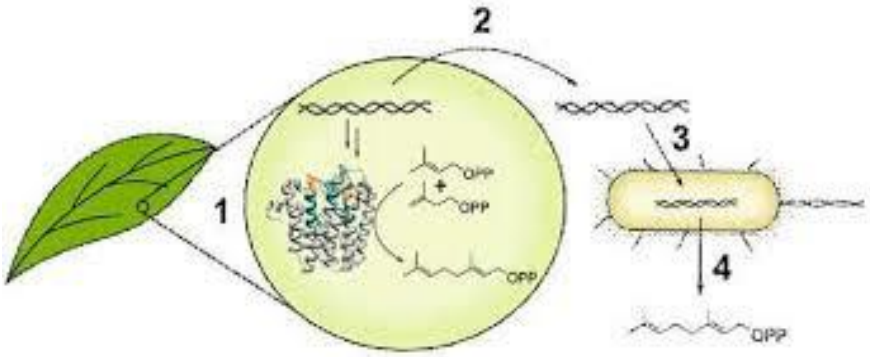


Figure 2. Any example of metabolic engineering by heterologous expression of a plant enzyme into bacteria involves
(1) identification and (2) isolation of the gene encoding the enzyme catalyzing the desired reaction and (3) transfer and expression of the gene in the host which results in production of the desired chemical (4).

5- The commonly used strategies to enhance the metabolite flux through a pathway can be clubbed under the following categories a) Increase the flux through rate limiting steps in the pathway; b) Increase the supply of precursors; c) Block branched chain pathways which lead to by-product formation and d) Remove feedback controls in the pathway (Figure).

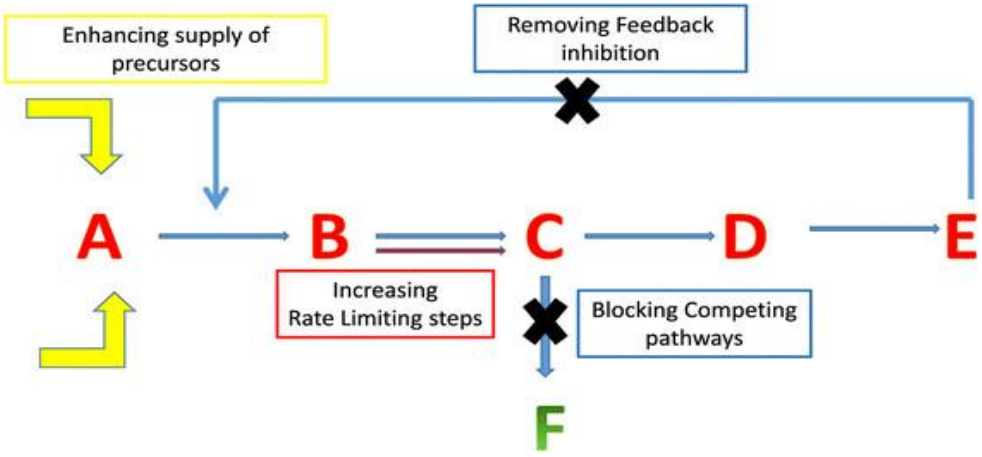


Fig : Metabolic engineering strategies to enhance the flux through a pathway. The various strategies used to improve the flux from a cellular intermediate to the desired product is shown. This includes enhancing pathways leading to the formation of intermediates (shown in yellow) and pathways which are rate limiting (B to C). Additionally branched chain pathways and feed back controls need to be blocked.

Challenges

• Difficult to target the gene (or genes) and to predict the consequences of the changes in the metabolic pathway

• Uncertain results due to complicated metabolic pathways that are highly regulated by a myriad of genes and enzymes of which many may still not known

• Success usually came from many trials after long research and hard development efforts – costly and time consuming

• It is more challenging when there is limited knowledge on the organism and its genomics and metabolic pathway

Some of the most successful examples of commercial metabolic engineering strategies include production of drugs , amino acids , biofuel related chemicals such as ethanol, alkanes and fatty acid esters . In recent controversial research, scientists have discovered an enzyme which catalyses the production of morphine in plants. Such genes have huge potential for the production of similar drug molecules in yeast by metabolic engineering. Nonetheless, it is also feared that such genes can be misused for illegal production of the abusive drugs.

Some examples of successful metabolic engineering are the following:

1- Production of glutamic acid and several other amino acids in large quantities is now carried out using mutants of *Corynebacterium glutamicum* that lack, or have only a limited ability to process, the TCA cycle intermediate _&-ketoglutarate to succinyl-CoA as shown in **figure 42.12.** A controlled low biotin level and the addition of fatty acid derivatives and β-lactam antibiotics. These treatments affect the cell surface structures of *C. glutamicum.*, results in increased membrane permeability and excretion of high concentrations of glutamic acid. The impaired bacteria use the glyoxylate pathway to meet their needs for essential biochemical intermediates, especially during the growth phase. After growth becomes limited because of changed nutrient availability, an almost complete molar conversion (or 81.7% weight conversion) of isocitrate to glutamate occurs.

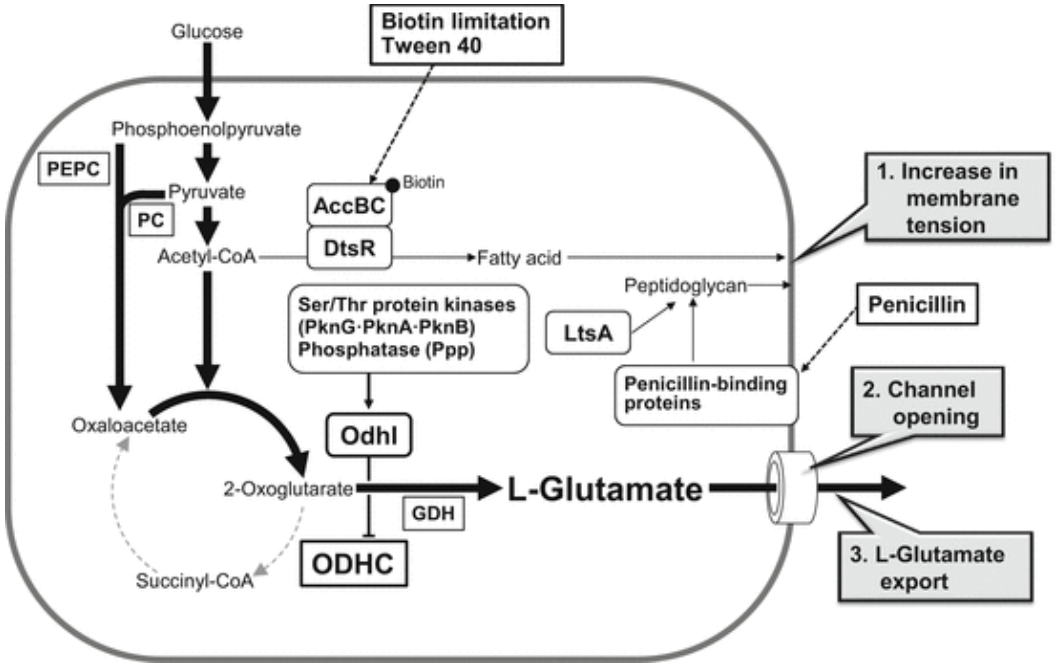


Figure 42.12 Glutamic Acid Production. The sequence of biosynthetic reactions leading from glucose to the accumulation of glutamate by *Corynebacterium glutamicum.* Major carbon flows are noted by bold arrows.
(a) Growth with use of the glyoxylate bypass to provide critical intermediates in the TCA cycle. (b) After growth is completed, most of the substrate carbon is processed to glutamate (note shifted bold arrows). The dashed lines indicate reactions that are being used to a lesser extent.

2- **Identification of constraints to lysine production in *Corynebacterium glutamicum*** and insertion of new genes to relieve these constraints to improve production. Lysine, was originally produced in a two-step microbial process. This has been replaced by a single-step fermentation in which the bacterium *Corynebacterium glutamicum*,

blocked in the synthesis of homoserine, accumulates lysine. Over 44 g/liter can be produced in a 3 day fermentation.

3- **Engineering of a new fatty acid biosynthesis pathway**, called reversed beta oxidation pathway, that is more efficient than the native pathway in producing fatty acids and alcohols which can potentially be catalytically converted to chemicals and fuels

4- **Improved production of DAHP an aromatic metabolite produced by *E. coli*** that is an intermediate in the production of aromatic amino acids. It was determined through metabolic flux analysis that the theoretical maximal yield of DAHP ((3-deoxy-D-arabino-heptulosonate 7-phosphate)per glucose molecule utilized, was 3/7. This is because some of the carbon from glucose is lost as carbon dioxide, instead of being utilized to produce DAHP. Also, one of the metabolites (PEP, or phosphoenolpyruvate) that are used to produce DAHP, was being converted to pyruvate (PYR) to transport glucose into the cell, and therefore, was no longer available to produce DAHP. In order to relieve the shortage of PEP and increase yield, Patnaik et al. used genetic engineering on *E. coli* to introduce a reaction that converts PYR back to PEP. Thus, the PEP used to transport glucose into the cell is regenerated, and can be used to make DAHP. This resulted in a new theoretical maximal yield of 6/7 – double that of the native *E. coli* system.

5- Succinate production with *Corynebacterium glutamicum*

Succinate can serve as a precursor for the production of a great variety of important bulk chemicals, which are currently produced petrochemically . Therefore, succinate was identified as one of the top 12 building block chemicals from biomass by the U.S. Department of Energy. *C. glutamicum* strains was developed by metabolic engineering for the synthesis of succinate both under aerobic and anaerobic conditions.

For succinate production from glucose under aerobic conditions, the succinate dehydrogenase genes *sdhCAB* were deleted first, leading to an accumulation of succinate as well as high amounts of acetate as by-product. By deleting genes for all known acetate-producing pathways (*pta-ackA*, *pqo*, and *cat*) acetate production could be reduced by 83% and

succinate production increased up to 7.8 g l^{-1} (66 mM). Whereas, simultaneous overproduction of pyruvate carboxylase and PEP carboxylase resulted in a strain that produced 9.7 g l^{-1} (82 mM) .

Besides glucose, also glycerol could be used as renewable carbon source for aerobic succinate production. For this purpose, strain BL1 was transformed with plasmid pVWEx1-glpFKD coding for glycerol utilisation genes of *Escherichia coli* . This plasmid enables growth of *C. glutamicum* with glycerol as sole carbon source.

C. glutamicum wild type is known to produce L-lactate, acetate, and succinate under oxygen-limiting conditions, whereby succinate is formed by the reduction of oxaloacetate to succinate. To eliminate acetate formation, a derivative of the type strain ATCC 13032 was constructed (strain BOL-1), which lacked all known pathways for acetate and lactate synthesis (Δ*cat*, Δ*pqo*, Δ*pta-ackA*, Δ*ldhA*). Chromosomal integration of the pyruvate carboxylase gene pyc^{P458S} into BOL-1 resulted in strain BOL-2, which catalysed fast succinate production from glucose with a yield of 1 mol/mol and showed only little acetate formation. In order to provide additional reducing equivalents derived from the co-substrate formate , the *fdh* gene from *Mycobacterium vaccae* coding for an NAD^+-coupled formate dehydrogenase (FDH) was chromosomally integrated into BOL-2, leading to strain BOL-3. In an anaerobic batch process with strain BOL-3, a 20% higher succinate yield from glucose was obtained in the presence of formate. A temporary metabolic blockage of strain BOL-3 was prevented by plasmid-borne overexpression of the glyceraldehyde 3-phosphate dehydrogenase gene *gapA*. The strain BOL-3/pAN6-*gap* accumulated 134 g L^{-1} succinate in 53 h with an average succinate production rate of 1.59 mmol g $(cdw)^{-1}$ h^{-1}.

6- Itaconate production

Itaconic acid is an unsaturated dicarboxylic acid which has gained considerable interest in recent years as it was reported to be one of the top 12 building block chemicals that can be produced from biomass and replace fossil-based chemicals. *Aspergillus terreus* is the dominant production host for itaconate, which is synthesised via the decarboxylation of *cis*-aconitate by the enzyme *cis*-aconitate

decarboxylase. *C. glutamicum* was highly tolerant to itaconate and did not metabolise it.

Expression of genes encoding citrate synthase (gltA) and aconitase (acnA) from Corynebacterium glutamicum and cis-aconitate decarboxylase (cadA) from Aspergillus terreus or immunoresponsive gene 1 (irg1) from Mus musculus. in *E.coli* , increase the production of itaconate with titer up to 560 mg/L with irg1 and 700 mg/L with cadA (Fig.) .

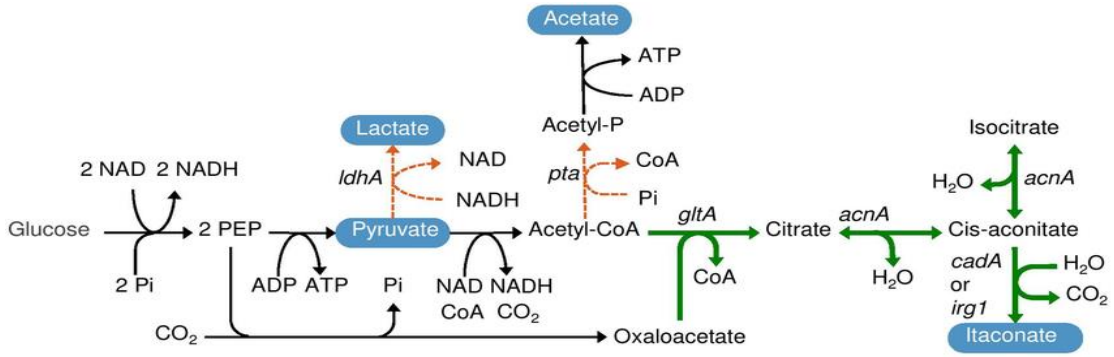


Fig : Itaconate production pathway in Escherichia coli. The bold arrows indicate the introduced pathway consisting of genes encoding citrate synthase (gltA) and aconitase (acnA) from Corynebacterium glutamicum and cis-aconitate decarboxylase (cadA) from Aspergillus terreus or immunoresponsive gene 1 (irg1) from Mus musculus. The dotted lines indicate that phosphate acetyltransferase (pta) and lactate dehydrogenase (ldhA) were deleted.

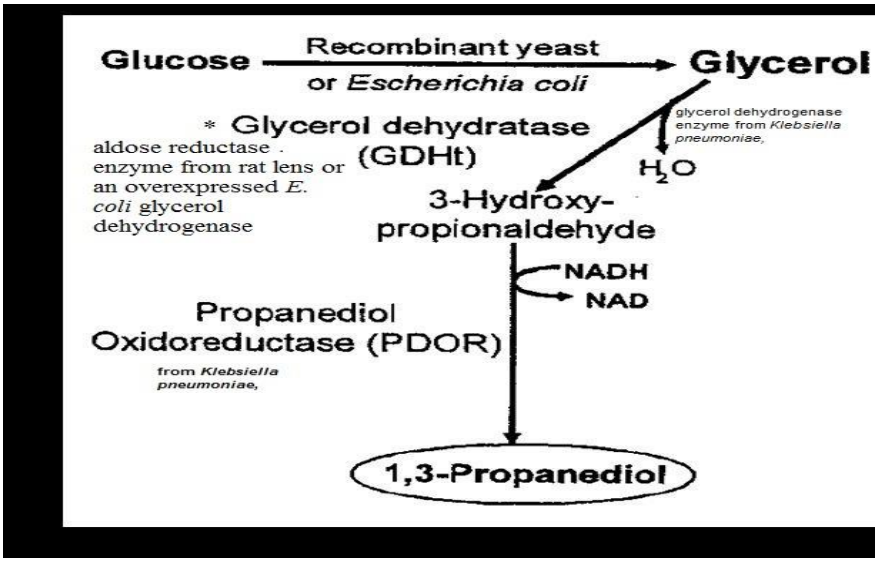
6- Production of L-leucine with *Corynebacterium glutamicum*

The branched-chain amino acids (BCAAs) L-valine, L-isoleucine, and L-leucine are essential amino acids and used as components of pharmaceuticals, in animal feed industry, and as additives of infusion solutions and dietary products. To developed an efficient L-leucine production strain of *C. glutamicum*. In the wild type of *C. glutamicum*, the *leuA*-encoded 2-isopropyl malate synthase (IPMS) is inhibited by low L-leucine concentrations , a feedback-resistant IMPS variant carrying two amino acid exchanges (R529H, G532D) was identified . The corresponding $leuA^{fbr}$ gene devoid of the attenuator region and under control of a strong promoter was integrated in one, two or three copies into the genome and combined with additional genomic modifications aimed at increasing L-leucine production. These modifications involved (i) deletion of the gene encoding the repressor LtbR to increase

expression of *leuBCD*, (ii) deletion of the gene encoding the transcriptional regulator IolR to increase glucose uptake, (iii) reduction of citrate synthase activity to increase precursor supply, and (iv) introduction of a gene encoding a feedback-resistant acetohydroxy acid synthase. The molar product yield was 0.30 mol L-leucine per mol glucose and the volumetric productivity was 4.3 mmol L^{-1} h^{-1}.

7- Produce Propanediol in *E. coli.*

Either an aldose reductase enzyme from rat lens or an overexpressed *E. coli* glycerol dehydrogenase enzyme and two enzymes from *Klebsiella pneumoniae,* glycerol dehydrogenase and 1,3-propanediol oxido reductase , are used to shift the intermediary metabolism of *E. coli* to the production of propanediols.



8- Like other industrially useful chemicals, drugs have also been the major target of metabolic engineering . Plant secondary metabolites that are of medicinal value, such as artemisinic acid, taxol precursor, and benzyl isoquinoline alkaloids have been successfully produced by metabolically engineered microorganisms.

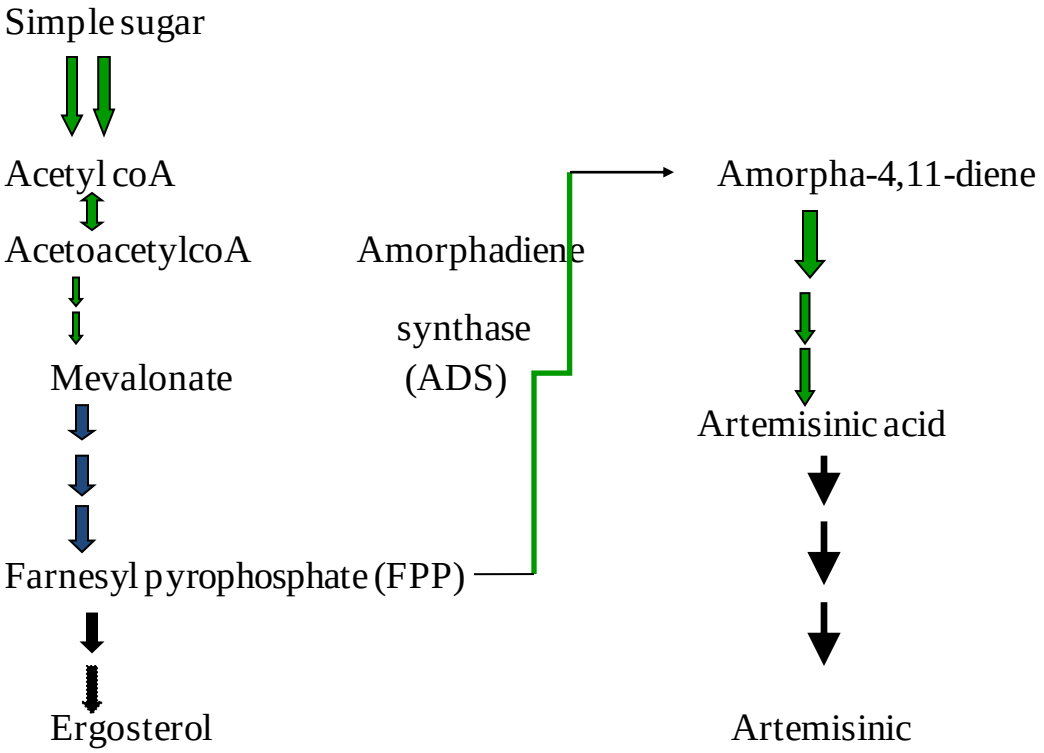
In 2006, a team from UC Berkeley reported they had engineered <u>*Saccharomyces cerevisiae*</u> yeast to produce small amount of the precursor artemisinic acid. The synthesized artemisinic acid can then be transported out, purified and chemically converted into artemisinin .

Strategy to engineer the yeast cell to produce the artemisinic acid at cheaper cost

-Engineering the farnesyl pyrophosphate (FPP) biosynthetic pathway to increase FPP production

-Introduction of the amorphadiene synthase (ADS) gene from *Artemisia annua* into the high FPP producer to convert FPP to amorphadiene,

-Cloning a novel cytochrom P450 that perform a three-step oxidation of amorphadiene to Artemisinic acid from *A. annua* and expressing it in the amorphadiene producer.



9- **Xylose utilization in S. cerevisiae** suffers from glucose repression .Transport of xylose into cytoplasm is mediated by hexose transporters which show much higher affinities to glucose than to xylose, with glucose having the highest priority, thus leading to ‘‘glucose repression’’. Such sequential utilization of xylose after glucose exhaustion presents several challenges to xylitol production. Firstly, xylose uptake is tremendously inhibited by glucose. Secondly, insufficient generation of cofactor such as NADPH (after glucose exhaustion) significantly reduces xylitol production since NAD(P)H is required for the conversion. Thus, xylitol production is dramatically impaired by delayed xylose utilization in recombinant S. cerevisiae . So far, no effective methods have been

proposed to overcome the problems resulting from glucose repression in S. cerevisiae . To increase xylose utilization by recombinant S. cerevisiae expressing XYL1 , repeated fed-batch fermentation was implemented to produce xylitol through continuously supplying glucose under aerobic or anaerobic conditions . A cellobiose metabolic pathway was incorporated into S. cerevisiae allowing for the simultaneous co- utilization of cellobiose (a dimer of glucose) and xylose . This approach facilitates faster xylose consumption and ethanol production compared with the traditional method of producing ethanol from mixtures of glucose and xylose, providing a solution to bypassing glucose repression. Furthermore, Jin and coworkers recently cloned the cellobiose metabolic pathway into a recombinant S. cerevisiae expressing xylose reductase (XYL1) from Scheffersomyces stipitis to produce xylitol at a high yield . In their study, they proposed a method to simultaneously produce ethanol and xylitol from mixtures of cellobiose and xylose under anaerobic conditions by construction of a recombinant S. cerevisiae strain through expression of a cellodextrin transporter (CDT-1) and an intracellular b -glucosidase (gh1-1) from Neurospora crassa and the xylose reductase (XYL1) from Sch . stipitis (Figure).

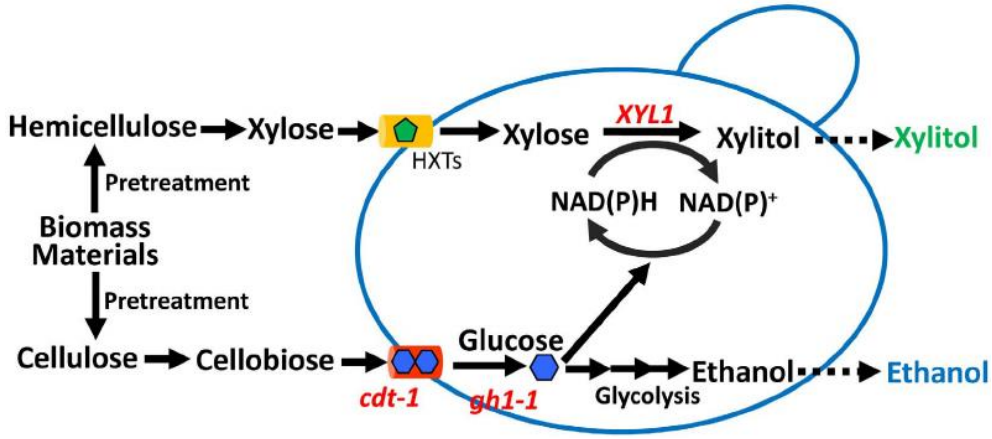


FIG: Construction strategy of the recombinant S. cerevisiae strain to achieve the co-production of ethanol and xylitol from ligonocellulosic biomass. The uptake and hydrolysis of cellobiose (derived from cellulose) were accomplished by a cellodextrin transporter (encoded by cdt-1) and an intracellular b -glucosidase (encoded by gh1-1), respectively. The uptake and conversion of xylose (derived from hemicellulose) was accomplished by endogenous hexose transporters and a xylose reductase (encoded by XYL1), respectively. Thus, ethanol and xylitol were produced simultaneously by this engineered yeast. NAD(P)H for xylitol production was provided by the cellobiose metabolism.

Lecture three

Microbial technology and Medicine Lec. 3

Microbial technology has wide range of applications in animal and human health care. Knowing the molecular basis of health and disease leads to improved methods for treating and preventing diseases .In human health care, biotechnology provides effective diagnostics, prevention and

treatment measures including production of novel drugs and recombinant vaccines . It gives effective drug delivery approaches, new methods for therapeutics. Parasitic and infectious diseases like Acquired Immunodeficiency Syndrome (AIDS) and tuberculosis (TB) have been diagnosed rapidly at relatively low cost.

Molecular diagnostic tools including polymerase chain reaction (PCR) , recombinant antigens and monoclonal antibodies have been used for this purpose. Biotechnology has offered modern diagnostic test kits, rickettsial, bacterial and viral vaccines along with radio labelled biological therapeutics for imaging and analysis. Vaccines have eliminated small pox, polio and other deadly diseases for the last hundred years.

Molecular diagnostics

Nearly 40% deaths are due to infectious and parasitic diseases (malaria, tuberculosis, and AIDS) each year. Spread of these diseases can be overcome by the development of quick and accurate diagnostic tools. These developments result in increase in survival rate as well as help to prevent the waste of resources on non-suitable treatment. Many conventional diagnostic tools are inaccurate, time consuming and expensive. Molecular diagnostics is based on following techniques; PCR, Monoclonal antibodies and microarrays. These are simple, quick, cost effective and have high sensitivity and specificity . PCR requires small volume of sample to amplify and identify the DNA sequence of pathogen (that cause the disease). It is identified in a very quick and accurate manner as compared to conventional diagnostics. The infectious or dangerous organisms (HIV, Mycobacterium and plasmodium) that are difficult to grow in culture are identified by the PCR . Multiplex PCR are used to detect the pathogens which cause the broad range of diseases at once and hence it saves both time and resources .

Development of simple and quick dipstick coated by antibody have been increased the applications of molecular diagnostics. It can be used anywhere without any laboratory facilities. “The program for appropriate technology in health (PATH)” have been developed the dipsticks for

identification of malaria, TB, Hepatitis C, HIV and pregnancy dipsticks. This test is rapid, accurate and easy to use .

Currently, in the diagnosis and treatment of diseases microarrays have become a powerful tool in contrast to the traditional DNA based tests. It is best for the study of causes of complex genetic disorders as it can identify and quantify the thousands of gene at the same time, **microarray** (also commonly known as DNA chip or biochip) is a collection of microscopic DNA spots attached to a solid surface. Scientists use DNA microarrays to measure the expression levels of large numbers of genes simultaneously or to genotype multiple regions of a genome. Each DNA spot contains picomoles (10^{-12} moles) of a specific DNA sequence, known as *probes* . These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA (also called anti-sense RNA) sample (called *target*) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target.

The core principle behind microarrays is hybridization between two DNA strands, the property of complementary nucleic acid sequences to specifically pair with each other by forming hydrogen bonds between complementary nucleotide base pairs. A high number of complementary base pairs in a nucleotide sequence means tighter non-covalent bonding between the two strands. After washing off non-specific bonding sequences, only strongly paired strands will remain hybridized. Fluorescently labeled target sequences that bind to a probe sequence generate a signal that depends on the hybridization conditions (such as temperature), and washing after hybridization. Total strength of the signal, from a spot (feature), depends upon the amount of target sample binding to the probes present on that spot. Microarrays use relative quantitation in which the intensity of a feature is compared to the

intensity of the same feature under a different condition, and the identity of the feature is known by its position.

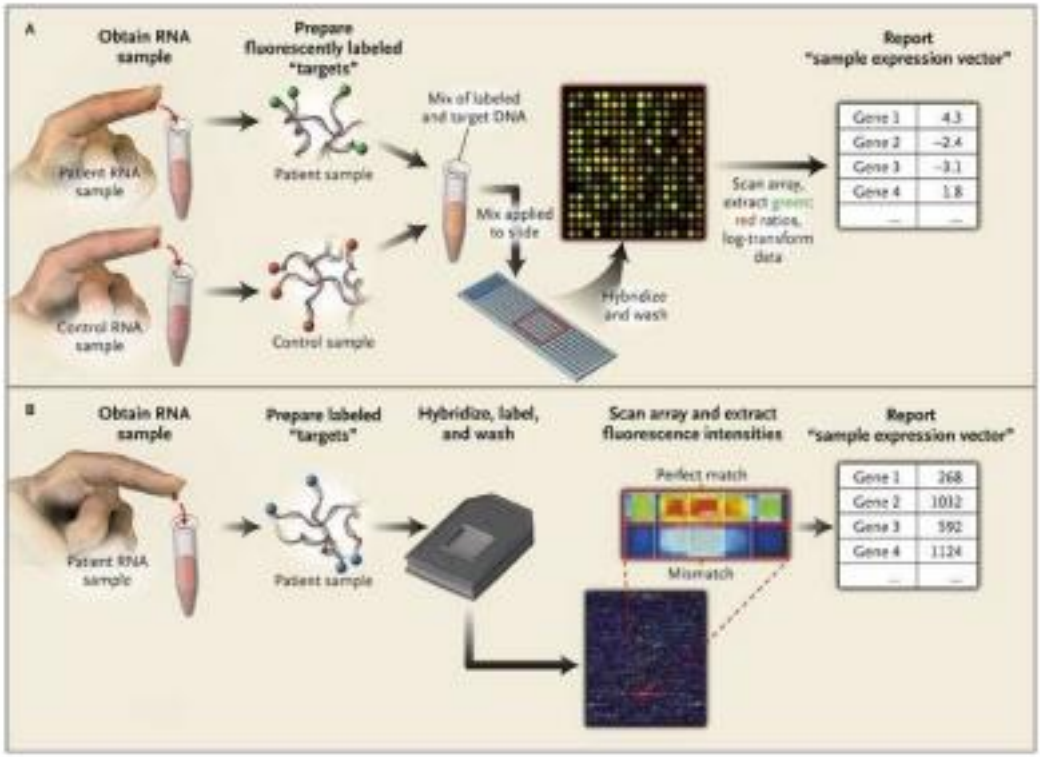


Figure : Overview of DNA Microarray Analysis: In a two-color analysis (Panel A), RNA samples obtained from patients and control subjects are individually labeled with distinguishable fluorescent dyes and hybridized to a single DNA microarray consisting of individual gene-specific probes. Relative levels of gene expression in the two samples are estimated by measuring the fluorescence intensity for each probe; a sample expression vector summarizes the level of expression of each gene in the sample obtained from a patient (as compared with a sample obtained from a control). A single-color analysis (Panel B), performed with the use of the Gene Chip (Affymetrix), hybridizes labeled RNA from each biologic sample to a single array in which a series of gene specific probes are arrayed. Gene-expression levels are estimated by measuring the hybridization intensity for a series of "perfect match" probes, and the background is measured with the use of a corresponding set of "mismatch" probes. Gene-expression levels are reported for each sample from a patient as a sample-expression vector that summarizes the difference between the signal and background for each gene.

The monoclonal antibodies (MAbs)

Monoclonal antibodies are monospecific antibodies that are the same because they are made by identical immune cells that are all clones of a unique parent cell. Monoclonal antibodies have monovalent affinity, in

that they bind to the same epitope. Monoclonal antibodies are prepared using **Hybridoma technology**, which is a technology of forming hybrid cell lines (called **hybridomas**) by fusing a specific antibody-producing B cell with a myeloma (B cell cancer) cell that is selected for its ability to grow in tissue culture and for an absence of antibody chain synthesis. The antibodies produced by the hybridoma are all of a single specificity and are therefore monoclonal antibodies.

Laboratory animals (mammals, e.g. mice) are first exposed to an antigen against which we are interested in isolating an antibody. Usually this is done by a series of injections of the antigen, over the course of several weeks. Once splenocytes are isolated from the mammal's spleen, the B cells are fused with myeloma cells. The fusion of the B cells with myeloma cells can be done using electrofusion. Electrofusion causes the B cells and Myeloma cells to fuse with the application of an electric field. **The myeloma cells are selected beforehand to ensure they are not secreting antibody themselves and that they lack the hypoxanthine guanine phosphoribosyltransferase (HGPRT) gene, making them sensitive to the HAT medium (hypoxanthine-aminopterin-thymidine medium). After fusion the cell cultured on HAT medium , the unfused B cells die and only the B cell-myeloma hybrids survive, then specific antibody-producing hybridoma is selected and propagated in culture vessels (in vitro) or in animal (in vivo) and monoclonal antibodies harvested.

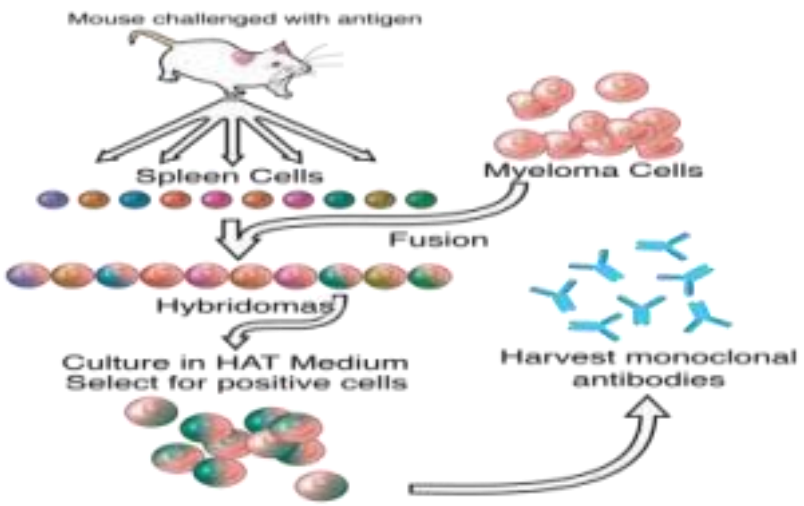


Fig: A general representation of the method used to produce monoclonal antibodies

MAbs as tools to diagnose the diseases

(A) MAbs in Biochemical Analysis:

Diagnostic tests based on the use of MAbs as reagents are routinely used in radioimmunoassay (RIA) and enzyme-linked immunosorbent assays (ELISA) in the laboratory. These assays measure the circulating concentrations of hormones (insulin, growth hormone, progesterone, thyroxine, thyroid stimulating hormone, gastrin, renin), and several other tissue and cell products (blood group antigens, blood clotting factors, interferon's, interleukins, tumor markers). In recent years, a number of diagnostic kits using MAbs have become commercially available.

Now possible to do the early diagnosis of the following conditions/diseases.

- Pregnancy diagnosis by detecting the urinary levels of human chorionic gonadotropin.

- Cancers estimation of plasma carcino embryonic antigen in colorectal cancer, and prostate specific antigen for prostate cancer. Besides diagnosis, estimation of tumor markers is also useful for the prognosis of cancers. That is a gradual fall in a specific tumor marker is observed with a reduction in tumor size, following treatment.

- Hormonal disorders analysis of thyroxine, tri iodothyronine and thyroid stimulating hormone for thyroid disorders.

(B) MAbs in Diagnosis of Infectious diseases

Infectious diseases diagnosis by detecting the circulatory levels of antigens specific to the infectious agent for example :

a) Diagnosis of diseases caused by protozoa and helminthes

The monoclonal antibodies used as tools to diagnose the diseases caused by protozoa and helminthes. ELISA test to detect various kind of diseases, such as **malaria**.

b) Diagnosis of sexually transmitted diseases (STD)and other bacterial diseases using monoclonal antibodies

The occurrence of sexually transmitted disease has gone up in recent years both in developed as well as developing countries. Some of the pathogens causing these diseases are- *Neisseria gonorrhoeae, Chlamydia trachomatis, Herpes simplex* virus etc.

The conventional methods for the diagnosis of these diseases are microscopic examination, culture method, Immunological method, and measurement of antibody. The use of monoclonal antibody has been very effective in the diagnosis of these diseases. The immune reaction is detected through radio-immunoassay that would distinguish between the different pathogens causing STDs.

ELISA tests also are used as in *in vitro* diagnostics in medical laboratories. The other uses of ELISA include: detection of *Mycobacterium* antibodies in tuberculosis , detection of rotavirus in feces , detection of hepatitis B markers in serum , detection of enterotoxin of *E. coli* in feces and detection of HIV antibodies in blood samples.

(C) MAbs in Diagnostic Imaging:

Radiolabeled-MAbs are used in the diagnostic imaging of diseases, and this

technique is referred to as immunoscintigraphy. The radioisotopes commonly used for labeling MAb are iodine—131 The MAb tagged with radioisotope are injected intravenously into the patients.

These MAbs localize at specific sites which can be detected by imaging the radioactivity. In recent years, single photon emission computed tomography (SPECT) cameras are used to give a more sensitive three dimensional appearance of the spots localized by radiolabeled- MAbs. Monoclonal antibodies are successfully used in the diagnostic imaging of cardiovascular diseases, cancers and sites of bacterial infections.

Monoclonal antibodies against many types of human cancers are now available. A selected list of tumor markers (along with the associated cancers) that can be used for MAb imaging is given in Table 17.2. Tumors can be located in patients using radioisotope labeled MAbs specific to the protein(s), particularly of membrane origin.

TABLE 17.2 Selected tumor markers along with the associated cancers used in MAb imaging

Tumor marker	*Associated cancer(s)*
Carcinoembryonic antigen (CEA)	Cancers of colon, stomach, pancreas
Alpha fetoprotein	Cancers of liver, and germ cells of testes
Human chorionic gonadotropin	Choriocarcinoma
Prostatic acid phosphatase	Prostate cancer
Epiderminal growth factor receptor	Melanoma
Tumor—associated cell surface antigens	Various cancers

It has been possible to detect certain cancers at early stages (lung cancer, breast cancer, ovariran cancer, melanoma, colorectal cancer) by employing MAbs. About 80 per cent specificity has been achieved for detecting cancers by this approach.

An iodine (^{131}l) labeled monoclonal antibody specific to breast cancer cells when administered to the patients detects (by imaging) the spread of cancer (metastasis) to other regions of the body. This is not possible by scanning techniques.

The imaging technique by using MAb can also be used to monitor therapeutic responses of a cancer. There are certain limitations in using MAb in cancer diagnosis and prognosis. These include the difficulty in the selection of a specific MAb and the access of MAb to the target site of the tumor which may be less vascularized.

MAbs in immunohistopathology of cancers:

The pathological changes of the cancerous tissue can be detected by immunohistochemical techniques. This can be done by using MAb against a specific antigen.

MAbs in hematopoietic malignancies:
Hematopoietic stem cells in bone marrow are the precursors for different blood cells, B- and T-lymphocytes which are produced in a stepwise transformation. During malignancy, transformation of lymphocytes stops at a particular stage of maturation. This can be detected by using stage specific MAbs.

Therapeutic Applications of Monoclonal antibodies : Monoclonal antibodies have a wide range of therapeutic applications. MAbs are used in the treatment of cancer, transplantation of bone marrow and organs, autoimmune diseases, cardiovascular diseases and infectious diseases.

The therapeutic applications of MAbs are broadly grouped into 2 types:
(A) Direct use of MAbs as therapeutic agents

(B) MAbs as targeting agents.

(A) MAbs as Direct Therapeutic Agents:

Monoclonal antibodies can be directly used for enhancing the immune function of the host. Direct use of MAbs causes minimal toxicity to the target tissues or the host.

In destroying disease-causing organisms:
MAbs promote efficient opsonization of pathogenic organisms (by coating with antibody) and enhance phagocytosis. In fact, MAbs were found to protect chimpanzees against certain viral (hepatitis B-virus) and bacterial (E. coli Haemophilus influenza, Streptococcus sp and Pseudomonas sp) infections.

In the treatment of cancer:
MAbs, against the antigens on the surface of cancer cells, are useful for the treatment of cancer. The antibodies bind to the cancer cells and destroy them. This is brought out by antibody—dependent cell-mediated cytotoxicity, complement-mediated cytotoxicity and phagocytosis of cancer cells (coated with MAbs) by reticuloendothelial system.

The patients suffering from leukemia, colorectal cancer, lymphoma and melanoma have been treated with MAbs. However, there was a wide variation in the success rate. A monoclonal antibody specific to the cells of leukemia is used to destroy the residual leukemia cells without affecting other cells. MAbs are used in vitro to remove the residual tumor cells prior

to autologous bone marrow transplantation (transplantation of the patient's own bone marrow cells, due to non-availability of a suitable donor).

Lec. 4

Limitations for direct use of MAbs in cancer:

1. The MAbs produced in mice and directly used for therapeutic purposes may lead to the development of anti-mouse antibodies and hypersensitivity reactions.

2. All the cancer cells may not carry the same antigen for which MAb has been produced. Thus, MAbs may not be attached to some cancer cells at all.

3. The free antigens (of target cells) present in the circulation may bind to MAbs and prevent them from their action on the target cells.

different monoclonal antibodies have to be made to target different types of cancer. Monoclonal antibodies work in slightly different ways. They may:

- Trigger the immune system
- Block signals telling cancer cells to divide

1-Trigger the immune system

Some monoclonal antibodies trigger the immune system to attack and kill cancer cells. Although cancer cells are abnormal, they develop from normal cells so they can be difficult for the immune system to spot. Some monoclonal antibodies simply attach themselves to cancer cells, making them easier for the cells of the immune system to find them. These include

- Rituximab (Mabthera) for non Hodgkin lymphoma (NHL) and some types of leukaemia
- Alemtuzumab (MabCampath) for chronic lymphocytic leukaemia (CLL)

Other types of monoclonal antibodies attach themselves to immune cells so that the cells carry on producing cells to attack cancer cells .
Ipilimumab for advanced melanoma skin cancer works by stimulating T cells in the body's immune system. T-cells help to fight cancer and disease. CTLA-4 is a molecule found on the surface of T-cells and it switches them off. Ipilimumab blocks CTLA-4 so that the T-cells stay switched on and active and can attack the cancer cells.

2-Block signals telling cancer cells to divide

Cancer cells often make large amounts of molecules called growth factor receptors. These sit on the cell surface and send signals to help the cell survive and divide. Some monoclonal antibodies stop growth factor receptors from working properly. So the cancer cell no longer receives the signals it needs. This type of MAB includes

- Trastuzumab (Herceptin) for breast cancer and stomach cancer
- Bevacizumab (Avastin) for advanced bowel cancer, breast cancer and some other cancers

- Cetuximab (Erbitux) and Panitumumab (Vectibix) for advanced bowel cancer .

Another monoclonal antibody researchers are looking at is pertuzumab (Perjeta). The growth factor receptor HER2 is found on some breast cancer and prostate cancer cells. Pertuzumab blocks (inhibits) this receptor.

In the immunosuppression of organ transplantation:

In the normal medical practice, immunosuppressive drugs such as cyclosporin and prednisone are administered to overcome the rejection of organ transplantation. In recent years, MAbs specific to T-lymphocyte surface antigens are being used for this purpose. The monoclonal antibody namely OKT_3, was the first MAb to be licensed by U.S.
Food and Drug Administration for use as immunosuppressive agent after organ transplantation in humans. OKT_3 specifically directed against CD_3 antigen of T-lymphocytes is successfully used in renal and bone marrow transplantations. In the normal course, CD_3 antigen activates T lymphocytes and plays a key role in organ transplant rejection (destroys the foreign cells in the host). This is prevented by use of MAb against CD_3 antigen.

In the treatment of AIDS:

Immunosuppression is the hall mark of AIDS. This is caused by reduction in CD_4 (cluster determinant antigen 4) cells of T-lymphocytes. The human immunodeficiency virus (HIV) binds to specific receptors on CD_4 cells by using surface membrane glycoprotein (gp120).
Genetic engineers have been successful to attach Fc portion of mouse monoclonal antibody to human CD_4 molecule. This complex has high affinity to bind to membrane glycoprotein gp120 of virus infected cells. The Fc fragment induces cell-mediated destruction of HIV infected cells (Fig. 17.7).

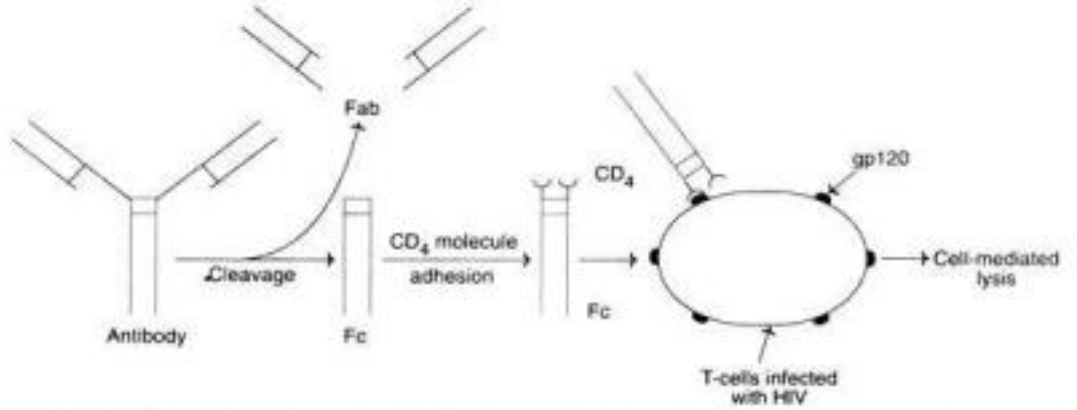


Fig. 17.7 : Modified monoclonal antibody in the treatment of AIDS.

In the treatment of autoimmune diseases:

Autoimmune diseases like rheumatoid arthritis and multiple sclerosis are of great concern. Some success has been reported in the clinical trials of rheumatoid arthritis patients by using MAbs directed against T lymphocytes and B-lymphocytes.

(B) MAbs as Targeting Agents in Therapy:

Toxins, drugs, radioisotopes etc., can be attached or conjugated to the tissue-specific monoclonal antibodies and carried to target tissues for efficient action. This allows higher concentration of drugs to reach the desired site with minimal toxicity. In this way, MAbs are used for the appropriate delivery of drugs or isotopes.

MAbs in use as immunotoxins:

The toxins can be coupled with MAbs to form immunotoxins and used in therapy e.g., diphtheria toxin, Pseudomonas exotoxin, toxins used for cancer treatment. Anti-Tac MAb raised against IL2-R (T-cell growth factor receptor) can be conjugated with exotoxin of Pseudomonas sp. This immunotoxin can be used to destroy the malignant T-cells in the patients suffering from T-cell leukemia (Note: IL2-R is expressed in abnormal T-cells with lymphoid malignancies).

Ricin is a cytotoxic protein derived from castor oil plant. It is composed of two polypeptide chains (A and B) held together by a disulfide linkage. The B-chain of ricin binds to the cell surface. This binding facilitates the A-chain of ricin to enter the cell and inhibit the function of ribosomes (i.e. biosynthesis of

all proteins is blocked).

This results in the death of cells (Fig. 17.8A).

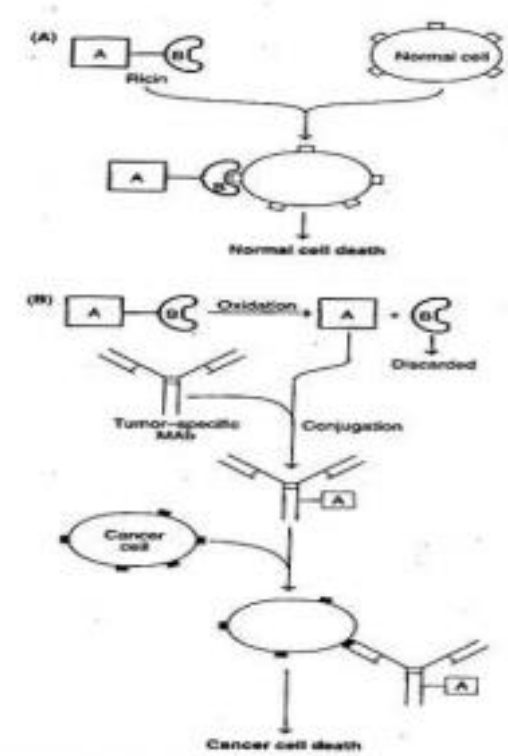


Fig. 17.8 : Use of ricin as a cytotoxic agent
(A) Action of ricin on a normal cell;
(B) A-chain of ricin conjugated to MAb causing cancer cell death
(Note : A-chain of ricin cannot enter normal cell).

MAbs in drug delivery:

In general, the drugs are less effective in vivo (in the living body) when compared to in vitro (in laboratory when tested with cultured cells). This is mainly due to the fact that sufficient quantity of the drug does not reach the target tissue. This problem can be -solved by using tissue-specific MAbs. The drugs can be coupled with MAb (directed against a cell surface antigen of the cells, say a tumor) and specifically targeted to reach the site of action (Fig. 17.9A).

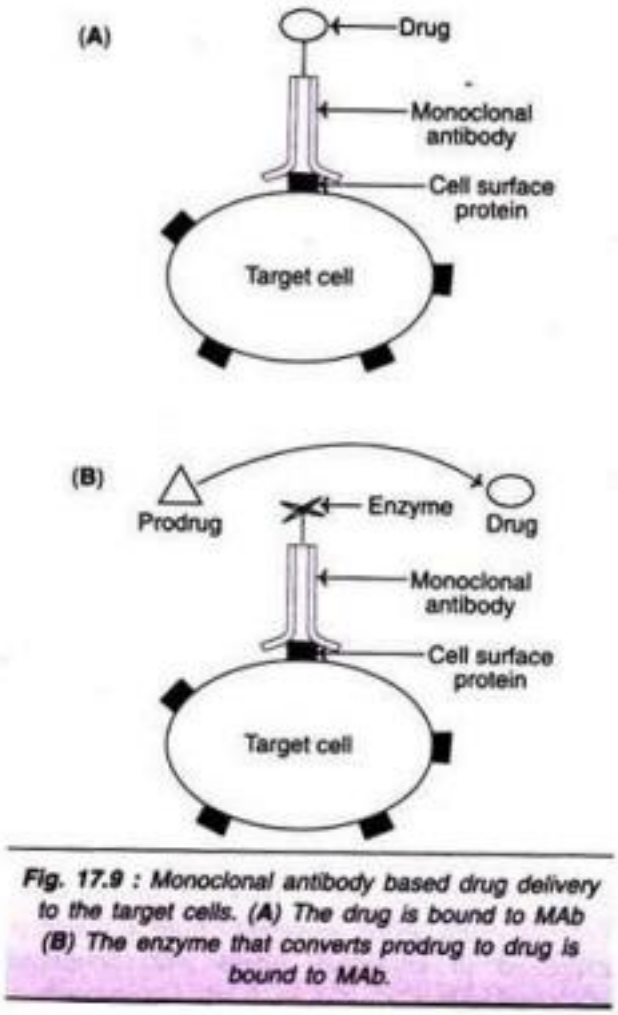


Fig. 17.9 : Monoclonal antibody based drug delivery to the target cells. (A) The drug is bound to MAb (B) The enzyme that converts prodrug to drug is bound to MAb.

In the treatment of certain diseases, a pro-drug (an inactive form of the drug) can be used. This can be enzymatically converted to active drug in the target tissues.

For this purpose, the enzyme (that converts pro-drug to drug) is coupled with MAb that is directed against a specific cell surface antigen (Fig. 17.9A). This approach, referred to as antibody directed enzyme pro-drug therapy (ADEPT), allows an effective delivery of the drug to the cells where it is required.

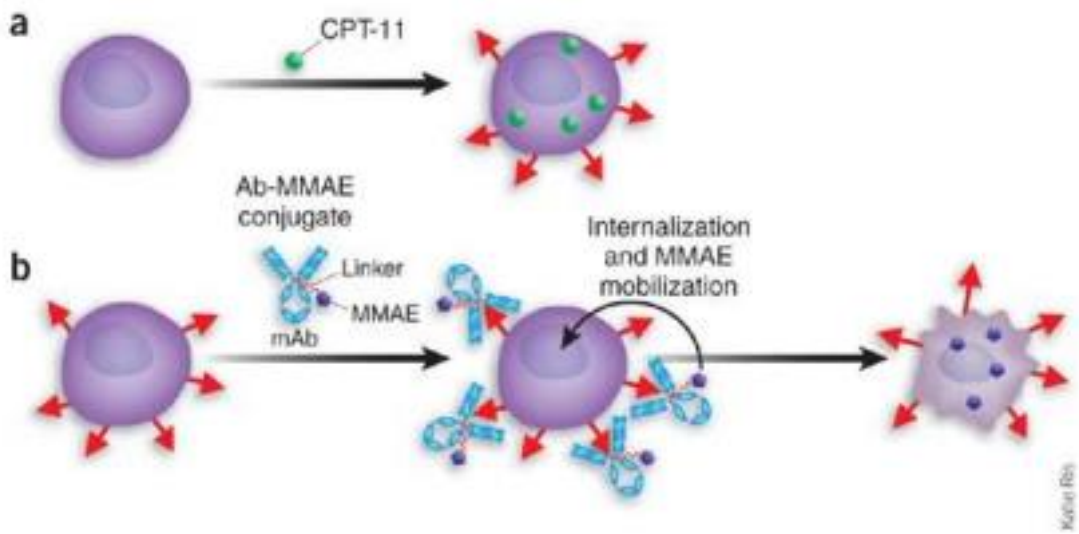
The following are some examples of enzymes that have been used in ADEPT:

i. Alkaline phosphatase for the conversion of phosphate pro-drugs. ii. Carboxy peptidase for converting inactive carboxyl pro-drugs to active drugs.

iii. Lactamase for hydrolyzing β-lactam ring containing antibiotics.

Some monoclonal antibodies have drugs or radiation attached to them. The MAB finds the cancer cells and delivers the drug or radiation directly to them. These are called conjugated MABs. MABs that have a radioactive substance attached include

- Ibritumomab (Zevalin) and Tositumomab (Bexxar)for non Hodgkin lymphoma (NHL) .



(**a**) Cancer cells are treated with the anti-cancer drug CPT-11 resulting in induction of a specific cell-surface antigen. (**b**) The antigen is targeted by a monoclonal antibody–toxin conjugate that is internalized into the cancer cells and kills them.

MAbs in the dissolution of blood clots:

A great majority of natural deaths are due to a blockage in cornary or cerebral artery by a blood clot (thrombus). Fibrin is the major constituent of blood clot which gets dissolved by plasmin. Plasmin in turn is formed by the activation of plasminogen by plasminogen activator. The blockage of arteries occurs due to inadequate dissolution of blood clots. Tissue plasminogen activator (tPA) can be used as a therapeutic agent to remove the blood clots.

A monoclonal antibody directed against fibrin can be coupled to tPA and used for degradation of blood clots. MAb-tPA complex due to a high affinity gets attached to fibrin (Fig. 17.10). Due to the concentration of tPA at the target spots, there is more efficient conversion of plasminogen to plasmin which in turn dissolves blood clot (fibrin). Good success of clot lysis has been reported by using MAb-tPA complex in experimental animals.

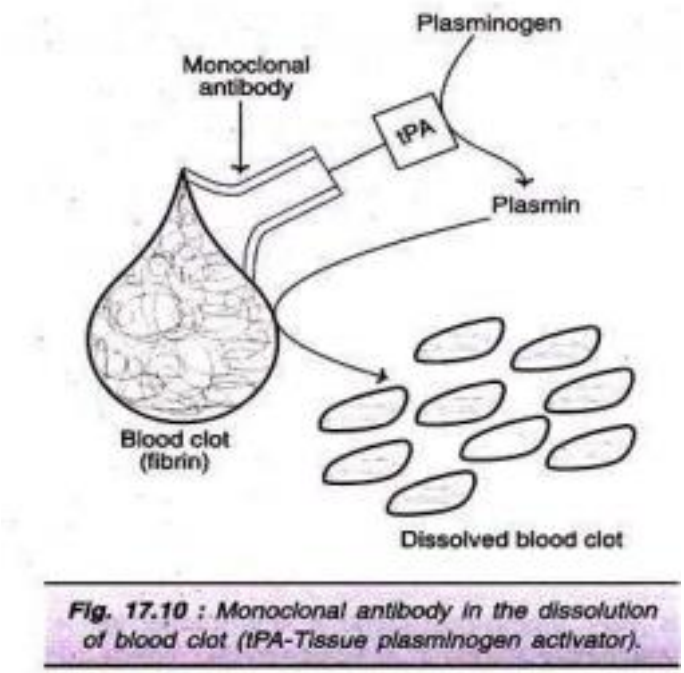


Fig. 17.10 : Monoclonal antibody in the dissolution of blood clot (tPA-Tissue plasminogen activator).

- Use of monoclonal antibodies for immunopurification of antigens

The method of immunopurification using monoclonal antibodies is used, to separate specific antigen from a mixture of very similar antigens. Once purified, the antigen is used for developing vaccine against a pathogen. Individual interferons have been purified using this technique. These interferons were later used for clinical trials and then commercially used.

Advantages: There are certain advantages include the specificity of the MAb to bind to the desired protein, very efficient elution from the chromatographic column and high degree of purification. Immunoaffinity chromatography is routinely used for the purification of recombinant interferon's. The efficiency of this technique will be obvious from the fact that by a single step, it is possible to achieve more than 5,000 fold purification of interferon-α_2.

Disadvantages: It is not possible to achieve 100% purity of the target protein by immunoaffinity. This is due to the fact that a small quantity of MAb leaks into the elution. Further, MAbs cannot distinguish between the intact target protein and a fragment of it with the antigenic site.

Lecture five

the majority of 'new' biotechnology investment over the last 30 years has been in healthcare and especially in the discovery of new drugs. However, the considerable time required to develop a modern pharmaceutical product must not be underestimated, and long periods of toxicological testing are necessary before the national regulatory bodies will grant approval for marketing.

The majority of pharmaceutical products are small-molecule compounds derived either from synthetic chemical processes, from naturally occurring sources (plants, microorganisms) or combinations of both.

1- Antibiotics

Many antibiotics are produced by microorganisms, predominantly by actinomycetes in the genus *Streptomyces* and by filamentous fungi. Antibiotics are produced industrially by a process of fermentation. Once the fermentation is completed, the broth is separated from the fungal mycelium and processed by absorption, precipitation, and crystallization to yield the final product. This basic product can then be modified by chemical procedures to yield a variety of semisynthetic antibiotics.

The field of antibiotic development continues to expand. At present, 6,000 antibiotics have been described, with 4,000 of these derived from actinomycetes. About 300 new antibiotics are being discovered per year.

Discovery and Production of antibiotics

I -Screening for antibiotic Producing Strains

The overall goal of screening is to reduce costs by identifying new microbial strains with antibiotic producing potential which increase productivity and have the ability to use cheaper or alternate substrates.
Below is a summary of what a screening programmed entails:

1) Isolation and fermentations of Potentially Producing Strains

2) Extraction and Purification of the Active Substance

3- Refining

Antibiotic products can take on many different forms. They can be sold in solutions for intravenous bags or syringes, in pill or gel capsule form, or they may be sold as powders, which are incorporated into topical ointments.

4- Quality Control

Quality control is very importance in the production of antibiotics. Since it involves a fermentation process, steps must be taken to ensure that absolutely no contamination is introduced at any point during production. To this end, the medium and all of the processing equipment are thoroughly steam sterilized. During manufacturing, the quality of all the compounds is checked on a regular basis.

II - Genetic modification of antibiotic producing strains

Microorganisms used in fermentation are rarely identical to the wild type. This is because species are often genetically modified to yield the maximum amounts of antibiotics. Mutation is often used.

-New techniques such as protoplast fusion and gene transfer technologies are leading to the development of new strains with higher productivity, improved stability and possible new products. These improvements have all resulted in continued decreases in overall costs of production.

Some examples are the following:

- The gene *cef* EF of the cephalosporin pathway amplified in *Acremonium chrysogenum* , this caused a decrease in the intermediate product (penicillin N) and a 30% increase in cephalosporin C production. Even better results (3 fold cephalosporin C production increase) were obtained when gene *cef* G(last step in the pathway) was amplified in *A. chrysogenum* .

Integration of additional copies of the second or the third gene of this three steps pathway , has not had an important effect on penicillin yields. However ,induction of additional copies of these two genes together in the original fragment caused a 40% increase in the penicillin low producing strain *P. chrysogenum*. Recently induction of the complete penicillin

cluster in the same strain was studied , transformants were isolated with production increase of 124-176%.

- Erythromycin A (Er-A) is the most potent and clinically important member in the Er family produced by *Saccharopolyspora erythraea*. Er-B and Er-C, which are biologically much less active and cause greater side effects than Er-A, serve as the intermediates for Er-A biosynthesis and impurities in fermentation processes of many industrial strains. Systematical modulation of the amounts of tailoring enzymes EryK (a P450 hydroxylase) and EryG (an *S*-adenosylmethionine-dependent *O*-methyltransferase) was carried out by genetic engineering in *S. erythraea*, including alterations of gene copy number ratio and organization and integrating the locus on the chromosome by homologous recombination. Introduction of additional *eryK* and *eryG* genes into *S. erythraea* showed significant impacts on their transcription levels and enhanced the biotransformation process from Er-D to Er-A with gene dose effects. At the *eryK*/*eryG* copy number ratio of 3:2 as well as their resultant transcript ratio of around 2.5:1 to 3.0:1, Er-B and Er-C were nearly completely eliminated and accordingly converted to Er-A, and the Er titer was improved by around 25% in the recombinant strain .

Table 1: Some economically important antibiotics

Antibiotic compound	**Producer microorganism**	**Activity spectrum**
Actinomycin D,Mitomycin C, Streptomycin , Bleomycin	***Streptomyces* sp.**	**Anti-tumor , Anti-bacterial**
Asparaginase	***Erwinia* sp.**	**Anti-leukaemia**
Bacitracin	***Bacillus* sp.**	**Anti-bacterial**
Cephalosporin	***Acremonium* sp.**	**Anti-bacterial**
Chloramphenicol	***Cephalosporium* sp.**	**Anti-bacterial**
Daunorubicin	***Streptomyces* sp.**	**Anti-protozoal**
Fumagillin	***Aspergillus* sp.**	**Amoebicidal**
Penicillin G ,Griseofulvin	***Penicillium* sp.**	**Anti-bacterial, Anti-fungal**
Nisin	***Streptococcus* sp.**	**Food preservative**
Rifamycin	***Nocardia* sp.**	**Anti-tuberculosis**

2)- Therapeutic proteins (biopharmaceuticals)

Therapeutic protein (biopharmaceutical) are protein-based and may either be derived from genetically altered bacteria or fungi (also called ***biotech drugs***), or may come from blood and blood plasma products (usually referred to as ***biologics***).

Therapeutic proteins are the proteins that has an effect of healing or use inside the body, e.g

- ❖ nutrition: the use of albumin.
- ❖ Globulins: the example is gamma globulin that boosts the defenses against infectious diseases (gamma globulin is a mixture of antibodies).
- ❖ Synthetic proteins: antibodies against inflammatory components (infliximab), or against tumor components (trastuzumab).

Advantages of Protein therapeutics:
• A highly specific and complex set of functions
• Less potential to interfere with normal biological processes and cause adverse effects
• Well tolerated and less likely to elicit immune responses
• Effective replacement treatment without the need for gene therapy
• A faster clinical development and FDA approval

It is possible to manufacture these drugs in large quantities by cloning the corresponding genes from human or animals through plasmid vectors in bacteria. This procedure brings down the cost of manufacturing the drugs.

<u>Classification of Protein therapeutics</u>

- ⏵ Group I: protein therapeutics with enzymatic or regulatory activity
- ⏵ Ia: Replacing a protein that is deficient or abnormal
- ⏵ Ib: Augmenting an existing pathway
- ⏵ Ic: Providing a novel function or activity
- ⏵ Group II : protein therapeutics with special targeting activity
- ⏵ IIa: Interfering with a molecule or organism
- ⏵ IIb: Delivering other compounds or proteins
- ⏵ Group III : protein vaccines

- IIIa: Protecting against a deleterious foreign agent.
- IIIb: Treating an autoimmune disease.
- IIIc: Treating cancer.
- Group IV : protein diagnostics

Group I: Protein therapeutics with enzymatic or regulatory activity

Ia: Replacing a protein that is deficient or abnormal

Therapeutic	Trade name	Function	Examples of clinical use
†Insulin[16-20]	Humulin, Novolin	Regulates blood glucose, shifts potassium into cells	Diabetes mellitus, diabetic ketoacidosis, hyperkalaemia

Ib: Augmenting an existing pathway

Therapeutic	Trade name	Function	Examples of clinical use
Teriparatide[213-216] (human parathyroid hormone residues 1–34)	Forteo	Markedly enhances bone formation; administered as a once-daily injection	Severe osteoporosis

Ic: Providing a novel function or activity

Therapeutic	Trade name	Function	Examples of clinical use
Hyaluronidase (recombinant human)[236]	Hylenex	Catalyses the hydrolysis of hyaluronic acid to increase tissue permeability and allow faster drug absorption	Used as an adjuvant to increase the absorption and dispersion of injected drugs, particularly anaesthetics in ophthalmic surgery and certain imaging agents

Group II: Protein therapeutics with special targeting

IIa: Interfering with a molecule or organism

Therapeutic	Trade name	Function	Examples of clinical use
Trastuzumab[84]	Herceptin	Humanized mAb that binds HER2/Neu cell surface receptor and controls cancer cell growth	Breast cancer

IIb: Delivering other compounds or proteins

Therapeutic	Trade name	Function	Examples of clinical use
Denileukin diftitox[89,90]	Ontak	Directs the cytocidal action of diphtheria toxin to cells expressing the IL2 receptor	Persistent or recurrent cutaneous T-cell lymphoma whose malignant cells express the CD25 component of the IL2 receptor

Group III: Protein vaccines

IIIa: Protecting against a deleterious foreign agent

Therapeutic	Trade name	Function	Examples of clinical use
Hepatitis B surface antigen (HBsAg)[93,94]	Engerix, Recombivax HB	Non-infectious protein on surface of hepatitis B virus	Hepatitis B vaccination

IIIb: Treating an autoimmune disease

Therapeutic	Trade name	Function	Examples of clinical use
Anti-Rhesus (Rh) immunoglobulin G[98]	Rhophylac	Neutralizes Rh antigens that could otherwise elicit anti-Rh antibodies in an Rh-negative individual	Routine antepartum and postpartum prevention of Rh(D) immunization in Rh(D)-negative women; Rh prophylaxis in case of obstetric complications or invasive procedures during pregnancy; suppression of Rh immunization in Rh(D)-negative individuals transfused with Rh(D)-positive red blood cells

IIIc: Treating cancer

Therapeutic	Trade name	Function	Examples of clinical use
–	–	–	Currently in clinical trials

Group IV: Protein diagnostics

Therapeutic	Trade name	Function	Examples of clinical use
Recombinant purified protein derivative (DPPD)[100–102]	DPPD	Noninfectious protein from *Mycobacterium tuberculosis*	Diagnosis of tuberculosis exposure
‡Growth hormone releasing hormone (GHRH)[103,104]	Geref	Recombinant fragment of GHRH that stimulates growth hormone release by somatotroph cells of the pituitary gland	Diagnosis of defective growth-hormone secretion
Capromab pendetide[108]	ProstaScint	Imaging agent; indium-111-labelled anti-PSA antibody; recognizes intracellular PSA	Prostate cancer detection
Imciromab pentetate[297]	Myoscint	Imaging agent; indium-111-labelled antibody specific for human cardiac myosin	Detects presence and location of myocardial injury in patients with suspected myocardial infarction
HIV antigens[109–111]	Enzyme immunoassay, OraQuick, Uni-Gold	Detects human antibodies to HIV (enzyme immunoassay, western blot)	Diagnosis of HIV infection

Challenges for protein therapeutics:

1-Protein solubility, route of administration, distribution, and stability.
2-Immune response.
3-Physiologically active – post-translational modifications.
4- Costs.
5- Ethics

Recombinant therapeutic proteins **Lec. 6**

A wide range of cellular forms including bacteria, yeasts, mammalian and human cells have been used for heterologous protein expression. Transgenic animals and plants can also be used . When a trans gene is introduced into a recipient animal, the expression of the gene product can occur in the milk, blood or urine of the animal.

With the advent of gene technology it is now possible to produce human therapeutic proteins in large quantities and high purity. As such, recombinant human proteins can now be used in rational therapy using the body's own substances, which will not be immunogenic.

The DNA sequences coding for the therapeutic proteins can also be modified by direct mutagenesis allowing further changes in protein structure. This is called *protein engineering* and the mutated proteins are termed *muteins* .

The first human gene sequences encoding important therapeutic proteins cloned into microorganisms were insulin , interferons and Human Growth hormone.

- The use of recombinant DNA technology to modify *Escherichia coli* bacteria to produce human ***insulin***.

a) Genetic researchers produced artificial genes for each of the two protein chains that comprise the insulin molecule.
b) The artificial genes were then inserted into plasmids among a group of genes that are activated by lactose. Thus, *the insulin producing genes were also activated by lactose*.
c) The recombinant plasmids were inserted into *Escherichia coli* bacteria, which were "induced to produce 100,000 molecules of either chain A or chain B human insulin.
d) The two protein chains were then combined to produce insulin molecules.

- Production of human growth hormone is done by inserting DNA coding for human growth hormone into a plasmid that was implanted in *Escherichia coli* bacteria. The gene that was inserted into the plasmid was created by reverse transcription of the mRNA found in pituitary glands to complementary DNA.

In 2008, about 633 therapeutic products for over 100 different diseases including cancers, bacterial diseases, immune diseases, HIV/AIDS virus were under development according to "the pharmaceutical research and manufactures of America (PhrMA)" , 31 noval recombinant drugs and further 12 new therapeutic proteins in 2009 are approved by FDA . Now a day, these drugs are commonly used for the cure of rare diseases which are impossible to treat by conventional therapies.

Table 1: Some of the 200 biopharmaceuticals approved in the USA and EU over the last 25 years.

Product	**Therapeutic indication**
Recombinant blood factors, e.g. Factor VIII	**Haemophilia A**
Recombinant thrombolytics & anticoagulants , e.g. tissue plasminogen activator	**Myocardial infarction**
Recombinant hormones, e.g. insulin, human growth hormone	**Diabetes mellitus, growth disturbances in children and adults**
Recombinant growth factors, e.g. erythropoietin	**Anaemia**
Recombinant interferons and interleukins, e.g. Interferon-α, Interferon-β	**Hepatitis B, C and various cancers**
Recombinant vaccines, e.g. Hepatitis B	**Hepatitis B**
Monoclonal antibodies, e.g. Herceptin, ProtaScint	**Breast cancer, prostate adenocarcinoma**
Recombinant enzymes, e.g. Myozyme	**Pompe disease**
Nucleic acid-based products, e.g. Macugen	**Macular degeneration**

4- Biotechnology Vaccine Production

Vaccines are the most effective form of disease prevention in the world .

Vaccines are considered possibly the best achievement of medical advance of the form century. Small pox has completely eliminated worldwide by vaccination. Research in the vaccine advances is being used effectively for communicable diseases as well as non communicable diseases for example cancer.

The principle for the use of vaccines is very simple because they mainly stimulate the patient's immune system against the infectious agents like viruses or bacteria without causing any disease itself . These vaccines include inactivated vaccines, toxoid vaccines, live attenuated vaccines, conjugate vaccines, subunit vaccines, recombinant protein vaccines and DNA vaccines.

Recombinant Vaccines

• Two types:

(i) *Recombinant protein vaccines*: This is based on production of recombinant DNA which is expressed to release the specific protein used in vaccine preparation

(ii) *DNA vaccines*: Here the gene encoding for immunogenic protein is isolated and used to produce recombinant DNA which acts as vaccine to be injected into the individual.

Recombinant protein vaccines:

• A pathogen produces its proteins in the body which elicit an immune response from the infected body.

• The gene encoding such a protein is isolated from the causative organism

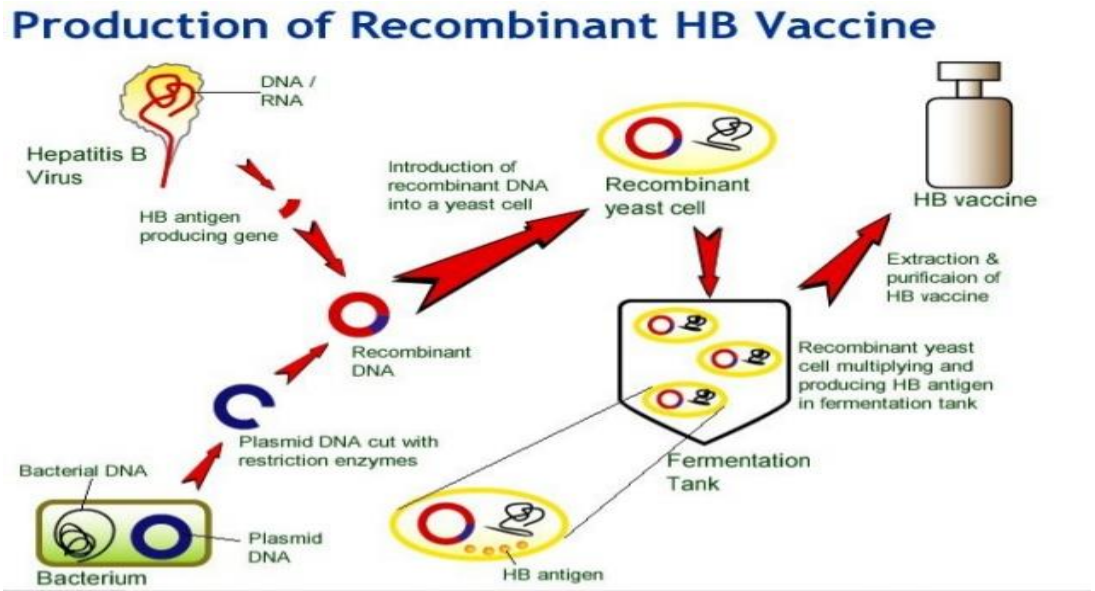
• This DNA is expressed in another host organism, like genetically engineered microbes; animal cells; plant cells; insect larvae etc, resulting in the release of appropriate proteins.

• These when injected into the body, causes immunogenic response against the corresponding disease providing immunity

Most of the new vaccines consist only of the antigen, not the actual microbe. The vaccine is made by inserting the gene that produces the antigen into a manufacturing cell, such as yeast. During the manufacturing

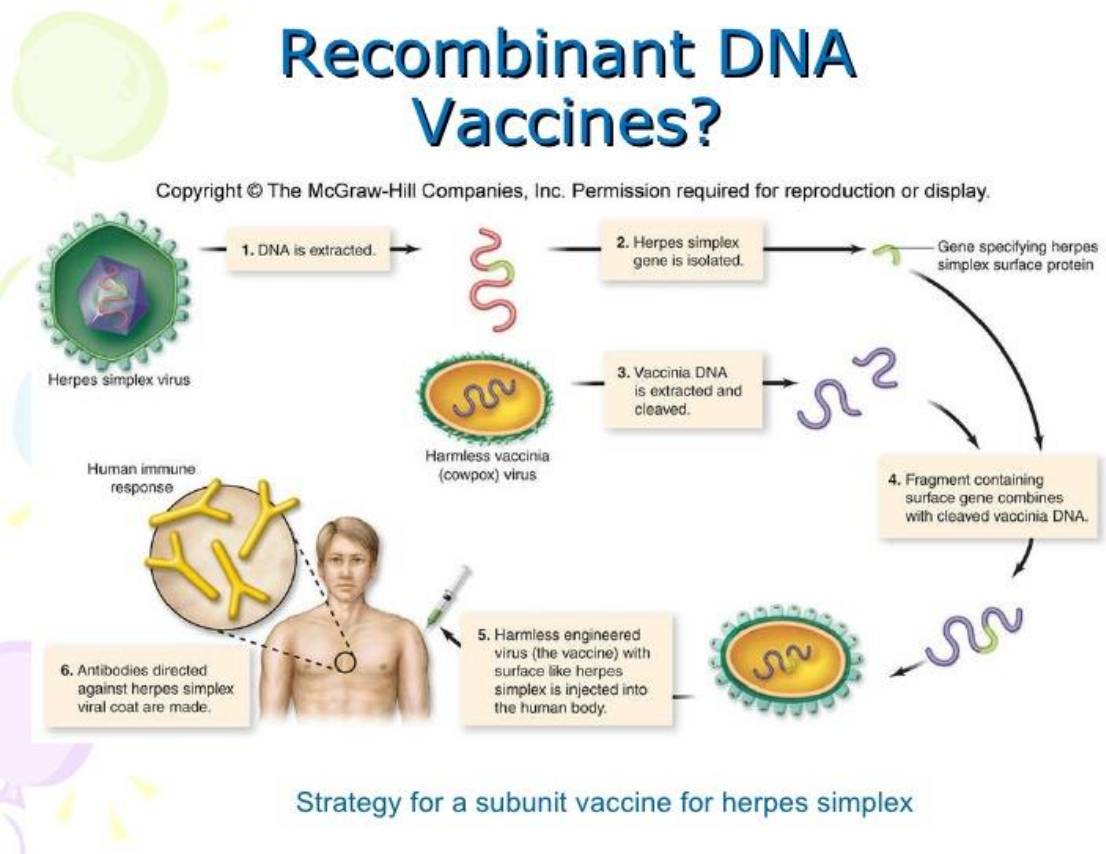
process, each yeast cell makes a perfect copy of itself and the antigen gene. The antigen is later purified from the yeast cell culture. By isolating antigens and producing them in the laboratory, it is possible to make vaccines that cannot transmit the virus or bacterium itself. This method also increases the amount of vaccine that can be manufactured because biotechnology vaccines can be made without using live animals. Using these techniques of biotechnology, scientists have developed antigen-only vaccines against life-threatening diseases such as hepatitis B and meningitis.

Hundreds of genes in eukaryotes have been cloned from genomic DNA or from cDNA. These clones genes included a number of genes for specific antigens and some have been used for the synthesis of antigens leading to the preparation of vaccines. A very good example of this is cloning of Hepatitis B virus (HBV) genome. The HBV genome was cloned in the plasmid pBR322 followed by its propagation in ***E.coli***. The antigens produced from this clone reacted with hepatitis B core antibody (HBAb) which has been used to produce hepatitis B vaccine.



The second method used to produce vaccine include determination which gene in a pathogenic, or disease-causing virus stimulates the production of antibodies in the human immune system, then the section of DNA containing this gene is isolated and then placed into a non-harmful virus. This is usually accomplished by the use of a plasmid, which acts as chromosomal DNA and replicates in the new bacterium where it has been

incorporated. The new virus, containing the recombinant DNA, is used as a vaccine and injected into patients . .



Strategy for a subunit vaccine for herpes simplex

Genetically modified vaccines are much safer than conventional ones because they do not expose the patient to the actual virus, as it may sometimes lead to accidental infection.

A diversity of recombinant vaccines is being used worldwide for the treatment of human diseases. It has been demonstrated that a subunit vaccine RTS, S/AS02 provides protection against malarial natural infection in adults . TB is another major growing concern in developing countries for which scientists are making numerous efforts to achieve a recombinant TB vaccine. Because each year, it privileges at minimum two million lives . Up till now research has a subunit vaccine having the surface antigen Mtb8.4 that is responsible for the protection of mice from the infection of tuberculosis . In viral-derived diseases, vaccines are being developed by recombinant DNA technology against the influenza virus, polio virus, hepatitis B virus, herpes virus and more recently the AIDS virus.

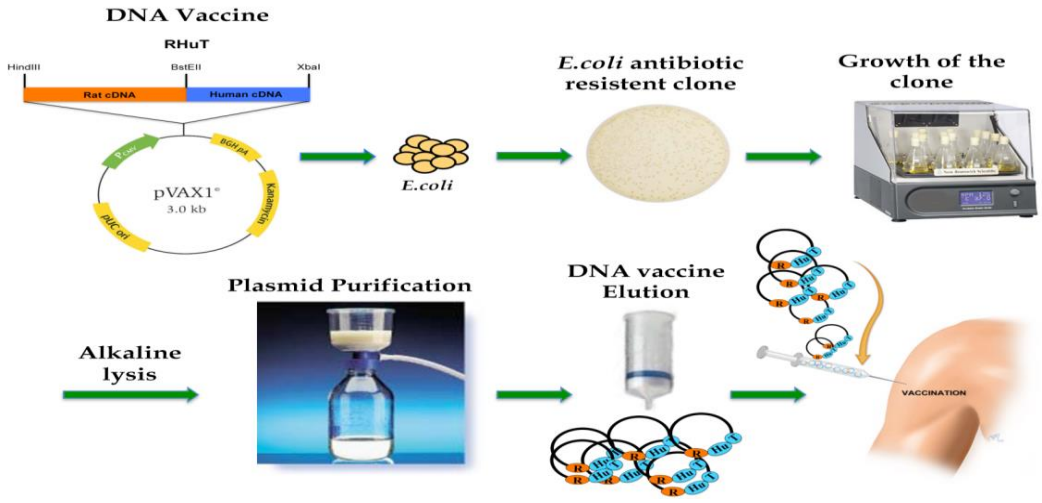
DNA vaccination

DNA vaccination is a technique for protecting an organism against disease by injecting it with genetically engineered DNA to produce an immunological response. Nucleic acid vaccines are still experimental, and have been applied to a number of viral, bacterial and parasitic models of disease, as well as to several tumor models. DNA vaccines have a number of advantages over conventional vaccines, including the ability to induce a wider range of immune response types. DNA vaccines have been introduced into animal tissues by a number of different methods.

The two most popular approaches are injection of DNA in saline, using a standard hypodermic needle, and gene gun delivery. Injection in saline is normally conducted intramuscularly (IM) in skeletal muscle, or intradermally (ID), with DNA being delivered to the extracellular spaces. Immune responses to this method of delivery can be affected by many factors, including needle type, needle alignment, speed of injection, volume of injection, muscle type, and age, sex and physiological condition of the animal being injected.

Gene gun delivery, the other commonly used method of delivery, ballistically accelerates plasmid DNA (pDNA) that has been adsorbed onto gold or tungsten microparticles into the target cells, using compressed helium as an accelerant. Alternative delivery methods have included aerosol instillation of naked DNA on mucosal surfaces, such as the nasal and lung mucosa, and topical administration of pDNA to the eye and vaginal mucosa. Mucosal surface delivery has also been achieved using cationic liposome-DNA preparations, biodegradable microspheres, attenuated *Shigella* or *Listeria* vectors for oral administration to the intestinal mucosa, and recombinant adenovirus vectors.

Another approach to DNA vaccination is expression library immunization (ELI). Using this technique, potentially all the genes from a pathogen can be delivered at one time, which may be useful for pathogens which are difficult to attenuate or culture. ELI can be used to identify which of the pathogen's genes induce a protective response. This has been tested with *Mycoplasma pulmonis*, a murine lung pathogen with a relatively small genome, and it was found that even partial expression libraries can induce protection from subsequent challenge.



Advantages of DNA vaccination

- Subunit vaccination with no risk for infection
- immune response focused only on antigen of interest
-Ease of development and production
- Stability of vaccine for storage and shipping with low cost
- avoid need for peptide synthesis, expression and purification of recombinant proteins and the use of toxic adjuvants
-Long-term persistence of immunogen
- In vivo expression ensures protein more closely resembles normal eukaryotic structure, with accompanying post-translational modifications.

Disadvantages of DNA vaccination

-Limited to protein immunogens (not useful for non-protein based antigens such as bacterial polysaccharides)
- Risk of affecting genes controlling cell growth
-Possibility of inducing antibody production against DNA
-Possibility of tolerance to the antigen (protein) produced

Synthetic peptides as vaccines

Vaccines can also be prepared through short synthetic peptide chains. There are several ways by which these can be used as vaccines.

As it is the three dimensional structure (not the amino acid sequence) of the protein which is responsible for the immunogenic response, it is essential to find out the protein region involved in immunogenic response. E.g. in Foot and Mouth Disease virus (FMDV), the amino acid 114-160 of

virus polypeptide can produce antibodies which can neutralize FMDV and provide protection. The region of 201-213 amino acids of the same protein also could neutralize FMDV hence it has been shown that small synthetic peptides representing these regions of proteins can show immunogenic response and can be used for the development of vaccine.

Vaccinations against cancer

Vaccinations against cancer has transformed into clinical studies aiming to optimally deliver vaccines based on defined antigens to induce anticancer immunity. This method of treating cancerous cells relies on vaccines consisting of peptides derived from the protein sequence of candidate tumor-associated or specific antigens . Tumor cells express antigens known as tumor-associated antigens (TAAs) that can be recognized by the host's immune system (T cells). Many TAAs have already been identified and molecularly characterized . These TAAs can be injected into cancer patients in an attempt to induce a systemic immune response that may result in the destruction of the cancer growing in different body tissues. This procedure is defined as active immunotherapy or vaccination as the host's immune system is either activated de novo or re stimulated to rise an effective tumor-specific immune reaction that may lead to tumor regression (Figure1). Any protein/peptide produced in a tumor cell that has an abnormal structure due to mutation can act as a tumor antigen. Such abnormal proteins are produced due to mutation of the concerned gene. Various clinical studies focus on the therapeutic potential of active immunization or vaccination with TAA peptides in patients with metastatic cancer

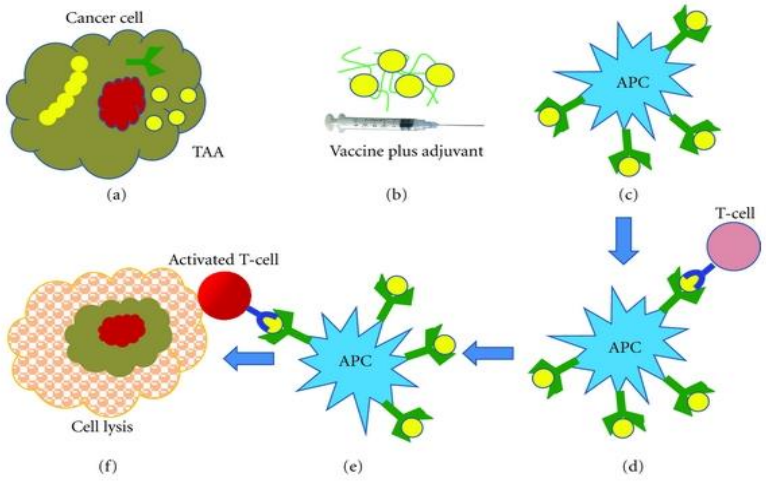


Figure1: Peptide-based cancer vaccines: tumor cells express antigens known as tumor-associated antigens (TAAs) that can be recognized by the host's immune system (a). These TAAs mixed with an adjuvant can be injected into cancer patients in an attempt to induce a systemic immune response (b). The antigen presenting cell (APC) presents the antigen to T cell ((c) and (d)), thereby the T cell is activated (e) which results in the destruction of the cancer cell (f).

Vaccine Delivery Systems

Whether the vaccine is a live virus, coat protein or a piece of DNA, vaccine production requires elaborate and costly facilities and procedures. And then there's the issue of injections, which can sometimes be painful and which many patients dislike. These problems can be avoided by the use of controlled-release delivery and injection free systems. In needle-free technologies through the use of high-speed jet of gas, vaccine is introduced inside the body. Besides these techniques, different solutions, skin patches and rubbing gels can also introduce agents into body by simple diffusion method. Nasal sprays and inhalers are also effective ways to administer drugs and vaccines through the respiratory tract. About 15% budget is required for the storage and refrigerated transport of vaccines that is the main cost in all vaccine programs . So, an ideal vaccine should have high immunogenicity and long lasting immunity as well as it should be heat stable, inexpensive, commercially available and affordable by maximum no. of people. There is a space and greater opportunities exist in the field of molecular biology and biotechnology for developing many "Edible vaccines" manufactured by plants and animals , that will be easily immunize people against infectious diseases .

Genetically modified goats have produced a possible malaria vaccine in their milk. Academic researchers have obtained positive results using human volunteers who consumed hepatitis vaccines in bananas, and *E. coli* and cholera vaccines in potatoes. In addition, because these vaccines are genetically incorporated into food plants and need no refrigeration, sterilization equipment or needles, they may prove particularly useful in developing countries Researchers are also developing skin patch vaccines for tetanus, anthrax, influenza and *E. coli.*

Lecture seven

c- Gene therapy lec 7

Gene therapy is the application of genetic engineering to insert enhanced genes into an individual in order to remedy a disease or dysfunction caused by a genetic defect. Through gene therapy it is possible to work directly at the base of a disorder rather than use. As the science of genetics advanced throughout the 1980s, gene therapy gained an established foothold in the minds of medical scientists as a promising approach to treatments for specific diseases.

There are two types of gene therapy: Germ line gene therapy and Somatic gene therapy.

-*Germ line* gene therapy is when germ cells, such as sperm or eggs, are modified by the introduction of functional genes. These functional genes are integrated into their genomes, therefore the change would be heritable and could be passed on to later generations.

-*Somatic* gene therapy is when therapeutic genes are transferred into the somatic cells of a patient, meaning any modifications and effects would be restricted to the individual and not heritable.

There are several approaches to go about gene therapy.

-A normal gene may be inserted into a nonspecific location within the genome to replace a nonfunctional gene. This approach is most common.
-An abnormal gene could be exchanged for a normal gene through homologous recombination.
-The abnormal gene could be repaired through selective reverse mutation, which returns the gene to its normal function.
-The regulation (the degree to which a gene is turned on or off) of a particular gene could be altered..

Patient therapy

In patient therapy the cells with healthy genes are introduced in the affected tissue but the inheritance trait of the patient is not affected or altered.

This therapy involves the following steps:
a) Identification of defective gene
b) Isolation or synthesis of normal healthy gene.
c) Isolation of the cells of the tissue where the normal healthy gene will

need to function.
d) The placement of normal gene into a cell where it can function.

Gene therapy holds great potential for treating disorders traceable to a single defective gene.

Viral vectors have been used to facilitate the replacement of defective genes with healthy, functional copies.

There are still technical problems to be solved before this becomes viable technology – First there is the possibility that the introduced gene may not function, and the second is when corrected cells are reintroduced, these maybe outnumbered by the diseased resident cells. The other problem is that there are only few diseases affecting only a single tissue.

Genes can be delivered into a group of cells in a patient's body in two ways.

1. **Ex vivo (outside the body)**

- Cells from the patient's blood or bone marrow are removed and grown in the laboratory. They are then exposed to a virus carrying the desired gene. The virus enters the cells, and the desired gene becomes part of the DNA of the cells. The cells are allowed to grow in the laboratory before being returned to the patient by injection into a vein.

2. In vivo (inside the body)

- No cells are removed from the patient's body. Instead, vectors are used to deliver the desired gene to cells in the patient's body.

Risks associated with Gene Therapy

1. Undesirable health effects (e.g. cancers / death)
2. If gene insertion occurs in the wrong location it may affect the functioning of pre-existing genes that are vital within the genome
3. Viral vectors may infect healthy cells or tissues
4. Virus may revert to original form (mutate) and become pathogenic
5. Virus entry may trigger an immune response leading to inflammation, toxicity and organ failure
6. Treatment must be repeated at regular intervals, increasing likelihood of adverse treatment response with time .

Gene Therapy Successes

- Immune deficiencies

Several inherited immune deficiencies have been treated successfully with gene therapy. Most commonly, blood stem cells are removed from patients, and retroviruses are used to deliver working copies of the defective genes. After the genes have been delivered, the stem cells are returned to the patient. Because the cells are treated outside the patient's body, the virus will infect and transfer the gene to only the desired target cells.

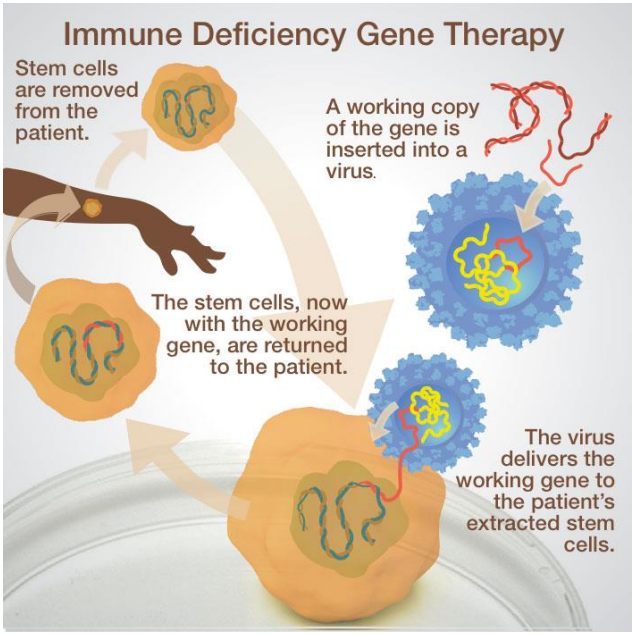
Severe Combined Immune Deficiency (SCID) was one of the first genetic disorders to be treated successfully with gene therapy, proving that the approach could work. This is a sex-linked disorder and involves dysfunctional versions of the Interleukin-2 receptor, which plays a very important role in how the body responds to infections. People who lack functional IL-2 receptors end up dying of infections due to compromised immunity in the early years of life. This is because IL-2 signaling is responsible for the maturation and differentiation of progenitors into T-Lymphocytes, functional B-lymphocytes and NK Cells. All of these are extremely vital components of the immune system and not having them opens the body to all kinds of pathogens.

SCID has been treated using ex-vivo gene therapy, where a retroviral vector containing a copy of a fully functional IL-2 receptors was used to induce the gene expression required to rescue immune function. The problem with using retroviral vectors is that they integrate randomly into host genomes, and this means it may affect normal gene expression and trigger cancers

However, the first clinical trials ended when the viral vector triggered leukemia (a type of blood cancer) in some patients. Since then, researchers have begun trials with new, safer viral vectors that are much less likely to cause cancer.

Adenosine deaminase (ADA) deficiency is another inherited immune disorder that has been successfully treated with gene therapy. In multiple small trials, patients' blood stem cells were removed, treated with a retroviral vector to deliver a functional copy of the ADA gene, and then returned to the patients. For the majority of patients in these trials, immune function improved to the point that they no longer needed

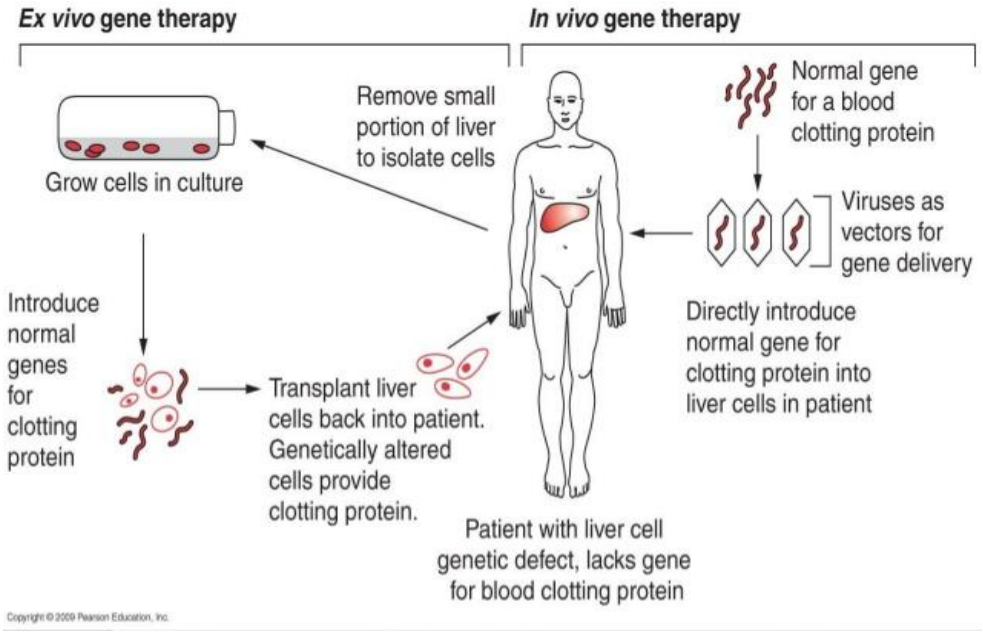
injections of ADA enzyme. Importantly, none of them developed leukemia.



-Hemophilia

People with hemophilia are missing proteins that help their blood form clots. Those with the most-severe forms of the disease can lose large amounts of blood through internal bleeding or even a minor cut.

In a small trial, researchers successfully used an adeno-associated viral vector to deliver a gene for Factor IX, the missing clotting protein, to liver cells. After treatment, most of the patients made at least some Factor IX, and they had fewer bleeding incidents.



-Blood disease

Patients with beta-Thalassemia have a defect in the beta-globin gene, which codes for an oxygen-carrying protein in red blood cells. Because of the defective gene, patients don't have enough red blood cells to carry oxygen to all the body's tissues. Many who have this disorder depend on blood transfusions for survival.

In 2007, a patient received gene therapy for severe beta-Thalassemia. Blood stem cells were taken from his bone marrow and treated with a retrovirus to transfer a working copy of the beta-globin gene. The modified stem cells were returned to his body, where they gave rise to healthy red blood cells. Seven years after the procedure, he was still doing well without blood transfusions.

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- **Cancer**

Most therapies simply add a useful gene into a selected cell type to:

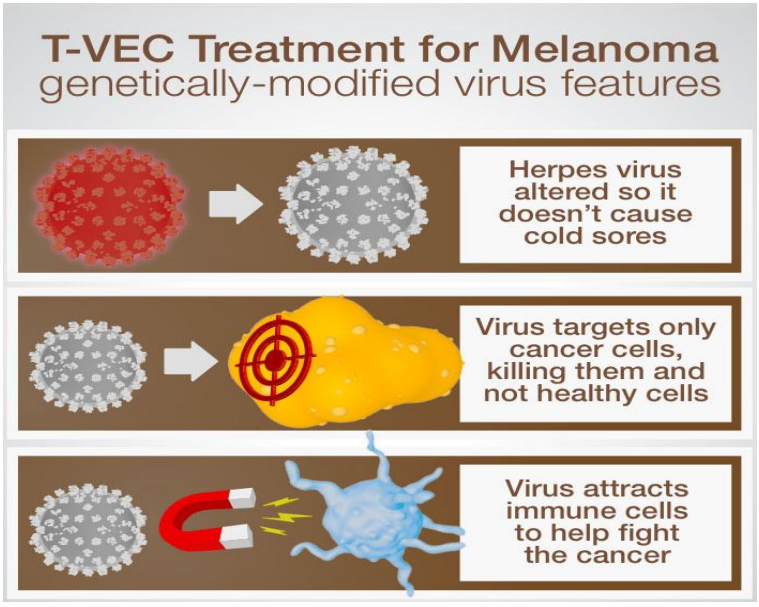
- inducing cancer cells to make a protein that will kill the cell(Direct approach).

- stimulating an immune response against selected cells or eliminating the blood supply(Indirect approach).

Several promising gene-therapy treatments are under development for cancer. One, a modified version of the herpes simplex 1 virus (which normally causes cold sores) has been shown to be effective against melanoma (a skin cancer) that has spread throughout the body. The treatment, called T-VEC, uses a virus that has been modified so that it will (1) not cause cold sores; (2) kill only cancer cells, not healthy ones; and (3) make signals that attract the patient's own immune cells, helping them learn to recognize and fight cancer cells throughout the body. The virus is injected directly into the patient's tumors. It replicates (makes more of itself) inside the cancer cells until they burst, releasing more viruses that can infect additional cancer cells.

A completely different approach was used in a trial to treat 59 patients with leukemia, a type of blood cancer. The patients' own immune cells were removed and treated with a virus that genetically altered them to recognize a protein that sits on the surface of the cancer cells. After the

immune cells were returned to the patients, 26 experienced complete remission.



probiotics Technology

Definition of Probiotics: a live microbial feed supplement which beneficially affects the host animal .

However the definition given by the Joint Food and Agriculture Organization/World Health Organization Working Group in 2001, i.e.; "Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host". The probiotic bacteria selected should have the following main characteristics:

• They should have "non-pathogenic' activity

• They should be "resistant to bile salts" and gastric acids

• They should have the "desired technological and organoleptic properties"

• They should have "biological efficiency" on humans, including 'adhesion to epithelial cells' in the intestine

• They should interact with "enteropathogenic" bacteria

• They should be able to "colonize" in the gut

• They should be able to "stimulate the immune system

Various yeasts and bacteria used as probiotics, including *Lactobacillus* (*L. salivarius , L.casei, L. acidophilus , L. rhamnosus , L. plantarum , L. reuteri*) , *Bifidobacterium , Lactococcus, Saccharomyces* (yeast) , *Streptococcus, Thermophilus* and *Enterococcus.*

Main Mechanisms of Action of Probiotics:

1-Inhibition of adhesion of pathogens

2-production of inhibitory compounds like antibiotics, bacteriocins, proteases, siderophores, lysozymes , hydrogen peroxide, organic acids (pH change)

3- Source of macro- and micro-nutrients

4- Enzymatic contribution to digestion

5-Modulation of the Immune System

6-Reduction in Inflammation

Important aspects of probiotics:

1.Viability , 2:Survival within the GI tract , 3: Human origin ,

4: Adhesion: Strains of probiotic bacteria with adherent properties are likely to stay longer in the intestine.

5:Safety: Mainly non pathogenic , Opportunistic.

Well-established probiotic effects are:

1- Prevention and alleviation of unspecific and irregular complaints of the gastrointestinal tracts in healthy people.

2-Lactic acid produce by *Lactobacillus* help enhancing the utilization of essential minerals such as calcium, phosphorus and iron .

3- Stabilization of gut microflora and competitive exclusion of enteric pathogen.

4-They can influence intestinal physiology either directly or indirectly through modulation of the endogenous ecosystem or immune system. The results that have been shown with a sufficient level of proof to enable probiotics to be used as treatments for gastrointestinal disturbances are

a) the good tolerance of yogurt compared with milk in subjects with primary or secondary lactose maldigestion.

b) the use of *Saccharomyces boulardii* and *Enterococcus faecium* SF 68 to prevent or shorten the duration of antibiotic-associated diarrhea and to prevent further recurrence of *Clostridium difficile*-associated diarrhea.

c*)Lactobacillus* controls the diarrhea due to antibiotics produced , probiotic consumption to be useful in the treatment of many types of diarrhea, including antibiotic-associated diarrhea in adults, travellers' diarrhea, treating acute viral gastroenteritis in healthy children. the use of fermented milks containing *Lactobacillus rhamnosus* GG to shorten the duration of diarrhea in infants with rotavirus enteritis (and probably also in gastroenteritis of other causes).

d) The probiotics were used to treat childhood *Helicobacter pylori* gastritis, irritable bowel syndrome, chronic ulcerative colitis, and infantile colic.

5- Enhance innate host defense by production of antimicrobial substance. Enhance both the specific and nonspecific immune response, possibly by activating macrophages, increasing levels of cytokines, increasing natural killer cell activity, and/or increasing levels of immunoglobulins , these results may be particularly important to the elderly, who could benefit from an enhanced immune response. There are also safety concerns with the use of probiotics in infants and children who are immunocompromised, or seriously ill with indwelling medical devices.

6-Evidence suggests that probiotics can also reduce the risk of hypo-salivation and feeling of dry mouth , possibly reducing demineralization of teeth. Probiotics should adhere to dental tissues to establish a cariostatic effect and thus should be a part of the biofilm to fight the cariogenic bacteria . *L. reuteri* and *L. brevi* are among the species able to affect gingivitis and periodontitis .

7- Prevention of respiratory tract infections (common cold, influenza) and other infectious diseases as well as treatment of urogenital infections. Several *Lactobacillus* species have shown clinical efficacy as a treatment for vaginal infections and urinary-tract infections.

8- Improvement of nutritional quality of food and feed, metabolic stimuliation of the vitamins synthesis and enzyme production(*Lactobacillus* produces mainly vitamin B complex) . Probiotics be combined with enzymes that help break down food substances into simpler forms to enhance nutrient digestion.

9- Reduction of serum cholesterol .

10- Reduction of the concentration of cancer-promoting enzymes and/or bacterial metabolites in the gut. Decrease risk of colon cancer by detoxification of carcinogens .

11- Some food products derived from probiotics bacteria could possibly contribute to blood pressure control. Two tripeptides, valine- proline-proline and isoleucine-proline- proline, isolated from fermentation of a milk-based medium by *Saccharomyces cereviseae and L .helveticus* have been identified as the active components and reduce blood pressure .

Product Design and Description of the Probiotic

Product design for probiotics starts with:

1- The selection of a strain of bacteria or yeast that has characteristics thought to be associated with healthy functioning of human body systems. Selected strains usually have been isolated from healthy individuals or animals. Selected strains should be characterized using scientifically valid techniques.

2- Strain identification, source, and history.

Probiotics intended as biological drugs must be accurately identified by genus and species. The particular strain identification should be included, with references to publications, technical reports, and other sources. If a known strain is altered in its genetic or phenotypic characteristics during its development as a **live bio-therapeutic**, it should be given a new strain name, and traceability to the parent strain should be documented.

In the case of combination products, each strain and its source should be characterized separately and their selection should be controlled by acceptance criteria before combination in a final product.

The complete strain history, from its isolation to the establishment of a master strain, should be documented as accurately as possible.

Testing of strain identity. There are many in vitro microbiological, biochemical, and immunological techniques available that may be used in appropriate combination to identify and characterize the selected strains, as follows.

1-Bacteria and yeast are typically identified to the species level by their fermentation patterns and products. Any end product associated with the indication should be identified in the master strain.

2-Inherent resistance to gastric acidity or bile-salt activity may be critical to the effectiveness of a strain intended for oral administration.

3-The antibiotic-susceptibility profile is an important and useful characterization of strain identity.

4-Antimicrobial activity against pathogens or competition for adhesion may be important.

5-Resistance to spermicides should be studied for vaginal indications.

6-Interactions with primary cultures of epithelial cells should be studied.

7-Induction of cytokines by epithelial cells, and spleen cells should be tested.

Definitive identification will rely on genetic techniques. DNA hybridization, 16S ribosomal RNA sequencing has proved useful in identifying differences between strains, as well as in identifying the species. Strains of the same species often can be differentiated by the fingerprints that result from PCR amplification of isolated DNA.

The presence of plasmids should be determined, and, if they are present, it should be determined whether the plasmid encodes antibiotic-resistance genes.

3- A selected strain's potential for pathogenicity should be assessed. Appropriate animal studies should be performed to determine the virulence with respect to the route of administration and the number of viable cells in the dose.

4- Cell banks. After selection of the strain, the identified strain is first prepared as a pure culture or clone. Once the strain is confirmed to be a pure culture with the desired characteristics, it is preserved in a qualified cell bank as a master cell bank (**master strain**). A cell bank provides a common source for production, to ensure consistency in the manufacturing process and in the resulting product .

5- Manufacture of the Live Biotherapeutic Product

In the United States, a strain intended to be developed and marketed as a therapeutic product or for prevention of a specific disease condition is defined as a **live biotherapeutic product** and will be regulated as a biological drug . Manufacture of the Live Biotherapeutic Product include:

a- Manufacturing process, A flow chart and text description should show the steps taken in culture expansion, propagation, harvest, and purification and should described all critical inputs and outputs that are used to control the process .

b- Formulation. The active ingredient of a live biotherapeutic is the harvested and concentrated live organism. This is formulated into a dosage form that is convenient for use. Usually, a concentrated harvest is adjusted by dilution, or a dried preparation is blended with other dry ingredients, such as stabilizers or fillers. Uniformity of the dosage form is ensured by monitoring of weights and volume during the filling of vials, capsules, or during compression into tablets. Tablets and capsules may be enteric coated to ensure passage through the stomach and dissolution in the intestine.

c- Control of quality and consistency. Because major process changes often result in changes in the product quality, the process should be designed to require minimal adjustments. Product integrity and quality is ensured by defining the conditions and times for in-process steps in which the product may be held and by specifying the storage conditions.

d- Control of contamination and cross-contamination. The manufacture of biological products uses principles of aseptic processing because the component materials and final product may be degraded by contaminating microorganisms.

6- Specifications for Live Biotherapeutic Products

Specifications are acceptance criteria to which active ingredients and drug product must conform the product quality before they can be released as products for human use.

a-Control of the harvested live biotherapeutic.

The harvested live organism is the active pharmaceutical ingredient. Before formulation, specific identity testing , microbiological purity should be assessed for the purified active pharmaceutical ingredient and after high-risk manufacturing steps that might allow extraneous contamination.

b- Control of dosage form. The regulations for biologics list the product qualities that must be evaluated for each lot of product: potency, general safety, sterility, purity, and Stability. For some qualities, the methodology is also specified.

Biofilm Technology

Biofilm forms when bacteria adhere to surfaces in moist environments by excreting a slimy, glue-like substance. Sites for biofilm formation include all kinds of surfaces: natural materials above and below ground, metals, plastics, medical implant materials—even plant and body tissue. Wherever you find a combination of moisture, nutrients and a surface, you are likely to find biofilm.

A biofilm community can be formed by a single bacterial species, but in nature biofilms almost always consist of rich mixtures of many species of bacteria, as well as fungi, algae, yeasts, protozoa, other microorganisms, debris and corrosion products. Over 500 bacterial species have been identified in typical dental plaque biofilms. Biofilms are held together by sugary molecular strands, collectively termed "extracellular polymeric

substances" or "EPS." The cells produce EPS and are held together by these strands, allowing them to develop complex three-dimensional, resilient, attached communities. Biofilms can be as thin as a few cell layers or many inches thick, depending on environmental conditions.

Biofilms appear to show aspects of both solids and liquids—much like slug slime—and fall into a category called "viscoelastic." However, as biofilms collect sediment, or become scaled with rust or calcium deposits, they become less fluid and more like a brittle solid.

In the 1990s, as the biofilm concept was being introduced to the medical community, doctors began to make the connection between chronic, low-grade infections and the biofilm mode of growth. Because recent research implicates biofilms as reservoirs for pathogenic organisms and sources of disease outbreaks, Peptic ulcers, once thought to be caused by stress, have been proved to be caused by bacterial communities of *Heliobacter pylori*. Dentists now understand, for instance, that dental caries (cavities) are the result of bacterial infection (and biofilms!).

Biofilms are implicated in **otitis media**, **bacterial endocarditis** ,**cystic fibrosis** and **Legionnaire's disease** (an acute respiratory infection resulting from the aspiration of clumps of *Legionnella* biofilms). Biofilms may also be responsible for a wide variety of nosocomial (hospital-acquired) infections. Sources of biofilm-related infections can include the surfaces of catheters, medical implants, wound dressings, or other types of medical devices.

biotechnology measures are being created to control biofilms and/or sever the routes by which pathogenic organisms are transmitted from biofilms to susceptible people.
- Some reactors designed to promote biofilm growth are very effective for treating environmental wastes such as sewage, industrial waste streams, or contaminated groundwater. Biofilms can be used to produce a wide variety of biochemicals that are then purified and utilized for public good, including medicines, food additives, or chemical additives for cleaning products.

Other biofilm technologies with promise

Microbial fuel cells(MFCs)

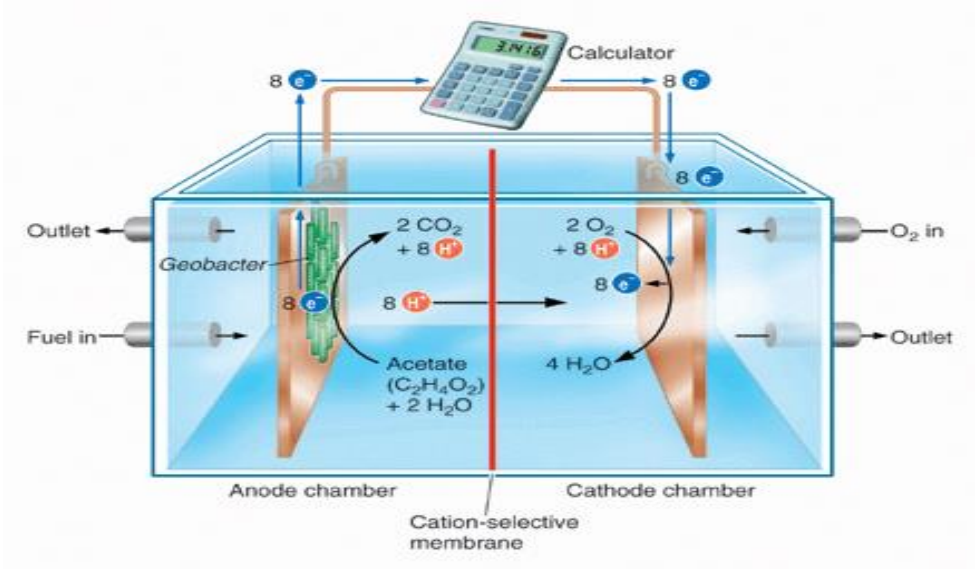
Bacteria gain energy by transferring electrons from an electron donor, such as glucose or acetate, to an electron acceptor, such as oxygen. MFCs operate using principles similar to normal fuel cells.

the most widely established MFC in terms of research and use, known simply as the mediator-free microbial fuel cell. It shares a lot in common with a standard hydrogen fuel cell in terms of setup, with an anode and cathode chamber and a proton exchange membrane between the two. The process moves along these steps:

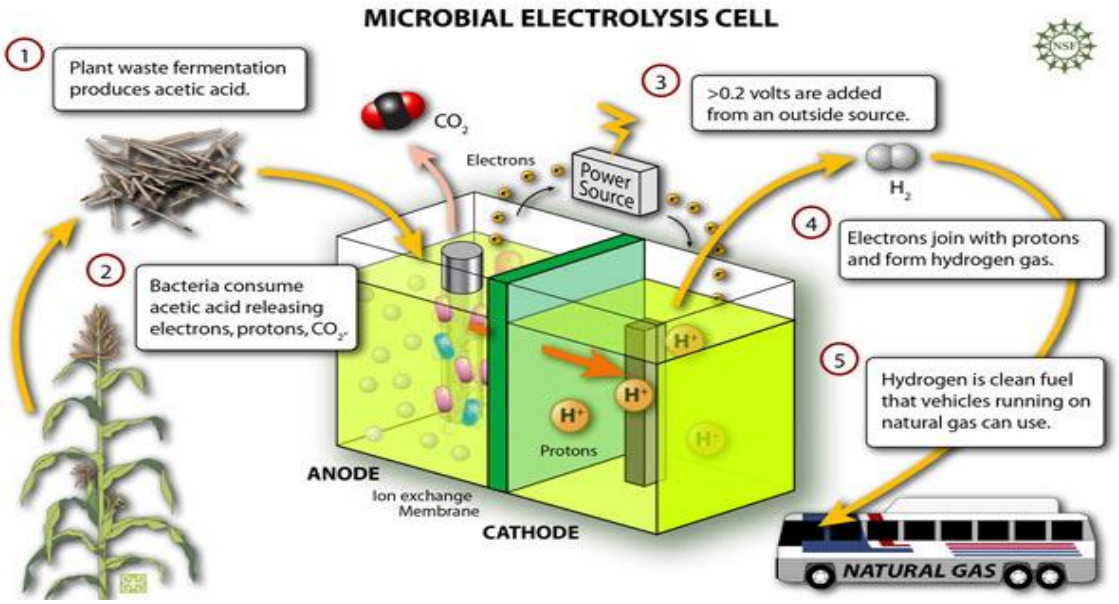
1- In anode chamber(anaerobic chamber) the microbes (normally a bacteria) consumes (oxidizes) fuel (organic matter) that passes into the anode, liberating electrons which it transfers into an electrode wire linking the anode with the cathode.

2- Hydrogen proton charges pass from the anode to the cathode via the proton exchange membrane.

3- The cathode chamber(aerobic chamber) contains oxygen or an oxidizing agent, and the hydrogen combines with the oxygen in electron charges for form water and completes the circuit, producing power.



One of the great things about microbial fuel cells is the wide range of "fuels" it can use to create electricity. Almost any organic material can use as fuel, and not only is energy created in the process, but also the fuel which consumed in the process is an advantage so that the microbial fuel cells could be applied for the treatment of liquid waste streams. By means of microbiological fermentation, a whole range of biofuels and related bioproducts can be produced from organic biomass present in solid waste and wastewater.



figuer : An example of uses plant waste to produce hydrogen via electricity partially generated from microbes.

Lecture eight

Bioremediation **Lec 8**

Is the use of living microorganism (M.O.) to degrade environmental pollutants or prevent pollution.

It is technology for using biological treatment to clean up contamination. The basic of bioremediation is the enormous natural capacity of M.O. to degrade organic compounds. This capacity could be improved by applying the **genetic modified microorganisms (GMMs).**

Microorganisms are used to remedy environmental problems found in soil, water and sediments.

Plant have also been used to assist bioremediation processes. This is called **phytoremediation**.

The idea of bioremediation has become popular with the onset of the twenty-first century.

In principle genetically engineered plants and microorganism can greatly enhance the potential range of bioremediation, for example; bacteria enzyme engineered into plants, it can speed up the breakdown of TNT and other explosives.

Bioremediation can occur on its own (natural or intrinsic bioremediation) or by addition of fertilizer to increase the bioavailability within the medium (**biostimulation**).

Natural bioremediation

The biodegradation of dead vegetation and dead animals is a kind of bioremediation. It is a natural part of the carbon, nitrogen and sulfur. Chemical energy present in waste material is used by M.O. to grow, while they convert organic carbon and hydrogen to carbon dioxide and water.

Intrinsic bioremediation

Conversion of environmental pollutants into the harmless forms through the innate capabilities of naturally occurring microbial population. The ability of M.O. dependent on the type and concentration of compounds, duration of M.O. that exposed to contamination.

Engineered bioremediation

Intrinsic bioremediation is satisfactory some places but it is slow process due to poorly adapted M.O., limited ability of electron acceptor and nutrients, cold temperature and high concentration of contamination, requires construction of engineered system to supply materials that stimulate microorganism.

Engineered bioremediation accelerate the desired biodegradation reaction by encouraging growth of more M.O. via optimizing physico-chemical condition.

Not all contaminants are easily treated by bioremediation using M.O., for example; heavy metals such as cadmium and lead are not readily absorbed by M.O.

Phytoremediation is useful in these circumstance because natural plants or transgenic plants are able to bioaccumulate these toxins in their above ground parts, which are then harvested incineration or oven recycled for industrial use.

Bioremediation of hydrocarbons

Petroleum and its products are hydrocarbons. These two have much economic importance. Oils constitute a variety of hydrocarbons like xylenes, naphthalene, octane, camphor, etc. if present in the environment these cause pollution.

In toxic environment, M.O. act only if the condition (temperature, pH and inorganic nutrients) are adequate. Oli is insoluble in water and is less

dense, it floats on water surface and form **slicks**. It should be noted that in bulk storage tank, microbial growth is not possible provided water and air supplied the M.O capable to degrade petroleum include *Pseudomonas*, various *Corynbacterium*, *Mycobacterium* and some yeasts. However, there are two methods for bioremediation of hydrocarbon. Oils spills by using mixture of bacteria and by using genetically engineered microbial strains.

A. Use mixture of bacteria

A large numbers of bacteria resides in interface of water and oil droplets. Each strain of bacteria consumes a very limited range of hydrocarbon, therefore methods have been devised to introduce mixture of bacteria.

Bacteria living in interface degrade oil at a very slow rate. The rate of degradation accelerated by adding inorganic nutrients such as phosphorous and nitrogen by pumped into the ground or applied to oil spill areas, this increase the rate of bioremediation significantly, for example; the fungal culture could degrade 0.4% sludge in 3 weeks. Degradation of petroleum sludge occurred within two weeks when the bacterial culture (Bacillus *circulans*) was used. Moreover, significant degradation of petroleum sludge was noticed in 10 days when the fungus and bacteria were added.

B. Use of genetically engineered bacteria strains

Strains of *Pseudomonas putida* contained the xylene (XYL) and NAH plasmids as well as a hybrid plasmid derived by recombining part of AM and OCT, these are incompatible and cannot co-exist as separate plasmids in the same bacterium.

This strain could grow rapidly on crude oil and metabolizing hydrocarbon more efficiency than any other one plasmid.

Bioremediation of industrial waste

A variety of pollutants are discharged in the environment from large number of industries, for example; textile industry alone contributes a significant amount of pollutants to water bodies such as enzyme, acids, alkali, alcohol, phenol, dye, heavy metals, trace of zinc, cadmium, copper, lead is found in dyes.

Maurya, 1996 have discussed about bioremediation of toxic textile effluents by free and immobilized cell and enzyme.

Actinomycetes show a higher capacity to bind metal ions as compared to fungi and bacteria. In addition, uptake mechanism of living and dead cells differ. Due to these differences, they have potential application in industries. The living microbial cell accumulate metal intracellularly at higher concentration, whereas dead cells precipitate metals in and around cell walls by several metabolic processes.

Immobilized *Aspergillus oryzae* cells on reticulated foam particles have been used for cadmium removal, dead biomass immobilized on polymeric membrane absorbs uranium well.

Bioremediation in the paper and pulp industry

Pulp and paper industry contain chromophoric substance and can be partly mutagenic and inhibitory to aquatic system. The presence of various pollutants produced during pulp and paper manufacturing necessitates the need for waste water treatment before discharge.

Certain soil inhibiting fungi, Streptomyces, bacteria and while rot fungi avarity of lignin – degrade substance these enzyme have been studied for their ability to decolorize chromophoric substance.

White rot fungi produce a variety of lignin-degrading enzyme, for example; peroxidase and laccase that degrade phenolic substance, these enzymes convert and bio-transform phenolic compounds used to treat wastewater.

Laccase= (p-diphenol-dioxygen oxidoreductase)

It is larger group of multi-copper enzyme (4-copper atom I, II, and III) called metal containing oxidase, produces naturally in plant, fungi and insect. The role of enzyme in plant is responsible for lignin synthesis or degradation. Laccase is capable of oxidizing phenol by reducing molecular oxygen to water by multi-copper.

Bioremediation of heavy metals

Due to discharge of industrial waste, various approaches have been made to detoxify and clean up these metal such as use of certain chemical which in turn cause secondary pollution. Another option is the use of different type of M.O. such as algae, fungi and bacteria that remove metals from the solution.

M.O. remove metals by following mechanism:

- Adsorption= negatively charged cell surface of M.O. bind to the metal ons.
- Complexation= M.O. produce organic acid, for example; citric acid, oxalic acid, gluconic acid, formic acid, lactic acid and malic acid which chelate metal ions.
- Biosorption of metals also take place due to carboxylic groups found in microbial polysaccharides and another polymer.
- Precipitation= some bacteria produce ammonia, organic base or H_2s which precipitate metals as hydroxides or sulfates, for example; *Desulfovibrio* and *Desulfotomaculu* transform So_4 to H_2s which promotes extracellular precipitation of insoluble metal sulfides.
  Klebsiella aerogenes detoxified cadmium sulfate which precipitates on cell surface.

- Volatilization= some bacteria cause methylation of Hg^{+2} and converts to dimethyl mercury which is a volatile compound.

Detoxification of $Hgcl_2$ take place by two enzymes

1. Lyase breakdown the $Hgcl_2$ ⟶ Hg^{+2}
2. Reductase which reduce Hg^{+2} ⟶ HgCo

Liquid Hg is useful but vapor Hg is toxic

The more metals toxicity are cadmium, leads, mercury, copper and chromium. Chromium occurs in oxidation state Cr(O) to Cr(VI), trivalent chromium Cr(III) is an essential micronutrient for humans and less soluble than hexavalent (VI) chromium which is toxic, mutagenic and carcinogenic.

The removal of Cr(VI) by physiochemical or by biological detoxification. Limitation of using physiochemical processes are high energy input, the use of chemical material result in secondary waste, and these are another problem. So using of M.O. to detoxification of Cr(VI) by bireduction and biosorption Cr(VI) by bacteria, fungi, yeast and plant biomass.

Bioreduction Cr(VI) by bacteria, for example; *Pseudomonas*, E. coli, Desulfovibrio, *Microbacterium* to Cr(III). Cr(VI) produced by manufacturing of pigment, leather, print films, in mining.

Fungi also are capable of accumulating heavy metals in their cells by several mechanisms.

Metabolism-in depended accumulation

The positivity charged ions in the solution are attached to negatively charged ligands in cell surface, but composition of biomass and other factors affect biosorption, for example in *Rhizopus arrhizus* adsorption

depends on ionic radicals of Li^{+3}, Mn^{+2}, Cu^{+2}, Pb^{+2} and Hg^{+2}, however binding of Hg^{+2}, Ag^{+2} and Cu^{+2} strongly depends on concentration of yeast cells.

Metabolism-depended accumulation

In fungi and yeast, heavy metal ions are transported into the cells through cell membrane. However, as a result of metabolic processes ions are precipitated around the cells and synthesized intracellularly by certain external factors such as pH, anions, cations and organic materials, growth phase etc. Metal uptake by growing batch culture was found maximum during lag phase and early phase in *Aspergillus niger*.

Extracellular precipitation and complexation Lec. 9

Fungi produce several extracellular products which can complex or precipitate heavy metals. For example, many fungi and yeast high affinity for binding compound that chelate iron. It is called **siderophores**. The fe^{+3} chelates which are formed outside the cell wall are taken up into the cell.

Saccharomyces cerevisiae remove metals by their precipitation as sulfides, for example as Cu^{+2} is precipitated as Cus.

Bacteria secrete extracellular polymers like capsule that have anionic character, externa biofilm associated with cell, complex with metals and accumulate substantially. *Bacillus subtilis* has shown to reduce gold from Au^{+3} to Au^{0} through extracellular enzymatic biotransformation.

Biosorption, it is a passive metabolism-independent physico-chemical interaction between heavy metal ions and microbial surface. Most biosorption phenomenon are combination of processes such as electrostatic interaction, ion exchange complex, ionic band formation, precipitation, nucleation.

Biosorption process can occur even with inactivated or dead cells. There are several M.O. involved in biosorption such as bacteria (*Pseudomonas*, *Bacillus*, *E. coli*, and *Streptomyces*) and fungi and yeast (*Saccharomyces*, *Aurcobasidium*) and algae (*Chlorella*, *Ascophyllum*) and fungal mycelia (*Aspergillus* and *Penicillium*).

Mycoremediation

Is a form of bioremediation in which fungi mycelia are used to decontaminate the area. The mycelium secretes extracellular enzyme and acid that breakdown lignin and cellulose, the two main building block of plant fiber.

Mycofiltration is a similar process of using fungal mycelium to filter toxic waste and microorganism from water in soil.

Phytoremediation

Plants have been used to assist bioremediation processes. Biological processes have been used for some inorganic materials like metals to lower radioactivity and to remediate organic contaminants with metal contamination. The usual challenge is to accumulate the metal into plant part, which must then dispose of in a hazardous waste landfill before or after incineration to reduce the plant to ash. The exceptions are mercury and selenium which can be released as volatile elements directly from plant to atmosphere.

In principle genetically engineered plant and M.O. can greatly enhance the potential range of bioremediation. For example bacterial enzyme engineered into plants, it can speed up the breakdown of TNT and other explosive. With transgenic poplar trees carrying bacterial gene methyl mercury may be converted to elemental mercury which is released to the atmosphere at extreme dilution.

Bioremediation

Herbicide

Also commonly as known weed killer, are pesticides used to kill unwanted plants. Selective herbicides kill specific targets, while leaving the desired crop relatively unharmed. Some of these act by interring with the growth of the weed and are often synthetic of plant hormones.

Some plants produce natural herbicides such as genus Juglans (Walnuts) or the tree of heaven, such as action of natural herbicides and other related chemical interactions are called **allelopathy**.

Classification of herbicides

Can be grouped by activity, use, chemical family, mode of action or type of vegetation controlled.

By activity

Contact herbicides destroy only the plant tissue in contact with the chemicals; these are the fattest action herbicides. They are less effective on perennial plants which are able to regrow from rhizomes and roots.

Systemic herbicides are translocated through the plant, either from foliar application down to the root, or from soil application up to the leaves. They are capable of controlling perennial plants and may be slower-action but more effective than contact herbicides.

By use

Soil applied herbicides: are applied to the soil and are taken up by the roots.

Accase inhibitors: are compound kill grasses. Acetyl co-enzyme A carboxylase is a part of the first step of lipid synthesis, thus accase inhibitors affect cell membrane production in the meristems of the grass plant. whereas the accase of dicot plants are not.

ALS inhibitors: the acetolactate synthase (ALS) enzyme is the first step in the synthesis of the branched-chain amino acids (Valine, leucine and isoleucine). These herbicides slowly lead to inhibition DNA synthesis. The

ALS biological pathway exists only in plants ant not animals, thus making the ALS inhibitors among safest herbicides.

EPSPS inhibitors: the enopyruvyl shikimate 3-phosphate synthase enzyme is used in the synthesis of amino acids (tryptophan, phenyl alanine and tyrosine).

Synthetic auxins mimic the plant hormones; they have several points of action on the cell membrane. For example, 2,4-D is a synthetic auxins herbicide.

Microorganisms play an important role in degradation of xenobiotic and maintaining of steady state concentration of chemical in the environment.

The complete degradation of pesticide molecule to its inorganic components than can be used an oxidative cycle to remove its potential toxicity from the environment.

The characters of pesticide of M.O. are located on plasmids and transposons, and are grouped in clusters on chromosome.

Microbial degradation of xenobiotic

Biodegradation of pesticides occurs by aerobic soil microbes. For example, biodegradation through hydrolysis of P-O-aryl bonds by *Pseudomonas diminuta* and *Flavobacterium* are significant step in the detoxification of organophosphorus compound.

The M.O. which associated with DDT degradation are *Aspergillus flavus*, *Fusarium oxysporum*, *Mucor aternans*. Environmental factors inducing pH, temperature, nutrient supply and oxygen availability affect biodegradation of pesticides.

Gene manipulation of pesticide-degrading microorganisms

Day by day the number of xenobiotic-degrading M.O. is increasing. However, pesticide-degrading gene of only a few M.O. has been characterized. They found that camphor degrading gene of *Pseudomonas putida* are located on plasmid. Soon after naphthalene (NAH) degrading plasmid was isolated. Discovery of these genes made it possible to construct a new genetically engineered strain of *P. putida* that alone was potent to degrade camphor, naphthalene, xylene, toluene and hexanes.

Bacillus thuringinesis a soil bacterium whose spore contain a crystalline (Cry) protein in the insect gut. The protein breakdown to release a toxin (endotoxin), this toxin binds to and created pore in the intestinal lining resulting in ion imbalance, paralysis of the digestive system and after a few days the insect death. The bacterial *Cry* gene has been incorporated into the plants own DNA. So those, the plants cellular machinery produces the toxin. When the insect chomps on a leaf or bores into a stem of a Bt-containing plant, it ingests the toxin and will die within a few days.

Microbial biodegradation

Microbial biodegradation is the chemical dissolution of materials by bacteria or consumed by M.O. and return to compounds, including hydrocarbons (oil), polyaromatic hydrocarbon, heterocyclic compound, pharmaceutical substance and metals.

Aromatic digestion

Series of processes in which M.O. breakdown biodegradable material in the absence of oxygen. It is used for industries or domestic purposes to manage wastes to release energy. Much of fermentation used industrially to produce food and drink products.

The digestion process begins with bacterial hydrolysis of the input materials to breakdown insoluble organic polymers such as carbohydrates and make them available for other bacteria. Acidogenic bacteria then convert the sugar and amino acid into $Co_2 + H_2 + NH_3$ and organic acid. Acetogenic bacteria then convert these resulting organic acid into acetic acid along with addition ammonia, H_2 and Co_2. Finally, methanogens convert these products to methane and Co_2, the methanogenic archaea population play the role in anaerobic wastewater treatment. Anaerobic digestion is one of the most useful sources of energy supply.

Ecological impact of GMMs released into the environment

GMMs refer to genetically modified microorganisms that released into the environment disturb the ecological by inhibiting promoting growth of indigenous microflora or replacing the natural strains as discussed below:

1. **Growth inhibitor of natural strain**

   A genetically modified of *Klebsiella plantica* constructed to produce ethanol from organic waste was found.

   a. To destroy mycorrhizal fungi
   b. To reduce plant growth
   c. To increase the population of parasitic nematode of plant.

A genetically modified strain of *P. putida* degraded in soil microcosm the herbicide 2,4-D and resulted in accumulation of 2,4-DCP which is toxic metabolite, the 2,4-DCP was more toxic than 2,4-D.

2. Growth stimulation of indigenous strains

The GMMs stain of *Erwinia carotovora* affected indigenous bacterial commonly. The total bacterial density increased. This increase is attributed to an inoculum nutrient effect the inoculated cell of *Erwinia* died and became a source of nutrients for indigenous soil M.O.

GMMs of *P. cepacia* released in soil microcosm containing the pollutant increased the taxonomic diversity of soil M.O. this is because the intermediate metabolites of genetically modified *P. cepacia* produced after biodegradation of pollutant which stimulated the growth of static cells. This has resulted in increased taxonomic diversity. Stimulation of plasmid transfer and recombination might cause an increase in genetic diversity.

3. Replacement of natural strains

A genetically modified strains of *Agrobacterium* with the deleted genes of tumor formation replaced the initial wild strain and produced plant from tumor formation. This ability of GMMs is useful in agricultural biotechnology for protection of plants against pathogen.

Environmental Biotechnology

1. Metal-binding peptides display onto the cell surface

Repetive metal binding modified consisting $(Glu\text{-}Cys)_n$ Gly, these peptides emulate the structure of phytochelatins, metal chelating molecules that play a major role in metal detoxification in plant and fungi.

The phytochelatin analogs were presented on the bacterial surface, enhancing cd^{+2} and Hg^{+2} bioaccumulation by 12-fold and 20-fold respectively than the nature metal-binding peptides.

2. Metallo-regulatory protein, another group of useful metal binding moiety with affinity and specificity-result of cleverly designed genetic circuit that is tightly under their condition. For example, MerR and ArsR1 which are regulatory protein used for controlling the expression of enzymes responsible for mercury and arsenic detoxification, respectively. The high affinity and selectivity of MerR toward mercury has been exploited for the construction of microbial biosorbents specific for mercury removal. Presence of surface-exposed MerR on an engineered strain enable 6-fold higher Hg^{+2} biosorption. Hg^{+2}-binding via MerR was very specific with no observed decline even in the presence of 100-fold excess cd^{-2} and Zn^{+2}.
3. Engineering microbes to function as designer biocatalyst, for example, neurotoxic organophosphate which is used as pesticides and chemical warfare agent, although an enzyme organophosphorus hydrolase (OPH) has been shown to degrade these pesticides effectively, the use of whole cell detoxification is limited by the transport barrier of substrate across the cell membrane.

Display of OPH resulting in 7-fold faster degradation compared to whole cell expression OPH intracellularly.

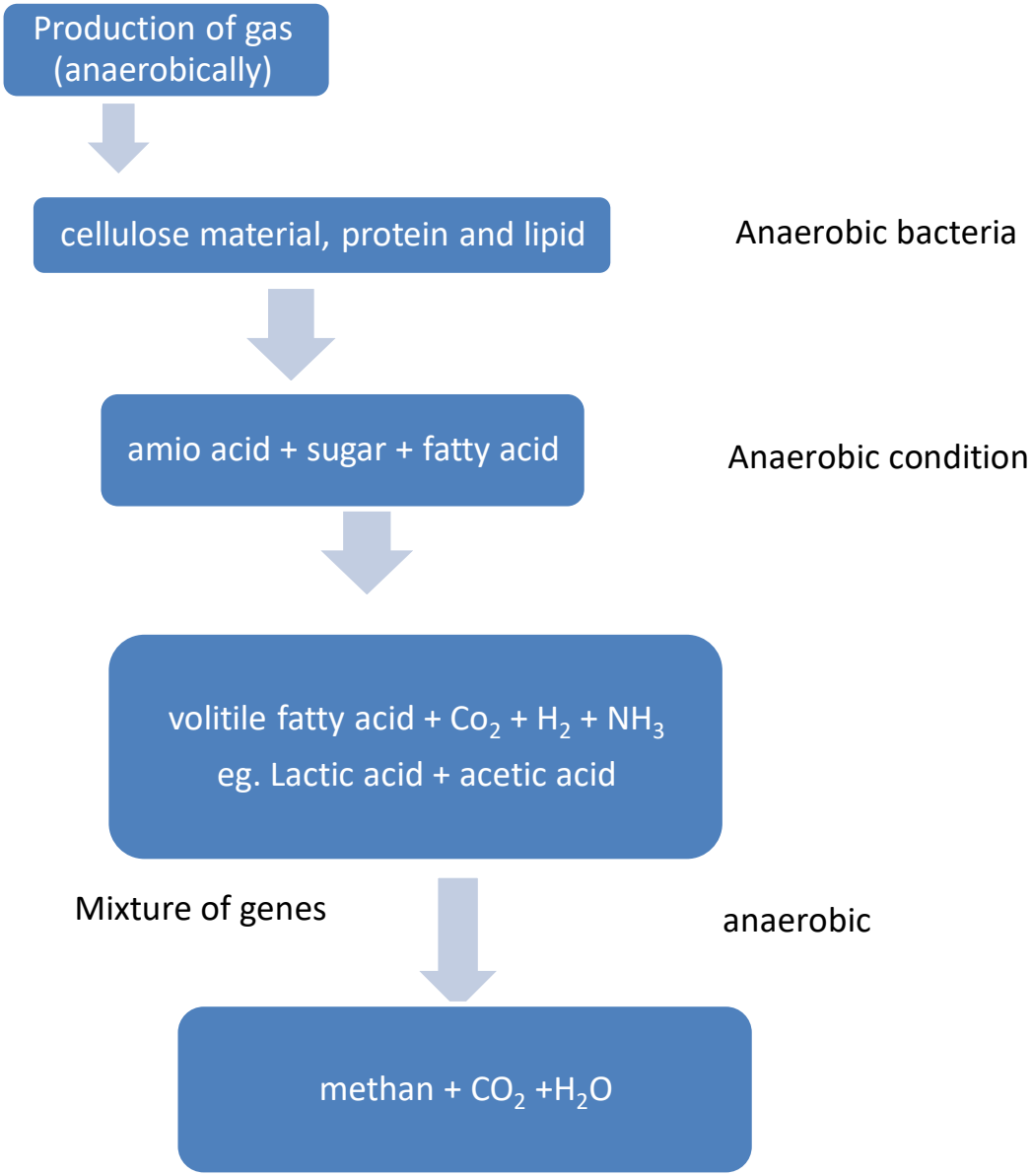
4. Production of hybrid strain which is capable of mineralization components of benzene, toluene and xylene mixture simultaneously was attempted by redesigning the metabolic pathway of *Pseudomonas putida*.

   A hybrid strain carrying both the TOD and TOL pathways was constructed and was found to mineralize a benzene, toluene and p-xylene mixture.

5. *Deinococcus radrodurans* is a soil bacterium that can survive acute exposure to ionizing radiation of 15000 Gy without lethality. A recombinant *D. radrodurans* strain expressing toluene dioxygenase was shown to effectively oxidize toluene, chlorobenzene and Trichloroethylene (TCE) in a highly irradiating environment.
6. Plant-microbe rhizoremediation: is synergistic plant-microbe relationship in a rhizosphere. By using a wheat rhizosphere system for the detoxification of soil-born TCE.

   The toluene-*O*-monooxygenase (*TOM*) gene was introduced into *Pseudomonas fluorescent*, bacterium that colonizes the wheat root, enabling the establishment of bacterium-plant-soil microorganism. Treatment of TCE-contaminated surface and near surface soil was demonstrated with more than 63% of the initial TCE removal within 4 days.

7. Production of valuable products and energy directly from waste water.


Production of gas (anaerobically)
cellulose material, protein and lipid
Anaerobic bacteria
amio acid + sugar + fatty acid
Anaerobic condition
volitile fatty acid + Co_2 + H_2 + NH_3
eg. Lactic acid + acetic acid
Mixture of genes
anaerobic
methan + CO_2 +H_2O

Lecture ten

Single cell protein (SCP)

Microorganism (M.O.) can be grown a whole range of cheap or waste materials, harvested and then purified or process to provide food for either human or livestock.

If the foodstuff produced is a protein, the product is called SCP. Many M.Os. are suitable for this process including algae (blue – green algae), fungi and bacteria.

Example of substrates available

- The cheese industry produces large amounts of whey much of which is wasted, whey contain protein and lactose then yeast can grow on this waste to produce protein and vitamin and used to supplement to cattle feed.
- The sugar refining industry produce molasses, yeast can be grown on molasses to give high yields of cattle feed.
- Sulphite liquor is a waste product from the wood pulping industry and contain a low levels of sugar. Fungi can grow on it.

Problems encounted with SCP

SCP is often contaminated with the substrate, as total purification is not feasible. This creates a problem where the substrate is toxic, such as sewage or methanol.

This problem can be overcome by adding an extra stage in the food chain. For example, the toxic substrate is used to grow organism A. organism A is then used as substrate for organism B, which is then harvested to yield SCP.

Sewage is converted to methane by methanogenic bacteria. The methane is used as the substrate on which to grow SCP.

Another problem is that prokaryotic microorganism used for SCP production have a high nucleic acid content. This happens where production is by fermentation.

Since growth is nucleic acid content is about 15% of the dry mass of bacteria, but only about 4% in meat and fish. Excess nucleic in human diets leads to health problem such as kidney stones, vomiting and

diarrhea. Using chemical methods or enzyme to reduce nucleic acid levels is prohibitively expensive. So prokaryote SCP is best used either as human food supplement or for feeding farm animals.

Eukaryotic SCP based on algae and fungi does not give such problems as the nucleic acid content is much lower.

Risk and concerns of genetic engineered food (GEF) and crops

The most important risks and concerns from GEF are:

- Toxic and poisons: GEF products clearly have the potential to be toxic and a threat to human health.

  In 1989 a GE brand of L-tryptophan, a common dietary supplement, killed 37 Americans more than 5000 others were affected with a potentially fatal and painful blood disorder, eosinophilia Myalgia syndromes.

  GE potato different in chemical composition from regular potatoes and when feed to lab rats damaged their vital organs and immune system these damages were caused by CaMV viral

  Cancer risks.

  In 1994 the FDA approved the (rBGH), this hormone is injected into dairy cows to force them to produced more milk. serious hazards such as human breast, prostate and colon cancer.
- Food allergies.

  Allergy affects approximately 8% of children and 2% of adults. Allergic reactions in human occur when normally protein enters the body and stimulates an immune response if the novel protein in GMF comes from the source that is known to cause allergies in human

  Stringent pre-market safety-testing (including long term animal feeding and volunteer human feeding studies) is necessary in order to prevent a further public health disaster.
- Antibiotic resistant.

  These new combination may be contributing to the growing public health dangers of antibiotics, for example new strain of *Salmonella*, *E. coli*, *Campylobacter* and *Enterococci* that not affect by antibiotic treatment.
- New viruses and pathogens.

Mixing viruses genes with plant for production of desirable characters created a new and more virulent viruses. Several years ago researchers experiment found that genetically altering plants to resist viruses

Genetically engineered farm animals

There are great possibilities for genetic engineering techniques in improving farm animals. One example is being developed in Australia to help control sheep tick.

Ticks are external parasites which suck a sheep blood. This can lead to serve secondary infections. At present tick infection is controlled by dipping the sheep in chemical solution which kills the ticks on contact.

Dipping is time consuming and harmful to farm worker, so the outer covering of an insect contain chitin which can be broken down by chitinase.

A chitinase gene occur naturally engineered into sheep and whether the ticks will be affected by the chitinase produced in this way.

Bovine Somatotropin (BsT)

BsT is polypeptide normally secreted by the pituitary gland of mammals. It act as grow stimulating hormone which increase the rate of protein synthesis, cell division and growth of bone.

In cow BsT increase milk production by diverting glucose, fatty acid and body fats to the mammary glands. Giving additional BsT can increase these effects.

The BsT for use in farming is produced by biotechnol, the gene for BsT synthesis has been isolated from cow and inserted into plasmid from a bacterium and used as vector to insert the gene in *E. coli*, then the transformed *E. coli* containing the recombinant plasmid and then allowed to multiply in a batch fermentation, these cloned *E. coli* produce large quantities of BsT as granules within their cells, then the bacterium (*E. coli*) are homogenized to split the cells, open, releasing granules alone, after that every complex purification process takes place.

Animal products

Animal product is any material derived from the body of an animals. For example fat., blood, milk, egg.

There products may go through a process known as (rendering) to be made into human and non-human foodstuff. Fat and other material that can be sold to make commercial product such as cosmetics, paints, cleaner polished, glue, soap and ink.

Bio-surfactants

Bio-surfactants are surface-active compounds from biological sources, usually extracellular, produced by bacteria, yeast or fungi.

Research on biological surfactant production has grown significantly due to their advantages, they present over synthetic compounds such as biodegradation, low toxicity diversity of applications and functionality under extreme conditions.

The majority of microbial bio-surfactants producers are *Pseudomonas* spp., *Acinetobacter* spp., *Bacillus* spp., *Arthrobacter*spp. and yeast. The study of bio surfactant producing by yeast has been growing in important by species *Candida* spp., *Pseudomonas* spp.

Low molecular weight compounds

1. **Lipopeptides**

   Surfactin, a cyclic lipopeptide produced by *Bacillus subtilis* considered the most active bio-surfactant discovered so far. Natural surfactins are mixture of isoforms A, B, C and D that classified according to the differences in their amino acids sequences and possess various physiological properties.

2. Glycolipids

High molecular weight bio-surfactants

There generally grouped together as polymeric bio-surfactant. They produced by number of bacteria and composed of lipoproteins, proteins, polysaccharides, lipopolysaccharides or complexes containing several of these structure types.

Advanced of bio-surfactant over chemical surfactants

1. Biodegradability: bio-surfactant can be easily degrade by many microorganism. Therefore, they do not represent any threat to the environment.

2. Generally low toxicity, make it safe to been used in food and cosmetic industries.

3. Biocompatibility and digestibility: this also ensures application in cosmetic pharmaceutical and as food additives.

4. Row material a viability: it can been produced from cheap row materials that are found in large amounts.

5. Economically efficient: it can also been produced from industrial waste and by product that is of particular important for the bulk production of bio-surfactants.

6. Specificity: bio-surfactant often specific in their action science it is complex organic compound with specific functional group and this of special important in detoxification of specific pollutants, de emulsification of industrial emulsion, specific cosmetics,

pharmaceutical and food application.