BIOTECHNOLOGY

(DZOO24)

(MSC - ZOOGOLOGY)

ACHARYA NAGARJUNA UNIVERSITY

CENTRE FOR DISTANCE EDUCATION

NAGARJUNA NAGAR,

GUNTUR

ANDHRA PRADESH

Lesson 8.1.1

Introduction and definition of Biotechnology

Objective

8.1.1.1 Origin

8.1.1.2 Definition

8.1.1.3 Historical background

8.1.1.4 Old and new biotechnology

8.1.1.5 An interdisciplinary activity

8.1.1.6 Biotechnology – a three component central core

8.1.1.7 Substrates for biotechnology

1. Natural raw materials
2. Availability of by-products
3. Chemical and petrochemical feed stocks

Objective : In this chapter origin and definition of biotechnology, its historical perspective and overall picture of biotechnology were discussed

8.1.1.1. Origin

The origin of biotechnology can be traced back to prehistoric times, when microorganisms were already used for processes like fermentation. Although a molecular biologist may consider cloning of DNA to be the most important event in the history of biotechnology, the latter has actually been rediscovered in 1970 for the 3rd time during the present century.

- In 1920 *Clostridium acetobutylicum* was used by Chaim Weizmann for converting starch into butanol and acetone, the latter was an essential component of explosives during World war I. This has raised hope for commercial production of useful chemicals through biological processes, and may be considered as the first rediscovery of biotechnology in the present century.

- Production of Penicillin (an antibiotic) on a large scale from cultures of *Pencillium notatum*, during World War II in 1940, marked as second rediscovery of biotechnology.

- The third rediscovery of biotechnology is its recent reincarnation in the form of DNA technology, which led to the development of a variety of gene technologies.

8.1.1.2. Definition

The term biotechnology is derived from a fusion of biology and technology. It concerns with the exploitation of biological agents or their components for generating useful products or services. By nature, the area covered under biotechnology is very vast and the techniques involved are highly divergent, this has often made a precise definition of the subject rather difficult. Some standard definitions of biotechnology are :

- Any technique that is used in living organisms to make or modify a product, to improve plants or animals or to develop microorganisms for specific uses. The document focuses on the development and application of modern biotechnology based on new enabling techniques of rDNA technology, often referred to as genetic engineering (United States Congress's Office of Technology assessment).

- Biotechnology consists of "the controlled use of biological agents, such as, microorganisms or cellular components, for beneficial use". (U.S. National Science Foundation).

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

- Biotechnology comprises the controlled and deliberate application of simple biological agents – living or dead, cells or cell components in technically useful operations, either of productive

manufacture or as service operation (Bulock, 1987).

- Biotechnology is "the application of biological organisms, system or processes to manufacturing and service industries" (British Biotechnologists).

- Biotechnology is "a technology using biological phenomenon for copying and manufacturing various kinds of useful substances" (Japanese Biotechnologists).

- Biotechnology may be defined as "the use of living organisms in systems or processes for the manufacture of useful products, it may involve algae, bacteria, fungi, yeast, cells of higher plants and animals or subsystems of any of these or isolated components from living matter (Gibbs and Greenhalgh, 1983).

It is very clear that the different definitions of biotechnology differ in their approach, content and emphasis. But the two main features common among them all are:

1. Utilization of biological entities (microorganisms, cells of higher organisms – either living or dead), their components or constituents (eg: enzymes), in such a way that

2. Some product or service is generated. This product or service should, obviously enhance human welfare.

8.1.1.3. Historical background

The oldest biotechnological processes are found in microbial fermentations, as born out by a Babylonian tabled dated 6000 BC; unearthed in 1881 in explaining the preparation of beer. The Sumerians were able to brew as many as twenty types of beer in the third millennium BC. In about 4000 BC, leavened bred was produced with the aid of yeast.

Year	Work
Before 6000 BC	: Yeast employed to make wine and beer.
Approx 4000 BC	: Leavened bread produced with the aid of yeast.
Before AD1521	: Aztecs harvested algae from lakes as a source of food
Before 1670-1680	: Copper mined with aid of microbes and spain Sntoine Van Leeuwenhoek first observed microbe with newly designed microscope.
1876	: Louis Pasteur identified extraneous microbes a cause of failed beer fermentation.
1877	Alcohol first used to fuel motors.

1897 : Edward Buchner discovered that enzymes extracted from yeast can convert sugar into alcohol.

Approx 1910 : Large scale sewage purification systems employing microbes was established.

1912-1924 : The important industrial chemicals (acetone, butnol and glycerol) were obtained from bacteria.

1928 : Alexander Flemming discovered penicillin.

1944 : Large scale production of penicillin.

1950 : Introduction of many new antibiotics.

1962 : Mining of Uranium with the aid of microbes begins in Canada.

1973 : Brazilian government initiated major fuel programme to replace oil with alcohol. First successful genetic engineering experiments.

1975 : Hydridomas which make monoclonal antibodies were first created.

US outline guidelined for genetic engineering.

1976 : US National Institute of Health Introduced guidelines on genetic engineering.

1980 : Rank Hovis Mc Dougall receive permission to market fungal food for human consumption in UK. Court has permitted that genetically engineered microbes can be patented.

1981 : Monoclonal antibodies received US approval for use in diagnosis.

1982 : Genetically engineered insulin approved for use in diabetes in UK and USA.

1983 : Animal inferferons approved for protection against cattle diseases.

Mid 1980 :

- Genetically engineered growth hormone approved for treatment of dwarfism.
- Interferon used to treat some viral diseases. Monoclonal antibodies widely employed in diagnosis.
- New antibiotics produced by cell fusion.
- Commercial production of dyes and industrial chemicals from algae.
- Genetically engineered proteins used to treat heart attacks.
- Monoclonal antibodies employed to boost the body' defence against cancer and other diseases,
- New vaeenies against foot and mouth disease.
- Growth hormones used to increase yield of meat and milk from cattle.

Late 1980 :

- Raw materials for plastic industry obtained from microbes.
- Interferons employed to treat certain types of cancer.
- More industrial chemicals produced by microbes.

1990 :

- An yeast strain capable of producing 12-4% ethanol from Institute of Microbial Technology, Chandigarh (India).
- Animal birth control product (Talsur)
- Aquaculture technology developed for prawn and major crabs, economically viable technology.
- Biofertilizer, improved Rhizobium particularly for legume crops and nitrogen fixation.
- Biopesticides (from viral, bacterial and fungal sources)
- Diagnostic kit for Filaria.
- DNA fingerprinting and BKM probe.
- Embryo transfer and split embryo technology.
- Genetically engineered microbes help in extracting oil from the ground.
- Microbes widely employed to extract metals from factory wastes.

- Monoclonal antibodies used to guide anti-cancer drugs to cancerous tissues.

- Number of vaccines, Oral polio vaccines

- Production of elite trees from micropropogation.

- Sericulture involving improvement in production and quality of Indian Silk.

- Small scale production of hydrogen from bacteria.

- Tissue culture propagation of cardamom and oil palm for commercial applications.

8.1.1.4. Old and new biotechnology

Although the term biotechnology is of recent origin, the discipline very old and being used in many ways. Man began employing microorganisms as early as 5000 BC for making wine, vinegar, curd, leavened bread etc… Some of these processes are so common and have become such an integral part of the usual kitchen technology of every home that we may even hesitate to refer to them as biotechnology. Such processes which are based on the natural capabilities of microorganisms etc… are commonly considered as old biotechnology.

The use of microorganisms for the production of chemicals at commercial scale was prompted, ironically by war efforts. During the first World War, Germans were forced to develop the technology for glycerol (Needed for the manufacturing of explosives) production when

their supply of vegetable oil was disrupted due to the British naval blockade. Similarly British resorted to acetone-butanol fermentation *clostridium acetobutylicum* due to the German interference with the normal supply of these chemicals. The first World War also left the citrus orchards of countries like Italy in ruins; this resulted in a great jump in the prices of citric acid which was extracted from citrus juice. As a result, the technology for citric acid production using *Aspergillus niger* was developed. The production of antibiotic by penicillin from *Penicillium notatum* was discovered in 1930 by Flemming, but its commercial production began, again only during the second World War. But subsequent developments in chemical and pharmaceutical production using microorganisms have been very rapid and have covered a very wide range in deed.

Man has continued his guest for

- improving the natural capabilities of microorganisms.
- Making them capable of novel processes and
- Discovering microorganisms with new capabilities.

This has led to the development recombinant – DNA technology which allows man to modify microorganisms and other organisms to create in them highly valuable, novel and naturally nonexistent ones.

For example, the human gene producing insulin has been transferred and expressed in bacteria like *E.coli*; the insulin produced by

these genetically engineered microbes (GEMs) is being used in the management of diabetes.

In addition, animal and plant-cells and their components are being employed to generate valuable products. Crop varieties and animal breeds with entirely new and highly useful traits are being created with the help of recombinant DNA technology. All these and many other similar examples constitute the new biotechnology.

The EFB definition is applicable to both old and new biotechnology. The aims of this federation are :

- To advance biotechnology for the public benefit.
- To promote awareness, communication and collaboration in all fields of biotechnology.
- To provide governmental and supranational bodies with information and informed opinions on biotechnology.
- To promote public understanding of biotechnology.

8.1.1.5. An interdisciplinary activity

In the early 1970s traditional biotechnology was not a popular or even common discipline. Biotechnology research was mainly centered in the department of chemical engineering and, occasionally, in specialized microbiology programs. Biotechnology is truly multidisciplinary in

nature and it encompasses several disciplines of basic sciences and engineering. The science disciplines from which biotechnology draws heavily are: microbiology, chemistry, biochemistry, genetics, molecular biology, immunology, cell and tissue culture and physiology.

Fig.

On the engineering side, it leans heavily on process, chemical and biochemical engineerings since large scale cultivation of microorganisms and cells, their down streaming processing etc… are based on them.

The interdisciplinary activity of biotechnology may be explained by using as example the production of a compound by micro-organisms, animal cells or plant cells. This biotechnological process can be separated into five major steps or operations.

a. Strain choice and improvement.

b. Mass culture

c. Optimization of cell responses

d. Process operation and

e. Product recovery or down stream processing.

a. Strain choice

The first step in a biotechnological process is the identification of a suitable biological agent (microorganism / animal cell / plant cell) capable of producing the desired compound. This would generally involve the isolation of such microorganism from an appropriate habitat and its improvement through suitable strain development strategies. These activities would require a knowledge of general biology and ecology of the organism to decide on what organism is to be isolated and from where, and then be able to assess its ability to perform the desired functions through appropriate chemical and biochemical tests. Often it may be necessary to produce genetically engineered microorganisms (GEMs) capable of production of the desired compounds.

Obviously, all these activities require major inputs form microbiology, cell biology, physiology, botany, zoology, genetics and molecular genetics.

b. Mass culture

Once a suitable strain has been developed, it needs to be maintained for as long as it is needed. Such strain can be used either to produce the biomass which per se is the desired product, eg: in case of single cell protein (SCP), or to recover some compounds from the biomass or the medium. In either case it is necessary to culture the strain on a large scale, the scale being much larger for biomass than that for chemical production. This operation, therefore, requires knowledge of cell physiology, genetics etc. in addition an understanding of the process engineering is necessary.

c. Optimisation of cell responses

The genetic machinery of cells is so geard that they perform a specified function only under specific conditions. In general, the conditions favoring rapid cell growth and biomass production are different from those conducive to the production of compound of interest, e.g. antibiotics. Therefore, in order to optimize the biochemical yields, the culture conditions have to be precisely regulated and, if needed, seguentially manipulated to fully exploit the intrinsic capabilities of cells obviously this would require inputs from cell biology, physiology, genetics and process engineering.

d. Process operations

The various steps of a biotechnological process need to be fully optimized for safety, reproducibility, control, and efficiency at all the scales of operation. In major part, this is the function of process engineering design developed with a full understanding of the biological, chemical and socioeconomic factors. The problems related to this aspect generally require fresh solutions for each new process and, often process environment. The practical exploitation of a biotechnological process chiefly depends on the successful implementation of the process operation.

Fig.

e. Product recovery

The essense and termination of any biotechnological process is the recovery of the concerned product in a useful form. The efficiency of product recovery is directly reflected in the product cost. The mode of this operation also determines the environmental friendliness of the process. In some cases, the process of product recovery or down stream processing may be either inefficient or costly preventing the commercial exploitation of the biotechnological process, eg, recovery of insulin, interferon etc. from seeds of transgenic plants. This generation derives processes from chemistry, biochemistry, chemical engineering and biochemical engineering.

8.1.1.6. Biotechnology – a three component central core

Many biotechnological processes may be considered as having a three-component central core, in which one part is concerned with obtaining the best biological catalyst for a specific function or process, the second part creates the best possible environment for the catalyst to perform, and the third part (Downstream processing) is concerned with the separation and purification of an essential product or products from a fermentation process.

In the majority of examples so far discussed, the most effective, stable and convenient form for the catalyst for a biotechnological process is a whole organism and it is for the reason that so much of biotechnology revolves around microbial processes. This does not

exclude the use of higher organisms, in particular, plant and animal cell culture will play an increasingly important role in biotechnology.

Microorganisms can be viewed both as primary fixers of photosynthetic energy and as systems for bringing about chemical changes in almost all types of natural and synthetic organic molecules collectively, they have an immense gene pool that offers almost unlimited synthetic and degradative potential. Further more, microorganisms can possess extremely rapid growth rates far in excess of any of the higher organisms such as plants and animals. Thus immense quantities can be produced under the right environmental conditions in short time periods.

These manipulated and improved organisms must be maintained in substantially unchanged form and this involves another spectrem of techniques for the preservation of organisms, for retaining essential features during industrial processes and, above all, retaining long-term vigour and viability. In many examples the catalyst is used in a separated and purified form, namely as enzymes, and a huge amount of information has been built up on the large scale production, isolation and purification of individual enzymes and on their stabilization by artificial means.

The second part of the core of biotechnology encompasses all aspects of the containment system or bioreactor within which the catalysts must function. Here the combined specialist knowledge of the bioscientist and bioprocess enginer will interact, providing the design and instrumentation for the maintenance and control of the

physicochemical environment, such as temperature, aeration, pH etc. all these allow the optimum expression of the biological properties of the catalyst.

After achieving the required end point of the biotechnological process within the bioreactor, eg, biomass or biochemical product, in most cases it will be necessary to separate the organic products from the predominantly aqueous environment. The third aspect of biotechnology, namely downstream processing, can be a technically difficult and expensive procedure, and is the least understood area of biotechnology.

Down stream processing is primarily concerned with initial separation of the bioreactor broth or medium into a liquid phase and a solid phase, and subsequent concentration and purification of the product. Processing will usually involve more than one stage. Down stream processing costs of fermentation products, vary considerably, eg, with yeast biomass, penicilling and certain enzymes processing costs as percentages of selling prices are 20%, 20-30%, and 60-70% respectively.

8.1.1.7. Substrates for biotechnology

1. Natural raw materials

Natural raw materials originate mostly from agriculture, food industry and forestry. These are mainly carbohydrates of varying chemical complexity and include sugar, starch, cellulose, hemicellulose and lignin.

Sugar bearing raw materials such as sugar beet, sugar cane and sugar millet are the most suitable and available to serve as feedstocks for biotechnological processing. Many tropical economics would collapse if the market for sugar were removed. Already cane sugar serves as the substrate for the Brazilian gasohol programme and many other nations are rapidly seeing the immense potential of these new technologies.

Starch-bearing agricultural products include the various types of grains such as maize, rice, and wheat, together with potatoes and other root crops such as sweet potato and cassava. A slight disadvantage of starch is that it must usually be degraded to monosaccharides or digosaccharides by digestion or hydrolysis before fermentation. However, many biotechnological processes using starch are being developed, including fuel production.

There can be little doubt that cellulose, both from agriculture and forestry sources, must contribute a major source of feedstock for biotechnological processes such as fuels and chemicals. However, cellulose is a very copmplex chemical and invariably occurs in nature in close association with lignin. The ability of lignocellulose complexes to withstand the biodegradative forces of nature is witnessed by the longevity of trees, which are composed mainly of lignocellulose.

Lignocellulose is the most abundant and renewable natural resource available to humanity throughout the world. However, massive technological difficulties must be over come before economic use may be made of this plentiful compound. At present, expensive energy –

demanding pretreatment processes are required to open up. This complex structure to wide microbial degradation. Pure cellulose can be degnaded by chemical or enzymic hydrolysis to soluble sugars, which can be fermented to form ethanol, butanol, acetone, single cell protein, methane and many other products. Time will surely show that lignocellulose will be the most useful carbon source for biotechnological developments.

2. Availability of by-products

While biotechnological processes will use many agricultural products such as sugars, starches, oils etc.., as substrates, the vast array of waste products derived from agricultural and forestry wastes come in many diverse types : cereal straws, corn husks and cobs, soy waster, coconut shelts, rice husks, coffee bean husks, wheat bran, sugar cane bagasse and forestry wastes including trimmings, saw dust, bark etc.…

A primary objective of biotechnology is to improve the management and utilization of the vast volumes of agricultural, industrial and domestic waste organic materials found throughout the world. The biotechnological utilization of these works will eliminate a source of pollution in particular water pollution, and convert some of these wastes into useful by –products.

Two widely occurring wastes that already find considerable fermentation uses are molasses and whey. Molasses is a by-product of the sugar industry and has a sugar content of approximately 50%.

Molasses is widely used as a fermentation feedstock for the production of antibiotics, organic acids and commercial yeast for baking, and is directly used In animal feeding. Whey, is used as a fermentation feedstock.

More complex wastes such as straw and begause are widely available and will be increasingly used as improved processes for lignocellulose breakdown become available. Wood wastes will include low grate wood, bark and saw dust, as well as waste liquors such as sulphite waste from pulp production, which already finds considerable biotechnological processing in Europe and communist countries.

3. Chemical and petrochemical feed stocks

With the development of commercial processes for the production of single cell protein (SCP) and other organic products, a number of chemical and petrochemical feed stocks have become particularly important for fermentation processes, since these materials have the advantage of being available in large quantities and in the same quality in most parts of the world. Thus natural gas or methane and gas oil have been preferred as raw material because of their easy processing and universal availability. Main commercial interest has been concerned with n-parafins, methanol and ethanol.

Model Questions :

1. Give the historical developments in the field of Biotechnology.

2. Discuss the origin of Biotechnology, and write your explanation for biotechnology as an interdisciplinary subject.

Lesson 8.1.2

Scope and Overview of Biotechnology

Objective

8.1.2.1 Scope of biotechnology

1. Tissue culture
2. Gene technology
3. Hybridoma and monoclonal antibodies
4. Medicine
5. Protein engineering
6. Metabolic engineering
7. Biosensors, Biochips, Biofilms and Biosurfectents
8. Agriculture
9. Industrial microbiology
10. Environment

8.1.2.2 Biotechnology in India

1. Bioinformatics
2. Plant molecular biology
3. Service oriented infrastructural facilities
4. Training and man power development

8.1.2.3 Commercial potential

8.1.2.4 Biotechnology and intellectual property rights

Objective : In this lesson the scope of biotechnology, various areas of biotechnology, its progress and potential of biotechnology were discussed.

8.1.2.1. Scope of biotechnology

Biotechnology has rapidly emerged as an area of activity having a marked realization as well as potential impact on virtually all domains of human welfare ranging from food processing, protecting the environment, to human health. Biotechnology has now its roots in the science of molecular biology and microbiology. Advances in these two areas have been exploited in a variety of ways both for production of industrially important biochemicals and for various advanced studies in molecular biology. Therefore, a new commercial environment has been created, in which many famous scientists including a nobel laureats like Walter Gilbert opted to work for biotechnology companies. Scores of these companies are created and closed down every year. In USA alone there are more than 200 such companies including Genentech, Cetus, Hybritech, Biogen, etc. And USA, Japan, and Europe have become leaders in biotechnology research and development. Several and varied commercial projects are being undertaken by these companies and a range of these programmes is evident from the following list.

Some of the biotechnological programmer being undertaken by several companies

1. Automated bioscreening
2. Genetical improvement of pharmaceutical micro-organism
3. Engineering of a series of organisms for specific industrial use
4. Developing immobilized cell and enzyme systems for chemical process industries
5. Improved production of vitamin B_{12}
6. Manufacturing fructose from inexpensive forms of glucose
7. Bioprocessing alkene to valuable oxider and glycols
8. Production of ethanol by continuous fermentation
9. Upgrading hydrocarbons microbiologically
10. Production of Xanthan gum in oil fields for enhanced crude oil recovery

11. Production of human insulin microbiologically

12. Production of human interferons microbiologically

13. Developing a vaccine to prevent colibacillosis, a widespread disease of newborn calves and piglets

14. Production of monoclonal antibodies for organ transplant tissue typing

15. Production of diagnostic kits for toxoplasmosis identification

16. Production of plants resistant to herbicides, viruses, insects and other pests

17. Production of photosynthtically efficient plants

18. Production of transgenic animals as bioreactors for producing valuable drugs

19. Production of biopesticides and biofertilizers

20. Human gene therapy for control of diseases

1. Tissue culture

An important aspect of all biotechnology processes is the culture of either the microorganisms or plant and animal cells (or protoplasts in case of plants) or tissues and organs in artificial

media. These possibilities led to significant advances and novel possibilities in the field of biotechnology. The microbes are used in recombinant DNA technology and in a variety of industrial processes and the plant cells and tissues are used for a variety of genetic manipulations. For example, anther culture is used for haploid breeding, gametic and somatic cell/ tissue cultures are used for tapping gametoclonal and somaclonal variation or for production of artificial seeds. The transformation of protoplast in culture leads to the production of useful transgenic plants. Embryo culture technique has also helped in extending the range of distant hybridization for plant breeding purposes. Similarly animal cells are used for multiplication of superior live stock using a variety of techniques like cloning of embryonic cells, transformation of cultured cells leading to the production of transgenic animals, and *in vitro* fertilization and transfer of embryos to surrogate mother.

The earlier researchers explored the problems of academic interest, then emphasis shifted to applied aspect such as haploid from pollen, triploid from endosperms, somatic hybridizations, tissue culture of cereals, legumes and oil crops, and clonal multiplication of elite species of plants. Our knowledge of cell and tissue culture is developing with a rapid progress in many areas, like totipotency, differentiation, cell division, cell nutrition,

metabolism, radiobiology, cell preservation etc. Now our people are in a position to cultivate cell is quantity, or as clones from single cell, to grow whole plant from isolated meristems, to induce callus or even single cell to develop into complete plant by either organogenesis or directly by embryogenesis *in vitro*. The production of haploid through tissue culture from anthers or isolated microspores and of protoplasts from higher plant cells has served as the basic tools for genetic engineering and somatic hybridization. Protoplasts can also be used as genetic material present in nuclei and chloroplasts as well as isolated DNA molecules. This technique provides the opportunity to combine by fusion of the genotype of species which are sexually incompatible and to introduce foreign genetic material such as organelles or DNA into the genome.

2. Gene technology

Most of the biotechnology companies make use of the principle and technique of gene technology or genetic engineering which involves recombinant DNA and gene cloning. These two techniques, recombinant DNA and gene cloning are the most powerful tools developed in the field of biology. The technique of genetic engineering involves manipulation of the genetic material of an organism to give an altered expression of our choice. It deals with identification and isolation of desired gene and then joining

their gene of interest into another organism. The desired gene expresses in that new organism and the various steps of the genetic engineering are as follows:

1. **Gene isolation :** Desired gene is identified, isolated and purified. This DNA of interest is also called donor DNA or target DNA.

2. **Selection of vector :** a vector is a self-replicating molecule of DNA, or replicon to which desired gene is anchored. The Vector molecule inserted with a foreign DNA is known as chimeric DNA. The Vector acts as a carrier and transports the gene into the host cell. Thus, it is also known as a cloning vehicle or a carrier molecule. Suitable vector has to be identified for each system: commonly used vectors are plasmids and viral DNA molecule. However, recent techniques involve physical delivery of DNA by various methods.

3. **Cloning of desired gene :** Multiple copies of desired gene can be obtained by pacing them in a host cell with the help of vectors. Here, the desired gene along with the vector is amplified. Large number of identical copies of gene of our interest are produced for subsequent gene transfer into the

target cell. Now, gene cloning is fast and mechanized process, using polymerase chain reaction (PCR) method.

4. **Specific gene transfer :** The gene of our interest is finally transferred in to host cells using different techniques. Transformed cells are selected, multipled and used to produce transgenic plants by tissue culture technique.

5. **Expression of the desired gene :** The desired gene of our interest transferred in to a new environment of host, thus the desired traits were expressed in that organism.

Genetic engineering includes the propagation of chimeric DNA in to a different host organism. The ability to cross natural species barriers and place genes from one organism to an unaltered host organism is an important feature of gene manipulation. And also it is fact that a defined and relatively small piece of DNA is propagated in host organism. Thus the emergence of genetic engineering has opened the doors to a range of molecular biological opportunities including nucleotide sequence determination, site-directed mutagenesis, and manipulation of gene sequences to ensure very high-level expression of an encoded polypeptide in a host organism. If used wisely genetic engineering promises to enhance the quality of human life.

3. Hybridoma and monoclonal antibodies

It is another very important area in which rapid progress has been made, which is very extensively being utilized for human health care and for a variety of other purposes.

For the purposes of research and use in medicine, antibodies are often isolated by sacrificing animal after hyper-immunizing them with antigens. From such hyperimmunized animals, the blood serum will be collected and antibodies can be isolated from the serum. However, these are heterogeneous in nature and lack desired specificity, and therefore cannot be utilized for diagnosis or screening purpose. Hence the monoclonal antibodies, which can be used for diagnostic purpose are prepared and produced by using somatic cell fusion called hybridoma technology. Although, hybridoma technology enables us to obtain monoclonal antibodies or homogeneous antibodies of predefixed specificity, it cannot allow production of novel antibodies of desired specificity. Such novel antibodies can be obtained by redesigning the antibodies through the application of gene technology and thy is an area of research which has revolutionized the potential monoclonal antibodies.

Enzyme-conjugated antibodies are being used for detection of viruses both in plants and animals (including humans) using ELISA (Enzyme linked immunosorbent assay) test. Immunotoxins are being produced from gene fusions so that the toxic drugs meant

for killing tumor cells may be carried to the target sites with the help of specific antibodies.

4. Medicine

Biotechnology has found a wide range of applications in medicine. For dealing with several human diseases for prevention, diognosis and control, several biotechnology applications are being practical. Through human genetics, it has found use in genetic counseling, antenatal diagnosis, and gene therapy. In forensic medicine, it has been used for identification of individuals, who would be criminals, with the help of blood stains, semen stains, hair roots, tears, saliva and perspiration.

Insulin and interferon synthesized by bacteria have already been released commercially for human use. A large number of vaccines for immunization against dreadly diseases, DNA probes, monoclonal antibodies for diagnosis of various diseases, and human growth hormone and other pharmaceutical drugs for treatment of diseases are being released or in the process of release. In 1988, first time the lymphocyks containing bacterial gene has been approved for patients who were in the terminal stage of cancer and having no chance for survival for its treatment had no chance to survive. During 1990, patients suffering with some lethal diseases were successfully subjected for gene therapy and

these patients were now doing well. The DNA fingerprinting and autoantibody fingerprinting techniques are proving to be a boon in forensic medicine for identification of criminals, murderers and rapists through the study of DNA or antibodies from blood and semen stains.

5. Protein engineering

Another important area of biotechnology is protein engineering that will lead to the production of superior enzymes and storage proteins. It is a multidisciplinary approach which involves computer aided molecular modeling (CAMM) based on the study of 3-dimensional structures of receptors and functional macromolecules, and then preparation of gene that will produce this desired protein in a predictable manner. Thus, in future the proteins can be engineered in the desired manner for various applications. Biotechnology has provided us with a remarkable technique in the form of immobilized enzyme systems, which allow the production of a variety of substances eg. production of high-fructose corn syrup (a sweetening agent for soft drink industry) using an immobilized enzyme, glucose isomerase. The market for these immobilized enzymes is now of the order of billions of dollars per year and supports multibillion dollar industries. The cost of production of these enzymes is only a small fraction of the value of the product.

6. Metabolic engineering

One of the major objectives of biotechnology research is the use of living systems for the production of useful metabolites at the industrial level. However, the natural metabolic network of the cell is not optimized for practical applications. Hence the performance of metabolic pathways or bioprocesses has to be modified by genetic manipulation of the cell, so that the metabolites can be over produced. The technology involved in this is described as metabolic engineering which can be defined as "the improvement of cellular activities by manipulation of enzymatic, transport and regulatory functions of the cell with the use of recombinant DNA technology". However, there is a variety of limitations in metabolic engineering that need to be overcome. For instance, when alterations are made by genetic manipulations for flux distribution at key branch points (nodes) of metabolic pathway, this may be opposed by mechanisms evolved in the cell for its optimal growth. This is described as `network regidity' and efforts are being made to overcome problems like this.

7. Biosensors, Biochips, Biofilms and Biosurfactants

The natural biosensors are the sense organs primarily the chemical sensors of smell and taste. Biosensors, technically in their various forms share a reliance of biological materials as

sensing elements. The technical biosensors have been under intense development since 1960 with the prospects of commercial potential offered by the development of biotechnology. Biosensors are the combination of biologically active material displaying characteristic specifically with chemical or electronic sensor to convert the response into electrical signals. Biosensors are being very much used in military research, as means of detecting nerve gases and other chemical warfare toxins. Their applications it biosensors are branched out to use in medical and health monitors in drug screening and in environmental quality control.

Biochips are made from different biological material and can be used in control of computer as a replacement to silicon chips. Biomolecular computers thus made, promise are very promising and ten to thousand times to be smaller than the best super computers with much faster switching time and extremely low power dissipation is possible. They are made up of semi-conducting molecules inserted into the protein frame work and fixing the whole thing on to a protein support. They can be used in implanting of several sorts in human body, like regulation of heart beat, response to nerve impulses by artificial limbs, overcoming the blindness and deafness etc. It is important to note that the biochips may be infected by microbes since they are made up of proteins.

A biofilm is an accumulation of microbial cells and inorganic components held together in a polymeric matrix and firmly attached to a substratum. Accumulation of biofilms is encountered in many natural and modulated environments. It may be fundamental for determining the process performance i.e. for fixed-film biological waste water treatment, sudden deterioration of water quality and deterioration of substrata.

Biosurfactants are surface active compounds produced by microorganisms. Biological surfactants possess a number of potential advantages over their chemically manufactured counterparts, including lower toxicity, biodegradability, a wide variety of possible structures, and ease in synthesis from inexpensive and renewable feed stocks. Consequently, biosurfactants may have applications in numerous areas, particularly for enhanced oil recovery and in food, beverages, cosmetics, pharmaceutical preparations.

8. Agriculture

Biotechnology has also revolutionized research activities in the area of agriculture which includes the following.

- Plant cell, tissue and organ culture.

- Genetic engineering leading to transformation followed by regeneration of plants to give transgenic. Plants

carrying desirable traits like disease resistance, insect resistance and heribicids resistance and eventually this may also be used for increasing photosynthetic efficiency, nitrogen fixing ability, improved storage proteins, hybrid crops, crops for food processing.

- Somatic hybrids between sexually incompatible species permitting transfer of desirable traits from wild or unrelated crop species to our interested crop plants:

- Transgenic animals produced in mice, pigs, goats, chicken, cows, etc. are well know for high yield and production, further it is suggested that some of these will eventually be used as bioreactors to produce drugs through their milk, blood or urine, this area has also been described as molecular farming.

9. Industrial microbiology

Industrial microbiology is yet another area, receiving major attention of biotechnologists. A number of pharmaceutical drugs and chemicals are being produced using the techniques of biotechnology from microbes to increase both the quality and quantity of these drugs and chemicals.

10. Environment

Biotechnology is also very much being used for dealing with the environmental problems. Fears are also being expressed about the implication of advances in biotechnology in terms of release of harmful organisms developed through recombinant DNA technology. In view of this, rules and laws have been framed from time to time to safeguard against these predicted risks, which the recombinant DNA technology poses to provide clean and friendly environment.

Several methods have been devised for solving some environmental problems which are as follows :

- Pollution control
- Deletion of natural resources for non-renewable energy
- Restoration of degraded land and
- Biodiversity conservation

The microbes are being developed to be used as biopesticides, biofertilizers, biosensors and for recovery of metals, cleaning of spilled oils and for a variety of other purposes in environment. They are also being used for biomonitoring in industries, where employees are being exposed to a variety of risks. Biomass is being produced and used as a renewable source of energy, by capturing solar energy. Tissue culture and genetic engineering, mycorrhizae

(VAM fungi), root nodulation are also being used for reclamation of degraded lands.

8.1.2.2 Biotechnology in India

The leaders in biotechnology are countries like USA, JAPAN and those in Europe. However, in India also at the 69th session of Indian Science Congress held at Mysore in 1982, Indian Scientists emphasized the importance of gene technology. Consequently, on the recommendations of science Advisory Committee to Parliament, Government of India Constituted a National Biotechnology Board under the department of Science and Technology to coordinate and encourage research in this direction. National Biotechnology Board (NBTB) decided to compile a list of biotechnologists in India and abroad working in the following fields.

- genetic engineering
- tissue culture
- enzyme engineering
- alcohol fermentation and
- immunotechnology

In view of the significance which is attached to biotechnology, a separate Department of Biotechnology in the Ministry of Science

and Technology was created by Government of India in the year 1986.

An International Centre for Genetic Engineering and Biotechnology (ICGEB) for developing countries in the auspieces of United Nations has also been established. This center has two locations, one in New Delhi, India and the other in Triesta, Italy. The centre in India started operating in 1987.

An International Institution of Biotechnology (IIB) having objectives parallel to those of ICGEB, was also established in UK (Canterbury, Kent). It is a charitable institute and coordinates activities of multidisciplinary, multinational groups from both industrialized and industrialized and industrializing countries. It also provides educational training through different methods including a course for the degree of Master of Biotechnology.

Biotechnology centers in India, have also been established at (i) Indian Agricultural Research Institute (IARI), New Delhi.

ii. National Dairy Research Institute (NDRI), Karnal and

iii. Indian Veterinary Research Institute (IVRI), Izatnagar (UP).

With the inception of NBTB in 1982 and that of DBT in 1986, enoromous effort for growth and development of biotechnology in

India has been made to create scientific infrastructure in terms of both physical facilities and trained man power.

1. Bioinformatics

In the area of bioinformatics, nine Distributed Information Centres (DICs) and 14 user centers were established in various institutions in the country. The nine DICs are located at the following institutions.

1. Indian Institution of Science (IISc) Bangalore
2. Madhurai Kamraj University (MKU), Madurai
3. Bose Institute, Calcutta
4. Jawaharlal Nehru University (JNU), New Delhi
5. Poona University, Pune
6. Indian Agricultural Research Institute (IARI), New Delhi
7. Centre for Cellular and Molecular Biology (CCMB), Hyderabad
8. National Institute of Immunology, (NII), New Delhi
9. Institute of Microbial Technology (IMTECH), Chandigarh

These DICs are linked with each other and with the Central Information Network at DBT in New Delhi, which has access to international databases in biotechnology.

2. Plant molecular biology

To give the desired boost to programmer of crop improvement through biotechnology, six centers for plant molecular biology were recently established by DBT at the following institutions.

1. MKU, Madurai
2. JNU, New Delhi
3. Tamil Nadu Agricultural University (TNAU) Coimbattore
4. Osmania University, Hyderabad
5. Bose Institute, Calcutta and
6. National Botanical Research Institute (NBRI), Lucknow.

3. Service oriented infrastructural facilities

In order to develop strong research and development (R & D) and manufacture capabilities, DBT has set up service oriented infrastructural facilities in the following areas:

- Germplasm banks for plants, animals, algae and microbes
- Animal houses
- Oligonucleotide synthesis (DNA synthesizers)

- Production, import and distribution of enzymes, reagents, and radio-labelled compounds.

- Bioprocess optemization and pilot plants

- Genetic engineering R & D units.

- Centre for reproductive biology and molecular endocrinology

- Carbohydrate cell surface and cellular transport

- Protein and peptide sequencing

- NMR facility

- Marine cyanobacterial germplasm collection

- Antibody development consortium

4. Training and manpower development

There are now at least 30 institutions in the country, where a strong infrastructure for training and research in biotechnology has been established. With the help of DBT, following programmes are now in operation.

- Post-graduate (M.Sc/M.Tech) programmes and post doctoral programmes have been initiated in 25 selected universities and other institutions.

- Short-term training programmes of 2-4 weeks duration have been introduced for teachers and scientists in high-tech specialized area at institutions where expertise and facilities exist.

- Technician training programme for R&D persons in industry and two `one year diploma courses' for candidates with B.Sc degree have been started to produce freshly trained technicians.

- Biotechnology associate ships are awarded every year for advanced research or specialized training in identified priority areas of biotechnology.

- Visiting scientists from abroad is another programme, under which a foreign scientist can visit India and collaborate with a host institution in India.

The above programmer do not include all the programmes financed by DBT, but these programmes have ultimately helped in the development of expertise in the area of biotechnology in the country.

8.1.2.3. Commercial potential

Biotechnology seems to have an unlimited commercial potential in view of its capability to generate an unlimited range of valuable and useful products / services concerned with virtually every aspect of human existence. For example, in the mid-1991, over 130 biotechnologically derived pharmaceuticals, aimed at everything from hemophilia to AIDS, and from anemia to leukemia, were under regulatory review in the United States. The contribution of the biotechnological products to the global market is rising rapidly, it is expected to be between $60 and 100 billion / year by the year 2000.

The biotechnology industry in India is growing rapidly in the wake of liberalization of the economy that has fostered alliances between Indian companies and multinationals. The demand for various biotechnological products in India has been estimated at around Rs. 27 billion for 1995, which is expected to rise to about Rs. 55 billion by the year 2000. As may be expected, the major share (72% in 1995, 64% in 2000) of this demand would be in the areas of human and animal health, followed by that of for industrial products (21% 1995, 27% 2000); agriculture sector accounts for a very small portion (6% in 1995 & 7% in 2000) of the demand.

8.1.2.4. Biotechnology and intellectual property

In recent years, there have also been discussions on the protection of intellectual property rights emanting from the use of biotechnology. The intellectual property rights (IPRs) include patent, trade secrets, trademarks and copyrights, which can be protected through a variety of laws in different countries. However, not all developments in biotechnology can be protected as intellectual property rights. For instance, techniques used in medical science (bypass heart surgery, organ transplant, use of drugs, antibiotics, artificial limbs, and vaccines, etc.) are not patentable. For plant biotechnology also, a variety of culture methods, biological control of pests and weeds are not patented. There are other examples of the products of biotechnology, which can be patented. These include products like modified antibiotics, hormones and enzymes, synthetic steroids, immobilized enzymes, organ specific drug delivery, heart values, artificial teeth, plastic bags for blood storage, etc. A variety of products in the field of agriculture and animal husbandary are also patentable. There are also international agreements made o enforce protection of intellectual property rights generated in one country and needing protection in other countries.

Summary

Biotechnological developments ultimately contributed for the benefit of human kind. All the allied fields are grown

simultaneously with the technological advancements in biotechnology. The scope for this is very wide and requires good infrastructural facilities. The persons who are working in these felids should be well trained to get good potential processes and products from the biotechnology.

Questions

1) Discuss the scope and potential for Biotechnology subject

2) Give the overall picture of Biotechnology its growth in India

Author

N.Srinivasa Reddy
Centre for Biotechnology
Acharya Nagarjuna University

Lesson 8.1.3

Different fields of Biotechnology

Objective

8.1.3.1 Bioprocess technology

1. Introduction
2. Microbial growth
3. The bioreactor / fermenter
4. Scale-up
5. Media for fermentation processes
6. Downstream processing

8.1.3.2 Enzyme technology

1. Introduction
2. Genetic engineering and protein engineering of enzymes
3. The technology of enzyme production
4. Immobilised enzymes

8.1.3.3 Plant biotechnology

1. Introduction
2. Features of plants relavent to their genetic engineering
3. Methods of genetic engineering in plants
    a. Agrobacterium mediated transfer
    b. Direct transfer of plants

8.1.3.4 Animal biotechnology

1. Introduction

2. Manipulation of reproduction in animals
    a. Artificial insemination
    b. Embryotransfer
    c. Invitro fertilization
    d. Animal cloning

8.1.3.5 Immunotechnology

1. Introduction
2. Monoclonal antibody technology
3. Human monoclonal antibodies
4. Cytokines
    a. Production of cytokines in vitro

8.1.3.6 Environmental biotechnology

1. Introduction
2. Waste-water and sewage treatment
3. Land fill technologies
4. Composting
5. Bioremediation

Objective :

This chapter deals with various interdisciplinary fields of biotechnology and their applications

8.1.3.1. Fermentation technology / Bioprocess technology

1. Introduction

The very beginnings of fermentation technology or, as it is now better recognized, bioprocess technology were derived in part from the use of microorganisms for the production of foods such as cheeses, yoghurts, sauerkraut, fermented pickles and sausages and beverages such as beers, wines and derived spirits. Although the traditional forms of bioprocess technology related to foods and beverages still represent the major commercial bioproducts, new products are increasingly being derived from microbial fermentations, namely.

- to overproduce essential primary metabolites such as acetic acid, lactic acid, glycerol, acetone, butyl alcohol, organic acids, aminoacids, vitamins and polysaccharides.

- To produce secondary metabolites such as penicillin, streptomycin, cephalosporin, gibberellins, etc...

- To produce many forms of industrially useful enzymes, eg. Extracellular enzymes (amylase, pectinase, and proteases) and intracellular enzymes (invertase, asparginase, restriction endonucleases, etc…)

All of these types of microbial product now command large industrial markets and are essential to modern society.

The product formation stages in bioprocess technology are essentially very similar no matter what organism is selected, the medium is used and what product formed. In all processes, large no. of cells are grown under defined controlled conditions. The organisms must be cultivated and motivated to form the desired products by means of a physical / technical containment system (the bioreactor), the correct medium composition and environmental growth regulating parameters such as aeration and temperature. The same apparatus, with modifications, can be used to produce an enzyme, an antibiotic, an aminoacid or single cell protein. In its simplest form, the bioprocess can be seen as just mixing microorganisms with a nutrient broth and allowing the components to react.

2. Microbial growth

The growth of organisms may be seen as the increase of cell material expressed in terms of mass or cell number and results from a highly complicated and coordinated series of enzyme-catalysed biological steps. Growth will be dependent on the availability and transport of necessary nutrients to the cell and their subsequent uptake, and on environmental parameters such as temperature, pH and aeration being optimally maintained.

In (biotechnological) bioprocesses there are three main ways of growing microorganisms in the bioreactor, namely batch, semi-continuous or continuous. With in the bioreactor, reactions can occur with static or agitated cultures, in the presence or absence of oxygen, and in liquid or low-moisture conditions. The microorganisms can be free or can be attached to surfaces by immobilization or by natural adherence.

Batch culture

In a batch culture the microorganisms are inoculated into a fixed volume of medium and as growth takes place nutrients are consumed and products of growth accumulate. The nutrient environment with in the bioreactor is continuously changing and thus, inturn, enforcing changes to cell metabolism. Eventually, cell multiplication ceases because of exhaustion or limitation of nutrients (s) and accumulation of toxic excreted waste products.

The initial lag phase is a time of no apparent growth but actual biochemical analyses show metabolic turnover indicating that cells are in the process of adapting to the environmental conditions and that new growth will eventually begin. There is then a transient acceleration phase as the inoculum begins to grow, to be followed quickly by the exponential phase. In the exponential phase microbial growth proceeds at the maximum possible rate for that organism with nutrients in excess ideal environmental parameters are present and growth inhibitors are absent. However, in batch cultivations exponential growth is of limited duration and, as nutrient condition changes, growth rate decreases, entering the deceleration phase to be followed by the stationary phase when overall growth can no longer be obtained because of nutrient exhaustion. The final phase of the cycle is the death phase when growth rate has ceased. Most (biotechnological) processes are stopped before this stage, because of decreasing metabolism and cell lysis.

Fig.

In industrial usage, batch cultivation has been operated to optimize organism or biomass production and then to allow the

organism to perform specific biochemical transformations such as end-product formation (eg; aminoacids) or decomposition of substances (sewage treatment, bioremediation). Many important products such as antibiotics are optimally formed during the stationary phase of the growth cycle in batch cultivation.

However, there are means of prolonging the life of a batch culture and thus increasing the yield by various substrate feed methods, namely:

- Fed batch: gradual addition of concentrated components of the nutrient eg: carbohydrates, so increasing the volume of the culture – used for industrial production of baker's yeast.

- Addition of medium to the culture (perfusion) and withdrawl of an equal volume of used cell-free medium – used in animal cell cultivations.

Continuous culture

Continuous cultivation gives near-balanced growth, with little fluctuation of nutrients, metabolites, cell numbers or biomass. This practice depends on fresh medium entering a batch system at the exponential phase of growth with a corresponding withdrawl of medium plus cells. Continuous methods of cultivation will permit

organisms to grow under steady state (unchanging) conditions in which growth occurs at a constant rate and in a constant environment.

In a completely mixed continuous culture system sterile medium is passed into the bioreactor at a steady flow rate, and culture broth (medium, waste products and organisms) emerges from if at the same rate, keeping the volume of the total culture in the bioreactor constant. Factors such as pH and the concentrations of nutrients and metabolic products that inevitably change during batch cultivation can be held near constant in continuous cultivations. In industrial practice continuously operated systems are of limited use and include only single cell protein and ethanol productions and some forms of waste-water treatment processes. However, for many reasons batch cultivation systems represent the dominant form of industrial usage.

Fig.

3. The fermenter / bioreactor

For each biotechnological process the most suitable containment system must be designed to give the correct

environment for optimization of organism growth and metabolic activity. Bioreactors range from simple stirred or non-stirred open containers to complex, aseptic, integrated systems involving varying levels of advanced computer inputs. For many advanced fermentation processes there is a need to avoid contamination of the process by unwanted microorganisms and the same time to avoid undue release of the culture organism into the environment.

Bioreactors bioprocesses of are of two different types. In the first instance they are primarily non-aseptic systems where it is not absolutely essential to operate with entirely pure cultures, eg. Brewing, effluent disposal systems; in he second type, aseptic conditions are a prerequisite for successful product formation eg; antibiotics, vitamins, polysaccharides.

Fermentation reactions are multiphase, involving a gas phase (containing N_2, O_2 and CO_2), one or more liquid phases (aqueous medium and liquid substrates) and solid microphase (the microorganisms and possibly solid substrates). All phases must be kept in close contact to achieve rapid mass and heat transfer. In a perfectly mixed bioreactor all reactants entering the system must be immediately mixed and uniformly distributed to ensure homogeneity inside the bioreactor.

For the active optimization of the bioreactor system, the following operating guidelines must be closely followed adhered to:

- the bioreactor should be designed to exclude entrance of contaminating organisms.
- The culture volume should remain constant, i.e. no leakage or evaporation.
- The dissolved oxygen level must be maintained above critical levels of aeration and culture agitation for aerobic organisms.
- Environmental parameters such as temperature, pH, etc… must be controlled; and the culture volume must be well mixed.

Fig.

4. Scale-up

Fermentation processes are normally developed in three stages or scales. In the initial stage basic screening procedures are carried out using relatively simple microbiological techniques, such

as petridishes, Erlenmeyer flasks etc... This is followed by a pilot plant investigation to determine the optimal operating conditions in a volume capacity of 5 to 200 lit. The final stage is the transfer of the study to plant or production scale and final economic realization. Throughout all the stages the optimal environmental conditions must be maintained for good yields.

5. Media for fermentation processes

Liquid fermentation : Water is at the center of all biotechnological processes and in most cases will be the dominant component of the media in which microorganisms grow. After liquid fermentation processes have achieved production, the removal of water is a major factor in the cost of bioproduct recovery and down stream processing. The quality of water is highly relevant because it affects microbial growth and the production of specific bioproducts.

The basic nutritional requirements of micro organisms are an energy or carbon source, an available nitrogen source, inorganic elements and, for some cell types, specific growth factors. In most biotechnological processes carbon and nitrogen sources are more often derived from relatively complex mixtures of cheap natural products or by-products.

Solid state Fermentation

These are many biotechnological processes that involve the growth of microorganisms on solid substrates in the absence or near absence of free water. The most regularly used solid substrates are cereal grains, legume seeds, wheat bran, lignoullulose materials such as straws, sawdust or wood shavings, and a wide range of plant and animal materials. Most of these compounds are invariably polymeric molecules, insoluble or sparingly soluble in water, but are mostly cheap, easily obtainable and represent a concentrated source of nutrients for microbial growth.

In many solid substrate fermentations there is a need to pretreat the substrate raw materials to enhance the availability of the bound nutrients an also to reduce the size of the components, eg; pulverized straw, shredded vegetable materials to optimize the physical aspects of the process.

Bioreactor designs for solid substrate fermentations are inherently more simple than for liquid cultivations. They are classified into fermentations

a. without agitation

b. with occasional agitation, and

c. with continuous agitation

advantages

- simple media with cheaper natural rather than costly fossile-derived compounds.
- Low moisture content of materials gives economy of bioreacter space, low liquid effluent treatment, less microbial contamination, often no need to sterilize, easier downstream processing.
- Aeration requirements can be met by simple gas diffusion or by aerating intermittently, rather than continuously.
- Yields of products can be high
- Low energy expenditure compared with stirred tank bioreactor.

Disadvantages

- Processes limited mainly to moulds that tolerate low moisture levels.
- Metabolic heat production in large-scale operation creates problems.

- Process monitoring, eg. Moisture levels, biomass, O_2 and CO_2 levels, is difficult to achieve accurately.

- Bioreactor design not well developed.

- Slower growth rate of microorganisms.

6. Down stream processing

It is not enough to grow the required cells in a bioreactor: extraction and purification of the desired end-product (the so-called down stream processing) from the bioprocess is equally, if not more important.

Downstream processing will be primarily concerned with initial separation of the bioreactor broth into a liquid phase and to a solid phase and subsequent concentration and purification of the product. Processing will normally involve more than one stage. Methods in use or proposed range from conventional to the arcane, including distillation, centrifugation, filtration, ultrafiltration, solvent extraction, adsorption, selective membrane technology, reverse osmosis, molecular sieves electrophresis and affinity chromatography.

Final product of the down stream purification stages should have some degree of stability for commercial distribution. Stability

is best achieved for most products by using some form of drying. In practice this is achieved by spray-drying, fluidized-bed drying or by freeze-drying. The method of choice is product and cost dependent. Products sold in the dry form include organic acids, aminoacids, antibiotics, polysaccharides, enzymes, single cell protein and many others. Many product cannot be supplied easily in a dried form and must be sold in liquid preparation. Care must be taken to avoid microbial contamination and deterioration and when the product is proteinaceous to avoid denaturation.

8.1.3.2. Enzyme technology

1. Introduction

Enzymes are complex protein molecules present in living cells, where they act as catalysts in bringing about chemical changes in substances. An enzyme carries out its activity without being consumed in the reaction, while the reaction occurs at a very much higher rate when the enzyme is present. Enzymes are highly specific and are required only in minute quantities. Some enzymes need additional factors, termed cofactors, that can be metobolites, nucleotides, etc.... Enzymes catalyse reactions at relatively low temperature (0-110^{o}C) and in the pH range 2-14. In industrial application this can result in high quality products, fewer by-

products and simpler purification procedures. Further more, enzymes are non-toxic and biodegradable.

Enzyme technology embraces production, isolation, purification, use in soluble form and finally the immobilization and use of enzymes in a wide range of bioreactor but has drawn heavily on microbiology, chemistry and process engineering to achieve the present status of science. For the future, enzyme technology and genetic engineering will be two very closely related areas of study dealing with the application of genes and their products.

2. Genetic engineering and protein engineering of enzymes

Recombinant DNA technology has allowed the transfer of useful-enzyme genes from one organism to another. Thus when an enzyme has been identified as a good candidate enzyme for industrial use, the relevant gene can be cloned into a more suitable production host organism and an industrial fermentation carried out. In this way it becomes possible to produce industrial enzymes of very high quality and purity.

Fig.

Protein engineering or molecular surgery has been used to alter the performance of enzyme molecules. It involves the creation of a 3-dimensional graphical model of the purified enzyme obtained from x-ray crystallographic data. Changes to the enzyme structure can then be considered that might result in increased stability to, for example pH and temperature, and the requisite molecular changes made in the gene coding for the enzyme.

Two main avenues of research have been pursued inorder to alter the performance of enzymes. In one approach, mutagenesis of cloned-gene product, aminoacid residues at defined positions in the structure of the enzyme can be replaced by other suitably coded amino acid residues. The altered gene is then transformed into a suitable host organism and the mutant enzyme subsequently produced with requisite changes in position. This process is known as site-directed mutagenesis. The second method used involves the isolation of the natural enzyme and modifications to its structure carried out by chemical or enzymatic means-some times referred to as `chemical' mutation. A recent successful example of protein engineering is that of the enzyme phospholipase A_2, which was modified structurally to resist higher concentrations of acid. This enzyme is widely used as emulsifier.

3. The technology of enzyme production

Although many useful enzymes have been derived from plant and animal sources it is clear that most future developments in enzyme technology will rely on enzymes of microbial origin. The use of microorganisms as a source material for enzyme production has developed for several important reasons.

- There is normally a high specific activity per unit dry weight of product.
- Seasonal fluctuations of raw materials and possible shortages due to climatic change as political upheavals donot occur.
- In a microbes a wide spectrum of enzyme characteristics, such as pH range, and high temperature resistance, is available for selection.
- Industrial genetics has greatly increased the possibilities for optimizing enzyme yields and type through strain selection, mutation, induction and selection of growth conditions and more recently, by using the innovative powers of gene transfer technology and protein engineering.

Novel enzymes from unusual sources can now he produced by cloning the relevant gene into a well-characterised and easily grown microorganism such as `*Aspergillus oryzae*'.

The rationale for selection between different microorganisms is complex and involves many ill-defined factors such as economics of cultivation, whether the enzyme is secreted in the culture broth or retained in the cell, and the presence of harmful enzymes. Depending on the sources material, enzymes differ greatly in their stability to temperature and to extremes of pH.

Example : Bacillus subtilis proteases are relatively heat stable and active under alkaline conditions and have been most suitable as soap-powder additives. In contrast, fungal amylases, because of their greater sensitivity to heat, have been more useful in the baking industry.

When selecting for enzyme production the industrial geneticist must seek to optimize desired properties (high enzyme yield, stability, independence of inducers, good recovery, etc.) while also attempting to remove or suppress undesired properties (harmful accompanying metabolites, odour, color etc.,). A common feature of industrial production organisms is that their genetics are little understood. However, gene transfer technology together with

protein engineering will alter this and present new horizons to enzyme technology.

The raw materials for industrial enzyme fermentations have normally been limited to substances that are readily available in large quantities at low cost, and are nutritionally safe. Ex: Starch hydrolysate, molasses, corn steep liquor, whey and many cereals. The choice of fermentation medium is important since it supplies the energy needs as well as carbon and nitrogen sources.

Fig.

A typical enzyme producing bioreactor is constructed from stainless steel and has a capacity of 10-50 m^3. In most cases enzymes are produced in batch fermentations lasting from 30-150 hr; continuous cultivation processes have found little application in industrial enzyme production. Sterility of the bioreactor system is essential throughout production.

At the completion of the fermentation the enzyme may be present within the microorganism or exerted into the liquid or solid medium. Commercial enzyme preparations for sale will be in either a solid or liquid form, crude or highly purified. The final cost if an enzyme will depend on the degree of down-stream processing that is required to achieve a saleable product.

At present only a small number of micro organisms are used for enzyme production. Responsibility for the safety of an enzyme product remains with the manufacturer. In practice, a safe enzyme product should have low allergenic potential an be free from toxic materials and harmful microorganisms.

Fig.

In practice both physical and chemical methods are repeatedly used for enzyme immobilization. Physically, enzymes may be adsorbed onto an insoluble matrix, entrapped with in a gel, or encapsulated with in a microcapsule or behind a semipermeable membrane. Chemically enzymes may be covalently attached to solid supports or cross-linked.

Fig.

A large number of chemical reactions have been used for the covalent binding of enzymes by way of their non-essential functional groups to inorganic carriers such as ceramics, glass,

iron, zirconium etc., to natural polymers such as sepharose & cellulose, and to synthetic polymers (nylon, polyaerylamide). In many of these procedures the covalent binding of enzymes to the carriers is non-specific, i.e., the binding of the enzyme to the carrier is by way of the enzyme's chemically active groups distributed at random. More recent studies have attempted to develop technologies that immobilize enzymes without affecting their catalytic activity.

The entrapment of enzymes in gel matrices is achieved by carrying out the polymerization or precipitation / coagulation reactions in the presence of the enzyme. Polyacrylamides, collagen, silicagel, etc., have all proved to be suitable matrices but the entrapment process is relatively difficult and results in low enzymic activity.

Immobilized whole microbial cells are becoming increasingly utilized and tend to eliminate the tedious, time-consuming and expensive enzyme purification steps. Immobilization of whole cells is normally achieved by the same methods as for cell-free enzymes.

The greatest potential for immobilized (enzymes) cell systems lies in replacing complex fermentations such as secondary product formation, in the continuous monitoring of chemical processes (via enzyme electrodes), water analysis and waste treatments,

continuous matting processes, nitrogen fixation, synthesis of steroids and other valuable medical products.

As a consequence of successful immobilization techniques in the form of enzyme capsules, enzyme beads, enzyme columns and enzyme membranes, many types of bioreactor have been developed at a laboratory scale and to a lesser extent in industrial scale. These include batch-stirred tank bioreactors, continuous packed-bed bioreactors and continuous fluidized –bed bioreactors.

Looking to the future, it seems reasonable to expect that the production and application of enzymes will & continue to expand. The growing world concern about the environment and resources in particular the rising price of oil and other raw materials, is promoting new avenues of research and there is little doubt that enzymes will play a major role in solving some of these problems.

4. Immobilized enzymes

Immobilization is defined as imprisonment of an enzyme in a polymeric support or polymer matrix through which substrate can pass and converted to products. Substrate can pass into the matrix and is converted to products and is released but never the enzyme comes out.

Fig.

Advantages:

It can be reused

- can be continuously used
- has high thermal and storage stability
- has high affinity for substrates
- easy recovery of products
- enzymes can also be easily recovered
- enzyme activity increases

Disadvantages

- There is a chance for loss of enzyme activity
- There is a chance for bacterial contamination
- High cost of synthetic polymers
- Efficiency of conversion of high molecular weight substrates to products is reduced due to steric interactions with the polymeric matrix.

8.1.3.3. Plant biotechnology

1. Introduction

The oldest of all biotechnologies is perhaps plant biotechnology. This was essentially aimed at improving crop productivity. Classical, plant biotechnology is limited to the introduction of required characteristics into plants by genetic crossing during sexual reproduction. This classical approach is useful if the desired genetic improvement is encoded by a single gene. If a tract is determined by several genes, it may be extremely difficult to introduce all the genes responsible for the crop improvement using conventional breeding techniques. The most serious limitation of classical plant breeding technology is the time needed to develop a usable plant variety it is estimated that it takes about 15 years to introduce a new variety using this method. It is in this context that the newly emerging area of biotechnology viz, genetic engineering, assumes greater significance. Genetic engineering has provided the technology with which plants can be genetically modified by manipulating a plant's own genes and also by introducing genes from taxonomically unrelated plants and other organisms such as bacteria, fungi, viruses and even animals.

2. Features of plants relavent to their genetic engineering

Among living things, plants are unique in several respects. Among these, totipoteney is of special value to plant biotechnologists.

Every cell, when separated from the plant and incubated under appropriate conditions can give rise to a complete new plant. Totipotency is somehow lost in animals, perhaps because of the extreme developmental differentiation of cells during embryo development in animals. Plant cells in that respect, donot undergo extreme differentiation and consequent irreversible inactivation of certain genes.

Plants are photoautotrophic, converting CO_2 to carbohydrates using the process of photosynthesis. The photoautotrophic capabilities of plants make them useful life forms to study unitreaction between chloroplast & nuclear genomes. Exploration of this property could have great potential in the development of novel plant varieties with increase yield.

An important feature of plants relevant to genetic engineering in plants is generally achieved using the bacterium. Agrobacterium tumifaciens which has evolved a mechanism of introducing genes into the nuclear genomes of plants. The case of performing genetic engineering in plants has led to the generation of a steady stream of transgenic plants in the past few years. Plant genetic

engineering is being used for introducing a variety of useful genes into plants as well as to gain more insights into plant gene function.

3. Methods of genetic engineering in plant cells

a. Agrobacterium mediated gene transfer

Agrobacterium tumefaciens is a soil bacterium that incites tumor development in wounded plant and causes crown gall disease by a naturally evolved `genetic engineering' process. The causative agent of tumorigenesis is an exceptionally large (200 kbp) plasmid designated as tumor-inducing (T_i) plasmid. A part of the plasmid called transferred DNA (T-DNA) is transferred from agrobacterium into plant cells becomes integrated into the plant genome. The T-DNA genes, when expressed in plant cells, code for the production of: an auxin and o cytokinin hormone (which cause the development of a tumor by promoting uncontrolled growth of plant cells); an opine (such as octopine and nopaline) which acts as a growth-promoting nutrient for the infecting strain of Agrobacterium.

Fig.

The ecological advantage for Agrobacterium in inciting tumors on plant tissues is the availability of opines in the tumors (opines are made by plant cells but can be utilized only by Agrobacterium) and the expression of hormone biosynthetic genes in the tumor cells which causes the tumor cells to proliferate at a rate faster than the normal cells. Since Agrobacterium exploits plant cells by inducing them to produce compounds that are of specific use only to itself and does this by way of genetic engineering, this process is referred to as `genetic colonisation'.

Mechanism of T-DNA transfer

While cells of agrobacterium are necessary for Ti plasmid transfer to plant cells. The mechanism involve chemotaxis of the bacterial cell to the plant cell and firm attachment of the bacterial cell to the plant cell. Both these events are influenced by genes on the bacterial cell chromosome. The T-DNA portion of the Ti plasmid is selectively processed into T-DNA transfer intermediates and transferred to a plant cell where it becomes integrated into the plant genome.

The major component of the Ti plasmid involved in the T-DNA transfer process are : T-DNA borders and Ti plasmid virulence region.

T-DNA border

A set of 24 bp, direct repeat sequences flank the T-DNA region. Since the DNA sequences between the 24 bp repeats are invariably transferred to the plant cells, these sequences are referred to as T-DNA borders. The left repeat is termed the left border and the right repeat is termed the right border. The right border is more critical for T-DNA transfer and tumorigenesis.

Ti plasmid virulence (vir) genes

A 40 kbp region of the Ti plasmid encompasses about 24 vir genes that code for a large number of proteins that function in `trains' and mobilize T-DNA into plant cells. All the `vir' genes are organized into 8 operons termed vir A to Vir H, which are coordinately expressed and thus constitute a vir regulation. VirA, vir B, vir G, vir D and vir E are essential for T-DNA transfer to all plants while vir C, vir E and vir H are dispensible in a number of plants.

Agrobacterium Ti plasmid-based vectors

The ability of agrobacterium to introduce genes into plants has been successfully exploited to develop Ti plasmid-based vectors for introducing desirable foreign genes into plants. Two types of Ti plasmid vectors, namely, (i) cointegrate vectors and (ii) binary vectors, are currently used in plant transformations.

Cointegrate vectors

A derivative of the nopalins – coding Ti plasmid pTiC58 was constructed by retaining the left and right border regions but deleting the entire interveining DNA except for the nopaline synthase gene. In this process the T-DNA genes for hormone biosynthesis were removed (the Ti plasmid was `disarmed'). In place of the deleted DNA a pBR322 (bacterial plasmid) sequence was placed. The plasmid thus formed, called `receptor plasmid', has all the structures of the T-DNA necessary to transfer to plant cells, but it lacks a selection marker needed in plant formation.

The advantage of cointegrate vectors is that the foreign genes are easily cloned in the relatively small intermediate plasmid which has a number of single-restriction sites. After performing all the cloning in E.coli the intermediate plasmid can be mobilized into agrobacterium and placed between the T-DNA borders, for transfer to plant cells.

Fig.

Binary vector

Studies on the T-DNA transfer mechanism revealed that the T-DNA region between the T-DNA borders could be transferred to plants even if the T-DNA is not physically lined to the Ti plasmid virulence region. In other words, vir genes could mobilize T-DNA in trans. This finding led to the development of more convenient Ti plasmid vectors called `binary vectors'. A binary vector system consists of an agrobacterium strain with a disarmed Ti plasmid called vir helper plasmid (in which the entire T-DNA region inclusive of borders is deleted but the vir gene is present) and a binary vector with T-DNA that replicate in E. coli and agrobacterium.

A typical binary vector has the following sequences: left and right border repeats that delimit the T-DNA region of the binary vector; a plant transformation marker such as npt III gene that confers kanamycin resistance in transformed plant cells; a multiple cloning site for introducing desirable foreign genes; an E.coli origin of replication such as Cole I for replication in E.coli; bacterial resistance marker such as tetracycline resistance gene for selecting the binary-vector clones in E.coli and agrobacterium; a brood host-range origin of replication such as RK2 that allows the replication of the binary vector is agrobacterium, and an ori T sequence for

conjugate mobilization of the binary vector from E.coli to agrobacterium.

Fig.

Vir helper plasmid is a non- oncogenic derivative of Ti plasmid in which the entire T-DNA, inclusive of T-DNA borders, is deleted.

A foreign gene of interest is inserted into the multiple cloning site of the binary vector, thus placing it between the right and left border repeats, and cloned in E.coli. The binary vector is then mobilized from E.coli to agrobacterium (vir helper strain) by triparental mating. The vir gene-encoded proteins act on the T-DNA borders in trans and mobilize the T-DNA of the binary vector into the plant cells.

b. Direct transformation of plants

Most dicot plants can be efficiently transformed by agrobacterium. However, many important dicot crops such as pulses and the majority of monocot crops, particularly cereals, are highly resistant to agrobacterium-mediated transformation, and so direct transformation methods have been developed to transform such plants. Ex: $CaCl_2$ and PEG mediated transformation,

liposome-mediated transformation; electroporation; and tansformation using microprojectiles, Electroporation and microprojectils bombardment, methods have been found to be most effective in direct transformation.

Electroporation

Electroporation is a process whereby electrical pulses of high field strength are used to reversibly permeabilise cell membranes to facilitate uptake of large molecules, including DNA. It has been successfully used for transforming plants in which efficient regeneration of plants from protoplasts is possible. The advantages of electroporation are its convenience, low toxicity and equal efficiency in dicots and monocots. The disadvantage is that it is effective only in protoplasts, from which intact plantlets need to be regenerated; this is technically extremely difficult in many crop plant species.

Particle-gun bombardments

Bombardment of DNA-coated microprojectiles using particle guns allows the transformation of intact cells of embryos, shoot apices and other tissues. The technology basically involves coating tiny tungrton or glas spheres (microprojectiles) with DNA (gene(s) to be introduced) and spreading the particles on the surface of a mobile plate or plastic or nylon bullet called the `macroprojectile'.

Then under partial vacuum, the macroprojectile is fired against a retaining plate or mesh either by an explosive discharge or by using shock waves initiated by a high voltage electric discharge. The macroprojectiles are retained by the retaining plate or mesh, while the micro projectiles, normally 1-3μm diameter, pass through due to their small size and higher density and penetrate the target plant tissue.

The major advantage of micro projectile bombardement is its ability to introduce genes into intact tissues there by obviating the need for protoplast isolation and plantlet regeneration from protoplasts. The limitations of this method are the high cost of the equipment, the high level of variations in transformation efficiencies and the low frequency of achieving stable transformation.

Fig.

8.1.3.4. Animal biotechnology

1. Introduction

All living production systems have four intrinsic limitations: reproduction, genetics health and nutrition. Animal production

has to deal with constraints that donot apply to the plant production. The major practical difference between plant and animal production systems is in their reproduction. Plants produce large numbers of female gametes (ova) which develop into propagates ideal for farmers (i.e, seeds), where as female mammals just produce a few gametes (ova or egg). In addition crop plants complete their life cycle within one year where as livestocks require 3-4 years from conception to maturity.

Manipulating genetics, too is much easier with plants. In many cases we can produce fertile progeny from matings between different plant species so that genes from a wild relative can be introduced into a cultivated crop plant, and genes can be (and are) shuffled between different cultivated species by suitable crosses and back selection. The same cannot be done with mammals. The infinitely great number of individuals of plant species also provides a much greater source of genetic variation to use in breeding selection.

The most important role biotechnology in animal production is to make the reproduction and genetics of livestock more that of plants-to increase reproductive rate by increasing male and female fecundity, and to identify and use genes that determine desirable traits.

2. Manipulation of reproduction in animals

Reproduction has two critical roles in production systems: it must be at a rate that ensures replenishment of stock is greater than its use, and reproduction has been, until the advent of genetic engineering, the only means by which the genetic quality of stock can be improved. The goals of reproductive technologies are to increase the number of progeny that a particular selected male and a particular female can produce.

a. Artificial insemination (AI)

Technologies affecting male reproduction aim to increase the number of females that can be inseminated by a male by decreasing the amount of semen needed to produce a pregnancy and increasing the length of time for which semen can be stored.

- The semen ejaculate is collected, diluted (extended), and examined under microscope to determine the no.of and motility of sperm. The extended semen is dispensed into small plastic straws that are plugged at each end prolonged storage of sperm in liquid nitrogen allows a single male to inseminate many thousands of females, even if the male is in a different country or has dies.

- The breeder must then take the place of the sire by inseminateny the female artificially. Since fertilization and pregnancy can result only if insemination occurs when the females ovulates and is receptive, the breeder must replace nature further by ensuring that females in a herd ovulate together at a conveineint time. In practice, it is never possible to achieve total synchrony of oestrous but some 80% females usually respond as expected.

b. Embyo transfer

Where AI is an obvious way of increasing the number of progeny that a selected male can sire, the solution to the problem of limited female fecundity is not so simple. We must change the animal's function, increasing the no. of nature eggs that a selected female produces. After fertilization those eggs must be implanted into less important foster mothers (recipients), one for each egg, since each ruminant female ideally carries just one pregnancy. The total cost of tis embryo transfer procedure is very high, including drugs, labour and recipient females. Clearly, it should be used only for animals of high genetic or economic value.

The first pregnancy by embryo transfer was achieved using rabbits in 1890. By the 1930s it had been used on sheep and goats, while methods were developed for pigs and cattle by the early 1950s.

c. Invitro fertilization

Transfer of embryos into prepared recipients is no more difficult or expensive than artificial insemination. The only significant difference is that more care is needed in thawing frozen embryos than frozen sperm. Yet the genetic advantages of embryo transfer are far greater than AI since complete diploid cells are transferred, not just a haploid gamete containing half a genome. The embryo develops in a foster mother that can be a poor quality animal since she makes no genetic contribution to the offspring she carries. The produces is not restricted to breeding from his existing high quality females.

Embryo transfer is not used widely because of its cost, technical difficulty and the limited supply of embryos available from superovulated donors. These limitations would be removed if we could fertilise in vitro the thousands of oocytes that are present in a females ovaries. In doing so, domestic animals would indeed become similar to plants in the fecundity of both sexes. Breeding programme could select for female genetics as easily as for male

genetics. The genetic influence of a male would be increased further since the amount of semen required for fertilization invitro is a fraction of the amount needed for AI.

IVF is generally quite successful, resulting in about 70-80% of fertilized eggs. The practical difficulties arise in sourcing eggs for fertilization and in the development of fertilized zygotes to term.

d. Animal cloning

When complete animals are obtained from somatic cells of animal, it is called animal cloning. Cloning is routine in plants but in case of animals only a limited success had been achieved so far. Earlier, nuclei from a tadpole were transplanted into the cytoplasm of an enucleated fertilized frog egg, and normal frogs were obtained. But early in 1997, British scientists announced successful cloning of sheep by transferring the nucleus from an udder cell of an adult sheep into the cytoplasm of an enucleated fertilized egg. The egg was then transplanted into the uterus of a surrogate mother where it developed like a normal zygote into a normal lamb which has now grown into a normal adult sheep.

Cloning is, in many situations, highly desirable since this allows indefinite multiplication of an elite desirable genotype without the risk of segregation and recombination during meioses, which must precede sexual reproduction. Obviously, the technique

holds a great promise in genetic research, especially in understanding aging and curing genetic disease. The technique needs to be refined and expanded to other animals.

8.1.3.5. Immunotechnology

1. Introduction

The immune system can be thought of as the body's third line of defence against the entry of foreign substances. The first line of defence is formed by physical barriers such as the skin while the second consists of a variety of non-specific mechanisms that include low pH conditions, ciliary motions, sneezing, and secretion of mucus. These mechanisms are non-specific in that they seek only to prevent the entry of a pathogen into the body. Together they constitute what is known as innate immunity. The immune system, the 3rd line of defence, is specific both in its acquired recognition of targets and its action.

Non specific, innate immunity is not inducible by foreign antigens specific immune responses, on the otherhand, are acquired ; they are induced by foreign stimuli and generally react only against the stimulating molecule: the antigen or immunogen. An antigen or immunogen is defined as any molecule that induces a specific immune response. Bacteria, viruses, yeasts, parasites and filamentous fungi all exhibit different antigens. Tumar cells

are recognized as `non-self' by the immune system, because they often express antigens that are not present on the surface of normal cells.

The cells of immune system are called lymphocytes that are found in the blood and in lymphoid tissues such as the thymus, spleen and lymph nodes. Lymphocytes are broadly classified into T-cells and B-cells, depending on the site in the body in which they mature, their function and their profile of surface antigens. T- and B-cells proliferate in response to a primary antigenic stimulus within just a few days of the original encounter with antigen in a lymphoid organ, effector T-cells are generated and B-cells start producing antibodies.

Antigen + T-cells $\longrightarrow$ Teffector cells

(T_c, T_H cells)

Antigen + B-cells $\longrightarrow$ Antibody molecules

T_c : Cytotoxic T-cells

T_H : Helper T-cells

2. Monoclonal antibody technology

Molecular genetic studies on antibody synthesis by B-cells indicate that as a group they have the potential capacity to respond

to atleast ten billions different antibody specificities, which would be sufficient to recognize any antigen. The clonal selection theory recognizes that each B-cell is able to produce an antibody response only a single antigenic determinants. A typical antigen is a complex molecule with several antigenic determinants which therefore induces in an animal a polyclonal response, in which antibodies of differing specificity, affinity by a number of different lymphocyte clones. The response differs from animal to animal, has a finite life span, and requires induction by the presence of the antigen inn the host.

In the disease called multiple myeloma, which is a clonal tumor of B-cells, all malignant cells generally produce one type of Ig (of a single specifity). Production of Ig by myeloma cells, which have an infinite life span, does not require induction by the presence of an antigen. Most of the myelomas are secretory, but some non-secretary myeloma cell lines are also available, especially in rodents.

The antibody producing cell, the B-cell, has a definite life span, but it can be immortalized artificially by fusing it with a myeloma cell which, by virtue of its cancerous nature, can multiply indefinitely in vitro. The hybridization of these two cell types was achieved for the first time by Georges Kohler and Cesar Milstein inn 1975. The basic procedure involves the fusion of B-cells from an

immunized animal with mycloma cells which donot secrete immunoglobulins and which lack functional hypoxanthine guanine phosphoribosyl transferase (HGPRT, a key enzyme in the salavage pathway of DNA synthesis). Fusion of the two cell types to produce a hybridoma is carried out by stimulating fusion of the cell membranes using polyethylene glycol. Hybrid cells are selected from the myeloma cells in a medium containing aminopterin (which blocks the denovo pathway of DNA synthesis) thymidine and hypoxanthine.

Fig.

In this technique B-lymphocytes are immortalized randomly. Whether they secrete antibody against the required epitope or another epitope in the immunogen used to vaccinate the animal from which the B-cells were taken. There is thus a need to segregate clones of hybridoma which produce antibody against the required epitope. This can be achieved by using a limiting dilution technique in which the cell suspenson is diluted in tissue culture medium so that only one cell occurs in aliquots added to tissue culture medium so that only one cell occurs in aliquots added to

tissue culture dishes. After growth, the contents of each dish are screened for the presence of the required antibody. By repeating this procedure a population (clone) of cells producing antibody of required specificity (reacting against one epitope only) can obtained. The antibody secreted by a hybridoma clone is referred to as monoclonal antibody (MAb).

Hybriodmas can be grown up to produce MAbs in the laboratory on a small scale, or on a larger scale in a production facility in air-lift fermenters or other bioreactors. Alternatively, mouse hybridoma clones can be grown as ascites after injection into the peritoneal cavity of the same strain of mice from which the progenitor cells were obtained. This procedure produces high yield of antibody without the need for sophisticated tissue culture facilities. However, the mice need to be maintained in a germ free environment so that they are not producing antibody against other antigens.

Fig.

3. Human monoclonal antibodies

While mouse monoclonal antibodies have revolutionrized biology in general and immunology in particular, there are limitations when it comes to using them for injection into humans. To circumvent this problem attempts have been made to produce monoclonals from human cells. Despite this difficulty that this technology poses, the potential of this treatment to human therapeutic and prophylactic use has stimulated a great deal of research and development in this area.

To generate human MAbs the following strategies have been used:

a. Human-human hybrids

Attempts have been made to fuse sensitized human B-cells (from an individual exposed, naturally or by vaccination, to an epitope) with human plastocytoma or lymphoblastoid cell lines (immortal cells from tumors of lymphocytic cells).

Progress in the development of such human-human hybridomas has been slow owing to the lack of suitable non-antibody secreting human plasmacytomas or lymphoblastoid cell lines with which to fuse human B-cells. However, the first human MAb against an antigen has been produced by fusing B-lymphocytes with a human plasma cell line.

b. Interspecific hybrids

Attempts have been made to fuse human B-cells with non-secretary mouse or rat myeloma cells (the antibody produced by such interspecific hybridomas would be of human type as its production is coded for by the human B-cells). However, such interspecific hybrid cells are found preferentially to eliminate human chromosomes. Human B lymphocytes isolated from peripheral blood, bonemarrow, spleen, tonsil or lymphnode have been used for fusion. The number of B-cells secreting specific antibody is quite small in peripheral blood, but their number can be increased by stimulating growth of these cells in vitro with pokeweed mitogen or antigen or a combination of both.

c. EBV-transformation

The Epstein-Barr Virus (EBV) transforms cells and results in uncontrolled growth of the transformed cells. EBV transformation of sensitized B-cells has been used as a means of producing an immortalized line of B-cells. However, EBV-transformed cell lines often secrete low amounts of antibody and are unstable.

d. Humanising mouse monoclonals by means of genetic engineering

Using genetic engineering it is possible to produce chimeric antibodies. The term `chimeric' is derived from Greek mythology, in which the chimera was a monster with the head of a lion, the

body of a goat and the tail of a dragon. A chimeric molecule (such as monoclonal antibody) is thus one in which diverse segments are coded for by genes which are not normally associated with each other. These genes may be inter specific or intraspecific.

4. Cytokines

Cells of the immune system are also communicated by soluble messengers molecules that carry signals from one cell to another. These soluble messengers are called cytokines, and they play crucial role in initiation and maintenance of immune response. Many of the messenger proteins are growth factors for other cells, some are stimulators of activity in effector cells, and some are both growth factors and cell-stimulators. Cytokines promote activities aimed at (i) the elimination of invading microorganisms and parasites, and (ii) the repair of damaged tissues. They can act either as short-range messengers between cells, or as long-range messengers by circulating in the blood and affecting cells at remote sites, just as hormones do. Several cytokinins are named interleukins, abbreviated to IL.

IL-1	Monocytes, Macrophages	Stimulates T-cells: inflammatory, tumoricidal
IL-2	T-helper cells	Proliferationn & differentiation of T-

		& B-cells; activates NK cells
IL-3	T-helper cells	Stimulates growth of stem cells & mast cells
IL-4	T-helper cells	Stimulates growth of T & B cells and mast cells; stimulates IgE production
IL-5	T-helper cells	Eosinophil & B-cell growth and differentiation factor
IL-12	Trasformed B-cells	Stimulates NK cells
IL-7	Bonemarrow stromal cells	Production of B-cell precursors in bone marrow; induces proliferaction of thymocytes.
IL-8	Mononuclear leukocytes	Neutrophil chemotactic factor

a. Productionn of cytokines innvitro

1. Stimulated T-cells : Lymphocytes isolated from peripheral blood (in humans) or from spleen (in rodents) and cultured in

appropriate growth medium, when stimulated with mitogen or with specific antigen to undergo mitosis, become activated and release cytokines into the culture medium. The stimulation can be followed by cytokine purification and identification of cytokines released.

2. T-cell hybridomas: Mitogen-activated or antigen-activated T-cells can be cloned or fused with appropriate cells to form hybridomas. The advantage of these hybridomas is that the cytokines produced from them in culture medium are more clearly defined compared to the material produced by bulk culturing of blood cells.

3. Constitutive or inducible cell lines: There are established cell lines (leukarmic cell lines for instance) that contitutively produce certain cytokines in culture medium, or can be induced to do so.

4. Generally engineered cells containing genes coding for cytokines: Large amounts of pure cytokines can be produced by recombinant DNA methodologies, and is accomplished by the expression of the appropriate cytokine gene in an appropriate vector.

8.1.3.6. Environmental biotechnology

1. Introduction

Environment consists of the sum of all the factors outside an organism. It consists of both biotic abiotic factors. Thus for a given organism, other organisms constitute a component of the environment, while the organism itself becomes a factor of the environment of other organisms.

Man obtains from the environment his life support systems which include oxygen, energy, water, raw materials, nutrients and place to live. The 3 abiotic components of environment viz., air, water and soil contribute in various ways to the fulfillment of human needs. In turn, the various activities of man eg, domestic, agricultural, manufacture, transport accidents etc... generate wastes and pollutants which contaminate air, water and soil. In response man has strived to minimize the damaging effects of his activities on the environment by developing.

- technologies to clean up the pollution generated by other technologies (end of the pipe technologies).

- Production technologies which are cleaner and generates less pollution (front of the pipe technologies).

Both these approaches minimizes damage to the environment. However, the term environmental biotechnology is

applied only to the `end of the pipe' technologies using biological agents i.e., biotechnological approaches applied to the management of environmental problems.

Environmental biotechnology can be considered as the discipline that studies the application of biological systems and processes in waste treatment and management. Microorganisms found in soil & water will attempt to utilize any organic substances encountered as sources of energy and carbon by enzymically breaking them down into simple molecules that can be absorbed and utilized. Under suitable environmental conditions all natural organic compounds should be degraded (biodegradation). Organic chemicals that cannot easily be degraded by microorganisms, or are indeed totally resistant to attack lignin, for example, are termed recalcitrant. Xenobiotics are synthetic compounds not formed by natural biosynthetic processes and, in many casers, can be recalritrant. A xenobiotic compound is, therefore a foreign substance in our ecosystem and may often have toxic effects.

2. Waste-water and sewage treatment

Growth in human populations has generally been matched by a concomitant formation of a wider range of waste products, many of which cause serious environmental pollution if they are allowed to accumulate in the ecosystem. In rural communities recycling of

human, animal and vegetable wastes has been practised for centuries, providing in many cases valuable fertilizers or fuel. However, it was also a source of disease to humans and animals by residual pathogenicity of enteric (intestinal) bacteria. In urban communities, where most of the deleterious wastes accumulate, efficient waste collection and specific treatment process have been developed because it is impractical to discharge high volumes of waste into natural land and waters. The introduction of these practices in the last century was one of the main reasons for the spectacular improvement in health and well-being in the developed countries.

The biological disposal organic wastes is achieved in many ways throughout the world. The complex but highly successful system involves a series of three stages of primary and secondary processing followed by microbial digestion. An optional tertiary stage involving chemical precipitation may be included. The primary activity is to remove coarse particles and solubles, leaving the dissolved organic materials to be degraded or oxidized by microorganisms in highly aerated, open bioreactor. The microorganisms multiply and form a biomass or sludge that can either be removed and dumped, or passed to an anaerobic digester (bioreactor) that will reduce the volume of solids, the odour and the number of pathogenic micro organisms.

Another important means of degrading dilute organic liquid wastes is the percolating or trickling filter bioreactor. In this system the liquid flows over a series of surfaces, which may be stones, gravel, plastic sheets, etc., on which attached microbes remove organic matter for essential growth. Excessive microbial growth can be a problem, creating blockages and loss of biological activity. Such techniques are widely used in water purification systems.

A biotechnological innovation in waste-water treatment is the deep-shaft fermentation method. The deep shaft is, in fact, a hole in the ground (upto 150m in depth), divided allow the cycling and mixing of waste-water, air and micro-organisms. It is most economical in land use and power, and produces much less sludge than do conventional systems.

Fig.

3. Land fill technologies

Solid wastes account for an increasing poportion of the waste streams generated by modern urbon socities. While part of this material will be made of glass, plastics, etc., a considerable

proportion will be decomposable solid organic material such as paper, food wastes, sewage wastes, wastes from large scale poultry and pigfarms, and, in the USA in particular, cattle feed wastes.

In large urbanized communities, the essential disposal of such wastes is problematic and one well-used system is by low cost anacrobic land fill technology. In this procedure solid wastes are deposited in low lying, low value sites and each day's waste deposit is compressed and covered by a layer of soil. The complete filling of such sites and each day's waste deposit is compressed and covered by a layer of soil. The complete filling of such sites can take months or years depending on the size of site and flow of wastes.

Improperly prepared and operated landfill sites may result in toxic heavy metals, hazardous pollutants and products of anaerobic decomposition seeping from the site into underground aquifers and subsequently polluting urban water supplies. Properly constructed and sealed landfill sites can be used to generate methane gas for commercial use. Much efforts is now made to use strong, impermeable liners to avoid leachates damaging surrounding land and water courses.

Fig.

4. Composting

Composting is an aerobic microbially driven process that converts solid organic wastes into a stable, sanitary, humus-like material that has been considerably reduced in bulk and can be safely returned to the environment. It is, in effect, a low moisture, solid substrate fermentation process. To be totally effective it should use as substrates only readily decomposable solid organic waste. In large-scale operations using largely domestic solid organic wastes, the final product is mostly used for soil improvement but in more specialized operations using specific organic raw substrates (straw, animal manures, etc.) the final product becomes the substrate for the world-wide commercial production of mushroom *Agaricus bisporus*.

Composting has only recently become a serious waste management technology and both theoretical and practical development of the technology is still in its infancy. The primary aim of a composting operation is to obtain, in a limited time within a limited compost, a final compost with a desired product quality. A composting plant must function under environmentally safe conditions.

Fig.

4. Bioremediation

The contamination of soil normally results from a range of activities related to our industrial society. Contaminated land is viewed as land that contains substances that when present in sufficient quantities or concentrations can probably cause harm to human beings, directly or indirectly, and to the environment in general. Many xenobiotic industrially derived compounds can show high levels of recalcitrance and, while in many cases only small concentrations reach the environment, they can be subject to biomagnification. Biomagnification in essence implies an increase in the concentration of a chemical substance, eg, the insecticide DDT, as the substance is passed through the food chain.

The basic principles of bioremediation / bioreclamation / biorestoration / biotreatment are superficially sample : optimize the environmental conditions so that microbial biodegradation can occur rapidly and as completely as possible. Microbes that are naturally present in soil and water environments are potential candidates for the biological transformation of xenobiotic compounds that are introduced into the ecosystem.

Some companies now market microbial inocula that are claimed to increase significantly the rate of biodegradation of oil pollutants. Organisms that can degrade complex organic

molecules such as lignin have a wide range of enzymic activities capable of degrading many of the most dangerous industrial pollutants.

A further possibility in bioremediation is to genetically engineer microorganisms to be able to degrade organic pollutants molecules that at present they are unable to attack. While this has been achieved in some cases there are considerable technical problems including genetic stability and survival of the `new' microbe in a hostile environment. Further more, there are legislative, ethical and perceptional problems concerned with their release into environments such as sewage concerned with their release into environments such as sewage systems, soils and oceans. To date no genetically engineered microorganism has left the laboratory and been tested in the field.

5. Microbes and the geological environment

Microbes are increasingly recognized as important catalytic agents in certain geological processes, e.g; mineral formation, mineral degradation, sedimentation, weathering and geochemcial cycling. One of the most detrimental examples of microbial involvement with minerals occurs in the production of acid mine waters. In many mining communities the huge volumes of sulphuric acid produced have created pollution on an

unprecedented scale. Other examples of detrimental effects of microbes include the microbal weathering of building stone such as limestone, leading to defacement or structural changes.

In contrast to these harmful effects, microbes are increasingly used beneficially to extract commercially important elements by solubilisation (bioleaching). For example, metals such as cobalt, copper, zinc, lead or uranium can be more easily separated from low-grade ores using microbial agents-mining with microbes.

The biological reactions in extractive metal leaching are usually concerned with the oxidation of mineral sulphides. Many bacteria, fungi, yeasts, algae and even protozoa are able to carry out these specific reactions. Many minerals exist in close association with other substances such as sulphur, e.g., iron sulphide, which must be oxidized to free the valuable metal. A widely used bacterium thiobacillus ferroxidans can oxidize both sulphur and iron, the sulphur in the ore wastes being converted by the bacteria to sulphuric acid. Simultaneously, the oxidation of iron sulphide to iron sulphate is enhanced.

Large-scale bioleaching of uranium ores is widely practiced in Canada, India, the U.S.A and the countries of the former Soviet Union. By means of bacterial leaching it is possible to recover

uranium from low-grade are (0.01-0.05% U_3O_8) which would be uneconomic by any other known process.

Summary

Biotechnology have many interdisciplinary fields such as, fermentation technology which deals about the types of fermentation processes and steps involved in upstream as well as down stream processing. The plant biotechnology deals with the production of transgenic plants and plant tissue culture methods and their applications in agriculture. The animal biotechnology deals with the artificial insemination, embryo transfer technologies and cloning techniques to produce animals. Immunotechnology deals with the production of monoclonal antibodies and development of vaccines against various diseases.

Questions

1) Discuss the various fields of biotechnology and mention their significance
2) Write the various steps involved in fermentation technology

Reference Books

P.K.Gupta, Elements of Biotechnology, Rastogi Publications

Author

N.Srinivasa Reddy

Centre for Biotechnology

Acharya Nagarjuna University

Lesson 8.1.4

Importance and Applications of Biotechnology

Objective

Introduction

8.1.4.1 Biofertilizers

1. Rhizobium spp.
2. Azotobacler and azospirillum
3. Blue-green algae and Azolla
4. Large-scale production

8.1.4.2 Single cell protein

1. Production of SCP
2. Microorganisms
3. Substrates
4. Nutritional and safety evaluations
5. Advantages of SCP

8.1.4.3 Monoclonal antibodies

1. In diagnosis
    a. Biochemical analysis
    b. Infectious disease
    c. Cancer
2. In treatment

a. Treating bacterial blood infections

b. Preventing rejection of transplanted organs

8.1.4.4 Vaccines

1. Birth control vaccines

8.1.4.5 Seed protein quality

1. Introduction of an appropriate transgene
2. Modification of endogenous genes

8.1.4.6 Drug designing

8.1.4.7 Recovery of metals

8.1.4.8 Desulphurisation of coals

8.1.4.9 Lactase in dairy industry

8.1.4.10 Enzymes in brewing industry

8.1.4.11 Protein engineering

1. Adding disulfide bonds
2. Reducing the number of free sulfhydryl residues
3. Modifying enzymes specificity

8.1.4.12 Biochemical products

1. Hiruolin
2. Polyhydroxybutyrate

Objective :

In this chapter the importance and applications of biotechnology in various fields were discussed.

Introduction :

The advancements in the field of biotechnology have contributed a lot for the development of various fields. Use of biofertilizers and biopesticides in the field of agriculture have improved the crop yields and reduced the environmental pollution risks. Not only agriculture, in medicine, in industry and in our daily life biotechnology has contributed enormously for the welfare of human kind.

8.1.4.1. Biofertilizers

Micro organisms employed to enhance availability of nutrients, viz, nitrogen (by fixing atmospheric N_2) and phosphorus (by solubilizing soil phosphorus), to the crops are called biofertilizers. The various microorganisms having realized potential applications as biofertilizers are: bacteria (Rhizobium spp, Azospirillum, Azotobacter), fungi (mycorrhizae like Glomus), blue-

green algae or cyanobacteria (Anabena, Nostoc, etc.,) and Azolla (a fern containing symbiotic anabena azollae).

1. Rhizobium species

These are gram negative soil bacteria capable of forming root nodules in most leguminous plants and some non-leguminous plants. In some cases, stem nodules are also produced. Rhizobium is divided into several species chiefly on the basis of the legume species they are able to nodulate.

R. leguminosarum – nodulates pea

R. Lupini - nodulates lupins

R. Melilotti - nodulates melilotus sp.

R. Phaseoli - nodulates phaseolus sp.

R.trifolii - nodulates trifolium sp.

Brady rhizobium - nodulates cow pea.

Rhizobium cells contain genes for nitrogen fixation (nif genes) on a mega plasmid. The bacteria enter the roots through root hairs, the interaction being highly specific and progressing through several steps; it ultimately results in nodule formation. Many genes of Rhizobium as well the host legume are involved in the

process. In side the nodule many bacterial cells change into non dividing bacteroids which produce nitrogenase, the enzyme which reduces atmospheric nitrogen into ammonia. Nitrogenase is highly sensitive to O_2; It is protected from O_2 by the pink pigment leghaemoglobin, which binds to O_2, produced by the legume and present in the nodule.

Different strains of Rhizobium species differ in their ability to fix nitrogen: this trait is also affected by the genes of host legumes as well. Therefore, extensive screening for efficient N_2 fixers is undertaken on the basis of N_2-fixation occurring in association with the host legumes. Some mutants of Rhizobium are more efficient N_2- fixers than the wild type eg., nitrate reductase deficient mutants. Further hap^+ strains of Rhizobium are more efficient since they are able to recycle the N_2 produced by bacterial cells, which otherwise is released as gas.

Fig.

Recycling of the hydrogen gas that is produced as a byproduct of nitrogen fixation. Hydrogen is generated by nitrogenase at the expense of ATP but, by using this pathway, the hydrogen can be recaptured by hydrogenase.

The estimated N_2 fixed by rhizobium species range from 50-150 kg/ha or even more, especially in case of clovers. Field trials in India suggest a 10-15% yield increase in inoculated pigeon pea and chickpea over the un inoculated controls. Rhizobium sp. Are attaked by viruses called rhizobiophages and the inoculated strains have to complete with the native rhizobia. It is therefore important that the selected strain should be toluant to elevated temperatures, have long shelf life when the inoculum is prepared and should out-compete the native rhizobia present in soil.

2. Azotobacter and Azospirillum

Azotobacter uses the organic matter present in soil to fix nitrogen asymbiotically, it is capable of fixing upto 30 kg nitrogen / ha/yr. Azospirillum species occur in association with the roots of many plants of the grass family, eg., jowar, wheat, bajra etc. These bacteria are capable of fixing over 30 kg N/ha/yr. Field inoculation of crops with Azotobacter or Azospirillum is estimated to save 15-25 kg N/ha.

3. Blue-green Algae and Azolla

Blue-green algae (cyanobacteria) are photosynthetic, prokaryotic organisms which fix N_2 asymbiotically; some cyanobacteria are known to form symbiotic associations, eg., Azolla (afern) – Anabena azollae. Examples of cyanobacteria are

Anabaena, Nostoc, Plectonema et. Usually, composite cultures containing 2 or more genera are used for field inoculation since they are often superior to single strain inoculations cyanobacteria produce nitrogenase and N_2-fixation occurs in specialized structures called heterocysts in which the nif region becomes reorganized (this is essential for N_2 fixation. In addition, heterocysts act as O_2-proof compartments which produce nitrogenase from O_2 inactivation.

Azolla owes its N_2-fixing capabilities to the symbiont Anabaena azollae. Azolla is widely used in Vietnam as biofertilizer for rice. It has not become popular in India due to its sensitivity to high temperature (during summer), necessity for assured and adequate water supply and susceptibility to insects and diseases.

Cyanobacteria in addition to N_2-fixation, accumulate biomass which improves the physical properties of soil, produce growth promoting substances and are useful in reclamation of alkaline soils. They are used for rice, the inoculum being introduced in the field about 10 days ofter transplantation.

4. Large-scale production

Rhizobium inoculum is produced in shake flasks or fermenters using appropriate growth medium. After a culture attains the desired cell density, contamination is checked before it

is mixed with an appropriate inert and sterile carrier, eg., peat, which is subsequently used as inoculum for coating the seeds. Alternative methods of Rhizobium inoculation have also been developed.

Blue- green algae are multiplied in troughs or small tanks. The algal biomass is harvested, dried and stored in gunny bags. The algal flasks are used at 10kg/ha for inoculation in rice fields.

8.1.4.2. Single cell protein

A major problem facing the world, in particular the developing nations, is the explosive rate of population growth. The number of humans in the world now totals 5.7 billion, is increasing by approximately 94 million annually and could well exceed 10 billion by 2050 if left uncontrolled. Conventional agriculture may well be unable to supply sufficient food – in particular, protein – to satisfy such demands. The Food and Agriculture Organization (FAO) already predicts a widening of the protein gap between developed and developing countries.

The search for sources of protein is relextlessly pursued. New agricultural practices are widespread, high protein cereals have been developed, the cultivation of soya beans and ground nuts is ever-expanding, protein may be extracted from liquid wastes by ultrafiltration, and now the use of microbes as protein

producers has gained wide experimental success. This field of study has become known as single cell protein or SCP production, referring to the fact that most of the micro organisms used as producers grow as single or filamentous individuals rather than as complex multicellular organisms such as plants or animals.

Microorganisms produce protein much more efficiently than any farm animal. The protein-producing capacities of a 250 kg cow and 250 g of put on 200 g of protein per day, the microbes, in theory, could produce 25 tonnes in the same time under ideal growth conditions.

SCP may be used directly as human food supplement, or else it may be used in animal feed to atleast partially replace the currently used protein-rich soyabean meal and fish proteins, and even cereals, which can be diverted for human consumption.

1. Production of SCP

The process of SCP production from any micro-organism or substrate would have the following basic steps.

1. Provision of a carbon source; it may need physical and / or chemical pretreatments.

2. Addition, to the carbon source, of sources of nitrogen, phosphorus and other nutrients needed to support optimal growth of the selected microorganism.

3. Prevention of contamination by maintaining sterile or hygienic conditions. The medium components may be heated or sterilized by filtration and fermentation equipments may be sterilized.

4. The selected micro organism is inoculated in a pure state.

5. Scp processes are highly aerobic (except those using algae) Therefore, adequate aeration must be provided. In addition, cooling is necessary as considerable heat is generated.

6. The microbial biomass is recovered from the medium.

7. Processing of the biomass for enhancing its usefulness and / or storability.

Biomass production is ordinarly carried out in the continuous mode to maximize yields and economic returns.

2. Microorganisms

Algae, fungi, yeast and bacteria are used for scp production. The microorganisms used for SCP production must be

- non pathogenic to plants, animals and man

- of good nutritional value
- easily and cheaply produced on scale
- toxin-free
- fast-growing and
- easy to separate from medium and to dry etc..

Algae

Members of the genera chlorella, scenedesmus and spirulina are generally grown in ponds / tanks. They use CO_2 and sunlight as substrate which are without any cost. Algal SCP has about 60% crude protein, which is generally good in aminoacid composition except for some deficiency in sulphur-containing amino acid. They are suitable for animal feed as protein-rich supplement. The disadvantage of algal SCP are: (i) rich chlorophyll content which is not suitable for human use, serious problems when chlorella and Scenedesmus are used in human diet (spirulina is more suited for human use), low cell density, e.g 1-2g dry weight / L, serious risk of contamination, and costly recovery methods for unicellular algae (spirulina harvested by filtration or simply by skimming).

Filamentous Fungi

Filamentous fungi have been used to produce SCP mainly from polysaccharide hydrolysates eg: starch hydrolysates, sulphite liquor from wood pulp industries etc. These are usually grown as submerged cultures in which they grow as yeast-like cells, in filamentous form or pellet form. They have crude protein content of 50-55%; the protein is low in S containing aminoacids, but otherwise is excellent in aminoacid composition. The recovery of filamentous and pellet forms is rather easy by filtration.

Yeasts

Members of Saccharomyces, candida and Torulopsis have been widely studied for SCP production and those of the first two genera are used for some commercial process using various substrates. The SCP has 55-60% crude protein which has good aminoacid balance except for a deficiency in S-containing aminoacids. It is usually very rich in B-group of vitamins. The SCP is used for both human food and animal feed supplementation. The risk of bacterial contamination is low and recovery is easy (by continuous centrifugation).

Bacteria

A large no. of bacterial species have been evaluated for SCP production – using a wide variety of substrates

- some of these are used for production at commercial scales, e.g., methylophilus methylotrophus using methanol. SCP has very high crude protein (over 80%) of good aminoacid composition, although in some cases a small deficit of S-containing aminoacids is encountered.

Microorganisms have high DNA, RNA content, degradation of which produces uric acid which may accumulate to damaging levels in human since they do not possess uricase activity. It is therefore, necessary that nucleic acids be reduced to acceptably low levels especially in SCP intended for human use. In the rapidly proliferating microbial cells used for SCP, the bulk of nucleic acids are RNAs. The level of RNA in SCP is reduced using one of the following methods.

- activation of endogenous Rnases by, usually, a brief heat treatment, eg., 20 min at 64^{o}C reduces RNA from 10% to 1% of dry weight, in Fusarium graminearum SCP,
- alkaline hydrolysis
- chemical extraction, and
- suitable manipulation of growth and physiology of the microbial cells.

3. Substrates

Gaseous hydrocarbons, liquid hydrocarbons, pure –n-alkanes, Gas oil in combination with ammonia and mineral nutrients, methanol, ethanol, CO_2, molasses, whey, cellulose hydrolysates, starch hydrolysate, industrial effluents, cellulosic wastes etc..... can be used as substrates (i.e, carbon and energy source) for the production of SCP from microbes.

4. Nutritional and safety evaluations

1. The SCP chemical composition must be characterized in terms of protein, aminoacid, nucleic acid, lipid, vitamin etc., contents.

2. Analysis of substrate residues and toxic substances, e.g., heavy metals, mycotoxins, polycyclic hydrocarbons etc. Must be done.

3. Physical properties like density, particle size, texture, colour, storage etc. properties should be determined.

4. Microbiological description, e.g., species, strain, should be provided, and information on contamination be also given.

5. The nutritional value should be evaluated on the target species, and other species should also be included.

6. Possible toxic or carcinogenic compounds must be assayed for. These compounds may have been present in the substrate, may be synthesized by the organism or produced during the processing of SCP.

5. Advantages of SCP

The SCP processes and products offer several advantages.

1. The SCP is rich in high quality protein and is rather poor in fats, which is rather desirable.
2. They can be produced all the year round and are not dependent of the climate (except algal processes).
3. The microbes are very fast growing and produce large quantities of SCP from relatively very small area of land.
4. They use low cost substrates and, some cases, such substrates which are being wasted and causing pollution to the environment.
5. When the substrate used for SCP process is a source of pollution, SCP production helps reduce pollution.

6. Strains having high biomass yields and a desirable aminoacid composition can be easily selected or produced by genetic engineering.

7. Some SCPs are good sources of vitamins, particularly B-group of vitamins, as well, e.g., yeasts and mushrooms.

8. Mushrooms are considered as delicacy in the human diet.

9. At present, SCP appears to be the only feasible approach to bridge the gap between requirement and supply of proteins.

8.1.4.3. Monoclonal antibodies

1. In diagnosis

a. Biochemical analysis

In the last two decades, many varieties of monoclonal antibody have been produced. They have varied applications in medicine, including laboratory diagnosis. Mabs are routinely used in radioimmuno assays (RIA) and enzyme linked immunosorbant assays (ELISA) to measure circulating levels of enzymes, hormones and tissue and cell products. They are also used for blood group and typing, and for HLA matching in organ transplantation. A no. of diagnostic kits, using Mabs, are now commercially available.

Immuno assays rely on the quantitative binding of Mab to epitope, and the quantitation of bound Mab by enzyme activity (in ELISA) or level of radioactivity (in RIA), A technique such as ELISA can be performed in a variety of ways, and an example is given below.

1. The antigen, (parathyroid hormone) (given volume of serum sample) is immobilized on a solid surface (usually the well of a microtitre dish) antiparathyroid hormone MAb may be used for this.

2. The well is washed in buffer.

3. Anti-parathyroid hormone antibody: alkaline phosphatase enzyme conjugate is added to the well (Mab binds specifically to bound parathyroid hormone).

4. The well is washed in buffer.

5. Enzyme substrate (para-nitrophenyl phosphate) is added and the mixture incubated.

6. The amount of product formed by the enzyme (para-nitrophenol can be measured using a spectro photometer. The amount of product formed is proportional to amount of conjugate bound, which is proportional to the amount of parathyroid hormone.

b. Infectious diseases

Two approaches have been used –

1. to detect and quantify levels of antigens circulating specific to an infectious agent and

2. to identify infectious agents.

Detection of antigens is indicative of recently acquired infection, where as detection of antibodies indicates past episodes of infections. Circulating antigens can be assayed using ELISA or RIA. The presence of a pathogenic organism in the body can be diagnosed by the direct testing of clinical specimens for antigens using fluorescent labeled Mabs.

In the past, this approach was seldom used because of high levels of background fluorescence associated with polyclonal antiserum with the help of fluorescent labelled Mabs, herper simplex virus has been demonstrated in swab specimens, chlamydia trichomatis in urethral and cervical smears and cytomegalovirus in lung biopsies. Association of Epstun –Barr virus with two different texmores-Burkitt lymphoma and nasopharyngeal carcinoma, has been demonstrated with MAbs to the viral antigen. MAbs are also used to type and sub-type bacteria and viruses; a panel of MAbs that recognize different epitopes on the pathogen can be used in concert.

1. Coat wells of microtitre plate with a range of monoclonal antibodies, each directed against a specific virus, one monoclonal per well.

Fig.

c. Cancer

Although polyclonal antisera (produced in animals) have been used in diagnosis of cancers they are not very reliable since they cross-react with antigenic determinants of normal tissues. MAbs have helped to over come these problems. They are used in 8 different ways in cancer diagnosis. (i) to detect tumor related antigens or tumor markers in body fluids or on cells, (ii) to demonstrate tumor antigens in histological sections and (iii) to serve as imaging agents.

Tumor markers :

Blood levels of certain tumor products are used as tumor markers in the diagnosis and prognosis of cancers. Alterations in the levels of the markers are variable and inconsistent in early stages, but are often raised in advanced cancers. When the tumor load is reduced, the blood level of the tumor marker is also reduced

considerably. Hence this can be need to monitor the tumor burden and judge the efficacy of therapy.

Ex:

Carcinoembryogenic antigen - colorectal cancer

Prostate specific antigen - prostate cancer

α- Feto protein - hepatoma and germ cell tumors

Levels of such compounds in body fluids can be measured using ELISA or RIA.

Diagnostic tumor pathology

The study of pathological changes has been performed by microscopical examination of tissue sections stained with MAbs using immuno histochemical techniques. The sensitivity of such techniques is influenced by the stability of the antigen, the nature of the fixative and the methods used. A two – step immuno – peroxidase procedure, which gives a color reaction at the end, is commonly used to demonstrate location of epitopes in a cell.

Immunohistopathology often helps in assessing the grade of malignancy of tumors one such example is the use of MAbs directed against keratins, which are cytoskeletal proteins expressed

by squanous cell carcinomas; depending on the stage of differentiation, tumors express different sets of keratins. Such knowledge is useful in directing therapy.

Immuno scientigraphy

MAbs with specificities to many types of human cancers are now available. These can be tagged with radioisotopes such as iodine – 131 or technetium-99, and injected intravenously into the patients; the antibodies localize the tumor, which can then be detected by imaging the radioactivity. This technique of imaging is known as immunoscintigraphy. Although imaging techniques such as CT scanning, ultra sound scanning and magnetic resonance imaging can detect small lesions, they are unable to differentiate between cancerous and non-cancerous growths. Being tumor specific, antibody imaging has therefore certain advantages. Small metastatic lesions, which remain undetected by CT scan, can be detected successfully, in many cases, with radiolabelled MAbs. Since an antibody is a large molecule (150 Kda) and thus doesnot readily enter cells and tissues, attempts are being made to improve the efficiency of the procedure by using smaller Fab (12-15 Kda) or F_v fragments.

2. In theraphy

a. Treating bacterial blood infections

Blood infections caused by gram-negative bacteria account for 24-72 thousand deaths annually in U.S. hospitals, because the most common bacterial strains are resistant to treatment with antibiotics.

The toxicity of gram negative bacteria is due to the release of a lipopolysaccharide (LPS) component (Endo toxin) from the bacterial cell wall. The soluble form of this endotoxin triggers a set of excessive immunological responses that result in fever, cell lysis, increased heart beat, hyper ventilation, and possibly fatal organ failure. One approach to the control of gram negative bacteria has been to use antibodies against the endotoxin. Both human and mouse monoclonal antibodies that bind to a region of endotoxin common to many different gram-negative bacteria were tested in to extensive preliminary clinical trials. Both types of MAbs proved capable of controlling the gram negative bacteria. No adverse side effects were observed after treatment with the human MAb, the mouse MAb produced only a minor immunological response in few patients.

b. Preventing rejection of transplanted organs

In the 1970s passive immunization was reconsidered as a way to prevent immunological rejection of a transplanted organ. The rationale was to administer to patients a specific antibody that

would bind to certain lymphocytes and diminish the immune response directed against the transplanted organ.

The mouse monoclonal antibody called OKT3 was the first one to be approved by the U.S. Food and Drug Administration for use in humans as on immunosuppressive agent after organ transplantation. Lymphocytes that differentiate in the thymus are called T-cells. Various members of the T-cell population act as immunological helper and effector cells and are responsible for organ rejection. The OKT3 monoclonal antibody binds to a cell surface receptor called CD3, which is present on all T-cells. This action prevents a full immunological response and spares the transplanted organ from rejection. Immunosuppression by this means was reasonably effective, although, as anticipated, there were some side effects including fever and rash formation.

8.1.4.4. Vaccines

Birth control vaccines

Vaccines traditionally mobilize the body's immune system to react against external antigens, but an interesting variation on this theme is the induction of immunity against self antigens in order to intercept and control a normal process. One example is the GnRH vaccine. Its role is to inhibit reproduction by inducing antibodies against a hormone crucial to the reproductive system. The vaccine

againt GnRH can block fertility in both males and females but, since it also impairs the secretion of sex steroid hormones, it may not be an acceptable form of contraception unless complemented with hormone supplements.

Currently the most advanced birth control vaccine is the hCG vaccine, which induces antibodies against human chorionic gonodotrophin (hCG). The appearance of hCG is an early signal of pregnancy and it plays a vital role in its establishment and maintenance.

The strategy of targeting hCG does not interfere with a woman's normal pituitary and ovarian function. She ovulates normally and has regular menstrual cycles. The interception occurs only when she conceives. A draw back with the most commonly used steroidal contraceptives is that they block the pituitary-ovarian axis, the user does not make her normal hormones and these therefore have to be replaced by synthetic subunits in the contraceptive pill. This is not the case with the hCG vaccine.

A vaccine was initially divised in which the β-subunit of hCG was linked to tetanus toxoid in defined proportions. This structured cojugate vaccine overcame immunological tolerance to hCG (or its subunits) that can develop in women. An improved

formulation of the vaccine employs the β-subunit of hCG associated with a heterospecific α-subunit (non-human). The hetero specific dimer (HSD) is a tailored analogue of hCG which has correct conformation to recognize tissue receptors (where as the dissociated α-and β-subunits fail to do so). The heterospecific α-subunit being foreign to humans, enhances immunogenicity of the vaccine without provoking cross reaction with human follicle, stimulating hormones, human thyroid – stimulating hormone, since the hetero specific α-subunit is not immunologically cross reactive with the human α-subunit. Moreover, the bio-neutralising capacity of the antibodies induced by HSD vaccine is superior.

The HSD-hCG vaccine was approved by the Drug. Rgulatory Authorities and the Institutional Ethics Committees for phase II efficacy trails which have recently concluded in three major medical institutions in India. Sexually-active women of proven fertility received the immunization. The protocol required observations over 750 menstrual cycles to determine whether antibody levels above 50mg/ml were protective. These trials have demonstrated for the first time the feasibility of preventing pregnancy by a birth control vaccine. The reversibility of the vaccine (the ability to regain fertility once the antibody levels declines in the absence of a booster injection)has also been verified. In earlier phase I clinical trials, the safety of the vaccine and the lack of sideeffects were

demonstrated. This HSD-hCG vaccine is currently the most advanced fertility control.

8.1.4.5. Seed protein quality

Curl seed proteins are deficient in lysine, while those of pulses are deficient in sulphur containing aminoacids, e.g., methionine, and in tryptophan. This limits their nutritional value for man since these aminoacids are essential for man. Therefore, improvement of seed storage protein quality is an important and seemingly feasible objective. The approaches to achieve. This objective may be grouped into two broad categories.

- introduction of an appropriate transgene
- modification of the endogenous protein encoding gene.

1. Introduction of an appropriate transgene

In this approach a new gene encoding a storage protein which is rich in the deficient aminoacids is introduced into the crop to correct its aminoacid deficiency. The trasgene is linked to a seed specific promoter to ensure its expression only in seeds. Vicilin is the major seed storage protein of pea; it contains 7% lysine but no sulphur containing aminoacids (Met and Cys). Therefore, pea seed protein is generally low in sulphur-aminoacids, which needs to be

ameliorated. In contrast, a sunflower seed storage protein, sunflower albumin 8 (SFA 8), contains 23% Met plus Cys. The gene coding for SFA 8 has been isolated. The presence of introns in the transgenic constructs has been shown to enahance the expression of SFA8 gene. The SFA8 gene has been fused with the vicilin gene promoter. This promoter is known to confer on genes the correct developmental and tissue-specific expression and accumulation of protein in seeds of tobacco. Attempts are being made to transfer the vicilin gene promoter – SFA8 gene construct into pea. It has been estimated that the accumulation in pea seeds of SFA8 to a level of 4% extractable seed protein would result in a 40% increase in the sulphur containing amino acid content of pea seed protein. This is enough to correct the deficiency of pea seed proteins.

2. Modification of endogenous genes

This approach to improve seed storage protein quality is based on the isolation and modification of the concerned protein encoding gene sequence either by – replacing one or few codons with the selected codons or by-inserting one or few selected additional codons at appropriate sites. For example, prolamine storage protein e.g., zein, of cereals are deficient in the essential aminoacids lysine and tryptophan. Single lysine replacement in the N-terminal coding sequence as well as with in and between the

peptide repeats, and double lysine replacement constructs of prolamine genes have been prepared. In addition, short oligonucleotides encoding lysine-and tryptophan rich peptides were inserted separately at several different points in the coding sequence.

These constructs were shown to express well in xenopus oocytes and their polypeptides were able to form normal aggregates of protein bodies. Further, high levels of mRNA transcripts were produced from these chimaeric constructs in transgenic petunia and tobacco seeds, but zein proteins were barely detectable. However, both normal and lysine-containing zein polypeptides were found to be unstable in these plants. It is possible that β, δ and possibly δ-zeins are required for α-zeins to form protein bodies, or it may be that an accumulation of α-zeins is deleterious to dicot cotyledonary cells.

Examples

In a recent study, the 75 legume seed storage protein, β-phaseolin, gene driven by rice storage protein gene gt 1 (glutelin 1) promoter was transferred in rice. Transgenic rice plants expressed the gene in their endosperm, and some plants showed upto 4% of their total proteins to be β-phascolin. The 115 legumin protein

gene driven by gt 1 promoter has also been transferred, and expressed in rice endosperm.

In another study, Du pont (USA) scientists have synthesized and patented a gene encoding a protein, called CP 3-5, containing 35% lysine and 22% methionine. The CP 3-5 gene was coupled with seed specific promoters and transferred into maize; the gene was expressed by tissue culture cells.

Rice gt 1 gene encodes the major rice seed storage protein. It has been modified to encode higher levels of lysine, tryptophan and methionine. The modified gt 1 gene driven by its own promoter, was transferred into rice protoplasts; the resulting transgenic rice plants express the modified gene in their developing endosperm. Similarly, a modified zein protein gene encodes a protein having improved methonine. This gene was introduced in maize, rice and wheat by microinjection, microprojectile bombardements and electroporation. Transgenic plants containing the modified zein gene showed upto 3.8% methionine in their seed protein.

8.1.4.6. Drug designing

This approach aims at designing drugs which specifically and selectively fit into the critical sites of the target molecules, there by inactivating the latter. The target molecule may be an enzyme (concerned with either metabolism or DNA replication), a hormone

receptor or some other important molecules involved in a disease. The aim of drug designing is to develop highly efficient drugs which have little or no side effects. There are three main steps in drug designing.

- detailed knowledge of the critical sites of target molecules
- designing of drug molecules which will specifically fit into and bind to these critical sites, and synthesis of such molecules,
- evaluation of the interaction of the synthesized drugs with the target molecules, and further modifications in the former to make them `safe' for medical use.

A successful example of drug designing is provided by the drug `propranolol' used for the treatment of heart attacks and hypertension (J. Black was awarded the Nobel Prize for physiology and medicine in 1988 for this work). Heart ailments are mainly due to an excess of the homones `norepinephrine' and `epinephrine', which act through two receptors called α and β propranolol blocks the β receptor, thereby interfering with the action of these hormones.

Another drug, called `cimetidine', blocks the H_2 receptor (in stomach lining) of histamine; histamine induces the release of HCl

in stomach leading to the development of stomach ulcers. Cimetidine therefore, specificially cures peptic ulcers by blocking the concerned receptor. G. Elison and G. Hitchings have used this approach to develop useful drugs for the treatment of cancer, gout, malaria and viral infections like herper. Recently, a drug called Azidothymidine (AZT) has been developed for the treatment of AIDS; this drug selectively inhibits the reverse transcriptase of HIV.

Another drug, Ro-31-8959, designed to inhibit the HIV protease is in stage III clinical trials using oral delivery. Inhibition of protease results in the production of immature non-infectious viral particles.

8.1.4.7. Recovery of metals

Microbes can be employed to recover valuable metals from low grade ore and also from dilute solutions for which the conventional metallurgical processes are uneconomical and, generally, rather polluting. In 1947, it was discovered that the bacterium thiobacillus ferroxidans induces leaching of copper as copper sulphate from ares other species of bacterin, e.g., thiobacillus thioxidans.

Thiobacillus acidophilus and

Thiobacillus organoparus were discovered

To have similar capabilities although they are of lesser importance. These bacteria require extremely low pH for optimum growth and activity and derive their energy needs by oxidising metal sulphides, e.g., of copper, zinc, iron, etc. Another bacterium leptospirillum ferooxidans oxidizes ferrous ions and is also involved in microbial are leaching. Microbial are reaching consists of the processes involving bacteria, mainly T. ferroxidans, that lead to leaching of metal sulphates from low grade ores containing insoluble metal sulphides.

The metal solubilizing action of the bacteria is both direct as well as indirect. In direct conversion, the metal sulphide is itself converted into metal sulphate as follows:

$$CUS + 2O_2 \xrightarrow{\text{T. ferroxidans}} CUSO_4$$

In contrast, indirect, conversion is based on the reduction of ferric sulphate ($Fe_2 (SO_4)_3$) to ferrous sulphate ($FeSO_4$); in this reaction, metal sulphate ($CuSO_4$) and elemental sulphurs are produced as follows.

$$CUS + Fe_2(SO_4)_3 \xrightarrow{\text{T. ferroxidans}} 8\ H_2SO_4$$

T. ferroxidans also oxidizes sulphur to H_2SO_4 and converts $FeSO_4$ to $Fe_2(SO_4)_3$, both reactions providing energy used by the bacterium.

$$S_8 + 12O_2 + 8H_2O \xrightarrow{\text{T. ferrooxidans}} 8\ H_2SO_4$$

(crystalline sulphur)

$$2FeSO_4 + H_2SO_4 \longrightarrow Fe_2(SO_4)_3 + H_2$$

It may be pointed out that T.thiooxidans oxidizes reduced sulphur compounds only for obtaining energy, while T. ferroxidans obtain energy by oxidising both reduced sulphur compounds as well as ferrous salts (to ferric salts).

The process involving T. ferrooxidans is being used since 1960s in Canada for uranium recovery, and since 1970s in South Africa for recovery of gold. In USA and some other countries it is being used mainly for the recovery of copper; about 10-20% of the world copper supply is derived by this process. Application of the process based on thiobacillus to copper recovery has resulted in

about 2-3 fold reduction in copper prices, and has eliminated the release of SO_2 into the atmosphere produced from copper sulphide (CUS), present in the ores, during conventional recovery process, the process of copper recovery using thiobacillus is briefly outlined below:

1. these are collected in a huge pile preferably on a surface that would be impermeable to the resulting metal sulphate solution. The size of pile may be massive, one such pile in USA has billions of tonnes of low grade copper are.

2. The pile is periodically sprayed with a leaching solution having ferric ions (as $Fe_2(SO_4)_3$) and H_2SO_4. H_2SO_4 maintain a low pH conductive for leaching. The leaching solution slowly percolates through the are pile.

3. Copper is released into the solution as $CuSO_4$ by the chemical reaction (indirect conversion of CUS). Bacteria present in the are will also convert CUS directly into $CUSO_4$. $CUSO_4$ is readily water soluble, while CUS is not.

4. The leaching solution ultimately reaches the bottom of pile and is drained to a copper recovery plant; the leachate may contain about 1-3g cu/l of leachate. At present, copper recovery is done by passing the leachate over scrap iron; this

produces iron sulphate and elemental copper; the latter forms a precipitate and is earily recovered.

$$CUSO_4 + Fe \longrightarrow FeSO_4 + Cu$$

5. The solution remaining after copper recovery is pumped into a regeneration tank/pond. The bacterium T. ferrooxidans oxidizes $FeSO_4$ into $Fe_2(SO_4)_3$; aeration may be provided to facilitate this conversion. Additional H_2SO_4 may be added to maintain low pH. The leaching solution thus becomes recharged and ready for re use. The kennecott chino mine in New Mexico (USA) produces about 50 tons copper per day using this process.

Alternatively, holes may be blasted into the mine and the leaching solution pumped into these holes. After a period of time, the solution is pumped out and copper is extracted from the leachate; the remaining solution is recharged and re used. This is called in sites are leaching.

In addition many bacteria, e.g., Barillus, streptococcus, proteus etc., adsorb metals to their cell surfaces and some bacteria even accumulate them within their cells. Mn, Fe are adsorbed to the cell surfaces of Bacillus, Leptothrix etc. The metal particles are attached to and bound by the negatively charged molecules in the microbial cell wall, e.g., polymers. This phenomenon is thought to

be responsible for the gold deposits in Alaska and Venezuela. Such microorganisms can be employed to recover precious metals from dilute solutions. For example, non-growing mycelium of fungus Rhizopus arrhizus can accumulate uranium upto 18% of its mycelial dry weight.

8.1.4.8. Desulphurisation coals

Coal contains usually 0.2-8% sulphur but in cases it may exceed 11%. Coal burning, therefore, liberates large amounts of SO_2^- and SO_3^- which dissolves in atmospheric moisture giving rise to `acid rain'. The sulphur is present both in inorganic (iron pyrite Fe_2S) and organic forms. Microbes can be used for desulphurisation of coal. Thiobacillus spp. May be used on pulverized coal to oxidize iron pyrite, but organic sulphur has to be oxidized using a bacterium like sulfolobus acidocaldarius which can grow upto 90^{o}C in acidic environments. S. acidocaldariurs can oxidize both iron pyrite as well as organic sulphur compounds. A two-stage process may be developed for desulphurisation of coal. In the first stage, conditions are chosen to maximize oxidation of iron pyrite, while in the second stage conditions optimum for organic sulphur utilization are provided.

8.1.4.9. Lactose in dairy industry

Milk and whey (the clear fluid obtained during cheese making) contain about 4.7% (w/v) lactose. Adults having their origins in Northern Europe and Indian subcontinent are tolerant to lactose. But a great majority of the adults of remaining regions are intolerant to lactose, eg., 97%, 90% and 70% of Thai, Chinese and black American populations, respectively. In addition, some rare individuals are intolerant to lactose due to lactose deficiency as some other metabolic defects. Lactose intolerant individuals suffer from diarrhoea, abdominal cramps, flatulence and in severe cases, dehydration and even death, when they consume normal milk. Clearly such persons need low lactose milk/milk preparations.

In addition, lactose is rather poorely soluble in water (crystals form above 11% w/v at 4^{o}C). This hampers the use of concentrated whey syrups in many food processes, makes the foods prone to microbial attack, and the disposal of such whey is rather expensive (due to the presence of lactose). These problems are readily overcome by hydrolysis of lactose using the enzyme lactase, this produces monosaccharides D-glucose and β-D-galactose.

$$\text{Lactose} + H_2O \xrightarrow{\text{Lactose}} \text{D – glucose} + \beta\text{-D-galactose}$$

The enzyme lactose is obtained from the following sources:

- from dairy yeast kluyveromyces fragilis – the enzyme has pH optimum of 6.5-7.0 and is suitable for treatment of milk.

- From Aspergillus niges or A. oryzae – these enzymes have pH optimum of 3.0-4.0 and 4.5-6.0, respectively, and are more suited for treatment of whey.

These lactases are subject to varying degrees of product inhibition by galactose. They are added to milk or whey at about 2000 u/kg and incubated for one day at 5^{o}C; about 50% of the lactose becomes hydrolysed making the milk or whey more sweet. In addition, when the milk/whey is condensed or frozen, lactose does not form crystals.

Lactase is used in the production of ice creams, and sweetened, flavoured and condensed milks. Lactase treated whey powder is used either in part or full, in place of the skimmed milk powder in ice cream recipes; this also improves the creaminess of ice creams. Small amounts of lactose (20 u/kg) may be added to ultra-high temperature sterilized milk; storage for one month at 20^{o}C produces low lactose milk. Alternatively, immobilized lactase may be used to process milk as well as whey.

8.1.4.10. Enzymes in brewing industry

Enzymes are widely used in fruit juices and brewing industries to active specific objectives which can not be otherwise achieved. In addition, brewing industry, employs varied but specific strains of yeast, e.g., saccharomyces cerevisiae, S.carisbergensis etc. for fermentation of the brew.

Beer

Beer is an undistilled alcoholic beverage produced from fermentation of barley malt of yeast, especially S. cerevisiae or S.carisbergensis. Generally, other materials rich in starch, e.g., wheat, maize, rice etc., are also added to increase the amount of fermentable sugars and to reduce the relative costs of fermentation; these are called adjuncts. Barley matt is prepared by soaking the grains in water, allowing them to germinate at 17^{o}C, and then drying them at 65^{o}C once the appropriate stage of germination is reached. For beer production the matt is powdered, mixed with warm water at 70^{o}C and pH 5.0; this is called mashing.

Mashing results in partial hydrolysis of starch and proteins. The mash is filtered and the filterate, called `beer wort', is used for fermentation after adequate saccharification. The wort is boiled with hops (papery scales of female flowers of Humulus lupulus); this stops saccharification, adds flavour and aroma and provides mild antibacterial activity. The wort is cooled and inoculated with

the appropriate strain of yeasts; fermentation is allowed at low temperatures (5-15^{o}C) for 5-10 days. The fermented wort is cooled to 0^{o}C and stored for several months to eliminate off-flavours and to improve the characteristics of the beer.

Generally, the enzyme activities needed for saccharification before fermentation are provided by the matt. Generally, starch is not saccharified completely since the nonferementable dextrins give the drink a quality called `body' and also stabilize its foam `head'. Therefore, ordinarly saccharification is stopped by boiling the wort when about 75% of the starch has been converted into fermentable sugars. The following enzymes are employed in beer production to achieve specific objectives.

1. Bacterial and fungal α-amylases and β-glucanase are used for increased and rapid saccharification, especially when adjuncts are added to the matt; this maximizes the economic returns.

2. Neutral proteases are added to the wart to hydrolyse proteins and to increase the fermentation rate in the later stages. For production of high-gravity beer, extra proteins are added to the wart; neutral proteases are used in such cases to digest the proteins.

3. Cellulases are used to digest barely glucans, particularly when wheat is used as an adjunct.

4. Proteins and tannis make the beer hazy when chilled, i.e., produce chill-haze. Papain is added to the beer during the later post fermentation stages to hydrolyse the proteins and prevent the occurance of `chill-haze'.

Fig.

5. More recently, the demand for low calorie or light beers has grown rapidly. These are produced by using lower levels of starch coupled with much higher levels of saccharification; this reduces both total solids and alcohol contents of the beer. This is usually achieved by using glucoamylase and / or fungal α-amylase during fermentation.

Dextrins are non-fermentable products of starch hydrolysis which may constitute upto 25% of the initial amount of starch. Recombinant DNA technology has been used to produce dextrin utilizing brewing yeasts (S.cerevisiae) which hydrolyse the dextrins into glucose, which is then converted into alcohol. Dextrins are hydrolysed by S.diasticus, which is not used in brewing. The

concerned gene from S.diasticus has been transferred into S. cerevisiae so that the recombinant yeast strains can now convert dextrins into alcohol; this reduces the calorie content of beer (by using up dextrins) and aldo enhances the alcohol content by about 1% v/v.

6. A variety of distilled alcoholic drinks, e.g., whisky, rum, vodka etc., are prepared by fermentation of a large number of substrates rich in carbohydrates. Some of these substrates, e.g., molasses (rum), grapes (brandy) etc., are rich in fermentable sugars; in such cases, saccharification is not required. Many of the substrates, however, contain starch, e.g., barley matt, maize, rye (whisky) etc. In such cases, saccharification of starch is essential. Saccharification may be effected by the native enzymes, e.g., in case of scotch matt whisky the barely matt is used exclusively. But in case of grain spirits, heat stable α-amylases from bacteria are used for saccharification. In case of distilled drinks, saccharification continues throughout the fermentation period.

8.1.4.11. Protein engineering

Only about 20 of the many thousands of enzymes that have been studied and characterized biochemically account for over 90%

of the enzymes that are currently being used industrially. A major reason that more enzymes are not used in industrial processes is that an activity evolved under natural conditions usually is not well suited for a highly specialized in vitro function. Most enzymes are easily denatured by exposure to the conditions, such as high temperature and the presence of organic solvents, that are used in many industrial processes. Althogh thermotolerant enzymes can be isolated from thermophilic microorganisms, these organisms often lack the particular enzyme required for industrial use. However, with the availability of directed mutagenesis and gene cloning, these constraints are no longer significant.

1. Adding disulfide bonds

The thermostability of a protein can be increased by creating a molecule that will not readily unfold at elevated temperatures. In addition, these thermostable enzymes are often resistant to denaturation by organic solvents and nonphysiological conditions such as extremes of pH. The addition of disulfide bonds can significantly increase the stability of a protein.

In one study, six variants of the enzyme T_4 cysoxyme were constructed by qligonucleotide – directed mutagenesis. To introduce internal disulfide bonds, two, four, or six aminoacid

residues were changed to cysteine, thereby generating one, two or three disulfide bonds respectively.

The aminoacid residues that were trargeted to become cysteine residues were spatially close to each other in the active enzyme. This proximity ensured that the overall conformation of the molecule would remain essentially unaffected by the formation of the new disulfide linkages. The targeted aminoacids were not involved in the active site of the enzyme. The newly introduced cysteins created disulfide bonds between position 3-97; 9-164; 21-142 of the enzyme.

Tab.

Wt: wild type

Pwt : Pseudo wild type

A-F: six engineered cysteins variants.

The results of this experiment indicate that the thermal stability of the enzyme increases as a result of the presence of disulfide bonds, with the most thermostable variant being the one with the largest number of disulfide bonds, and that some variants (C, E & F), which are more thermostable than wild type or pseudo wildtype, have lost their enzymic activity. The loss of enzymatic

activity in three of the variants probably reflects a distortion of the peptide backbone of the molecule containing a disulfide linkage between residues 21 & 142. In many instances, the engineering of a protein or a trial and error process. Hence, the precise aminoacid changes that yield the `best' variant are obvious.

2. Reducing the number of free sulfhydryl residues

Occasionally, an expressed foreign protein is less active than expected. Protein engineering can be used to increase this activity. For example, when CDNA for human β-interferon (IFN β) was cloned and expressed in E.coli, the protein product showed only a disappointing 10% of the antiviral activity of the authentic, glycosylated form. And although reasonable amounts of IFN β were synthesized, most of it was found to exist as dimers and higher oligomers, which were inactive.

Analysis of the DNA sequence of the IFN β gene revealed that the encoded protein has three cysteine residues, so one or more of these residues could be involved in the intermolecular disulfide bonding that produced the dimers and oligomers in E.coli but not in human cells. It was reasoned that conversion of one or more of the cysteine codons into serine codons might result in an IFN β derivative that would not form oligomers. Serine was chosen to replace cysteine, because the structure of the two aminoacids is

identical, except that serine contains oxygen instead of sulfur and, as a result, cannot form disulfide bonds.

This proved to be correct. No multimeric complexes were formed when a ser-17 variant of IFN β was expressed in E.coli. Moreover, the ser-17 IFNβ had a specific activity similar to that of authetic, native IFN β and was more stable during long-term storage than the native form.

Fig.

3. Modifying enzyme specificity

Although many of the protein engineering studies done with oligonucleotide- directed mutagenesis have focused on modifying native properties of specialized enzymes, it is conceivable that an enzyme could be redesigned to produce an entirely unique catalytic entity. For example, the gene for the relatively non-specific enzyme staphylococcal nuclease was specifically mutated. Then its protein product was covalently coupled to a short single-stranded oligonucleotide to create a sequence –specific single stranded DNA nuclease.

Staphylococcal nuclease is a single polypeptide of 149 aminoacids. It cleaves the phosphodiester bonds of single-stranded

RNA and of both single – and double – stranded DNA at A, U- or A+T- rich regions to generate 3^1-P and 5^1-OH termini. Detailed knowledge of the structure of staphylococcal nuclease from x-ray crystallographic studies enabled investigators to predict that it might be converted into a sequence specific enzyme by anchoring a short oligonucleotide of known sequence to an aminoacid near the active site. It was hoped that the bound oligonucleotide would basepair with specific DNA or RNA substrates.

The Lys 116 of staphylococcal nuclease was changed to cys-116 by oligonucleotide-directed mutagenesis. Then, an oligonucleotide that had been synthesized to contain a 3^1-sulfhydryl group was added to the modified enzyme to establish a mixed disulfide bond in which the oligonucleotide was covalently linked to the enzyme through cys-116. However, although staphylococcal nuclease doesnot have any other cysleine residues, there were three possible disulfide linkages.

- an enzyme dimer
- an oligonucleotide dimer
- an enzyme – oligonucleotide mixed disulfide

Gel filteration chromatography was used to separate the three reaction products on the basis of size.

Fig.

When enzyme-oligonucleotide complexes were added to a mixture of single-stranded DNAs, only the DNA molecules that had segments complementary to the oligonucleotide attached to the enzyme bound. After binding, the enzyme specifically cleaved the single – stranded DNA substrate adjacent to the region of base pairing.

This strategy can be used to develop a whole range of sequence – specific single-stranded DNA nucleases. For example, with such a repertoire, virtually any DNA sequence can be cleaved at a specific site after cloning into MB. In addition, if may be possible to design other unique enzymes by using the strategy of coupling some other specific moiety (other than an oligonucleotide) to an enzyme through a mixed disulfide linkage.

8.1.4.12. Biochemical production

Plants are the chief source of carbohydrates, eg., starch, sugar etc., lipids, proteins and a variety of unique biochemicals. Transgenes have been shown to introduce novel branches in the

biosynthetic pathways of plants, and there by, to generate valuable products or to produce new, valuable proteins. Virtually all the cases are promising and in developmental stages, except for the thrombin inhibitor protein hirudin, which is the first commercial example.

1. Hirudin

Hirudin is encoded by a synthetic gene, and is expressed in fusion with the oil-body protein oleosin which greatly facilitates the purification of heterologous polypeptides. The seed tissue expressing a heterologous protein fused with oleosin is extracted with water and the extract is centrifuged. Oil bodies containing the oleosin-fusion protein float on the surface, and are easily separated from the rest of seed proteins. The heterologous polypeptide is cleaved from oleosin at a protease recognition site located at their junction. Thus hirudin provides a successful example of transgene expression in plants for the isolation of a polypeptide of interest at a commercial scale.

2. Polyhydroxybutyrate

Polyhydroxy alkanoates (PHA), e.g., polyhydroxy butyrates (a biodegradable plastic substrate) (PHB), are synthesized from acetyl COA used as precursor, and are used for the synthesis of biodegradable plastics, with thermoplastic properties. At present,

PHAs are produced by bacterial fermentation and the cost of biodegradable plastic is substantially higher than that of synthetic plastics. Attempts are being made to produce PHAs in transgenic plants to reduce the cost. Genes encoding the two enzymes, aceto-acetyl-COA reductase (phb B) and PHB synthase (phb C), involved in the PHB synthesis from the precursor acetyl-COA have been transferred from the bacterium Alcaligenes eutropheis and expressed in Arabidopsu thaliana. When the two enzymes were synthesized located in the cytoplasm, a low level (100 μg/ PHB/g fresh wt., i.e., 0.14% of dry weight) for PHB was produced. It was reasoned that since the biosynthesis of fattyacids occurs in plastids, acetyl-COA will be available mainly plastids, and its supply in the optoplasm will be low and limiting to PHB biosynthesis. The two enzymes were, therefore, targeted into the plastids, and there was a 100-fold increase in PHB production (about 10mg PHB/g fresh wt., i.e., 0.1%). PHB production by transgenic plants provides an example of a novel compound synthesized in plants. Transgenic trees like popular expressing phb B and phb c accumulate in their leaves; the leaves are collected and used for PHB extraction.

Lesson 8.2.1

Tools and Techniques of Recombinant DNA Technology

8.2.1.1 Objective

8.2.1.2 Introduction

8.2.1.3 The solutions: basic techniques

8.2.1.3.1: Agarose gel electrophoresis

8.2.1.3.2: Nucleic acid blotting

(a) Southern blotting

(b) Northern blotting

(c) Western blotting

8.2.1.4 Transformation of *E. coli*

(a) Infection by vectors packaged as virions

(b) Transfection

(c) Calcium phosphate precipitation

(d) Electroporation

8.2.1.5 Tools for recombinant DNA technology

8.2.1.5.1 Plasmids

8.2.1.5.2 Restriction endonucleases

8.2.1.5.3 Alkaline phosphatases

8.2.1.5.4 T4 Polynucleotide Kinase

8.2.1.5.5 Reverse Transcriptase

8.2.1.5.6 Exonucleases

8.2.1.5.7 S_2 nucleases

8.2.1.5.8 DNA Polymerase

8.2.1.6 Summary

8.2.1.7 Model questions

8.2.1.8 Reference text books

8.2.1.1 Objective:

The objective of the lesson is to explain the importance of the tools and the different kinds of techniques applied in recombinant DNA technology.

`

8.2.1.2 Introduction :

The initial impetus for gene manipulation *in vitro* came about in the early 1970s with the simultaneous development of techniques for:

• genetic transformation of *Escherichia coli*;

• cutting and joining DNA molecules;

• monitoring the cutting and joining reactions.

In order to explain the significance of these developments we must first consider the essential requirements of a successful gene-manipulation procedure.

The basic problems

Before the advent of modern gene-manipulation methods, there had been many early attempts at transforming pro- and eukaryotic cells with foreign DNA. But, in general, little progress

could be made. The reasons for this are as follows. Let us assume that the exogenous DNA is taken up by the recipient cells. There are then two basic difficulties. First, where detection of uptake is dependent on gene expression, failure could be due to lack of accurate transcription or translation. Secondly, and more importantly, the exogenous DNA may not be maintained in the transformed cells. If the exogenous DNA is integrated into the host genome, there is no problem. The exact mechanism where by this integration occurs is not clear and it is usually a rare event. However this occurs, the result is that the foreign DNA sequence becomes incorporated into the host cell's genetic material and will subsequently be propagated as part of that genome. If, however, the exogenous DNA fails to be integrated, it will probably be lost during subsequent multiplication of the host cells. The reason for this is simple. In order to be replicated, DNA molecules must contain an *origin of replication*, and in bacteria and viruses there is usually only one per genome. Such molecules are called *replicons.* Fragments of DNA are not replicons and in the absence of replication will be diluted out of their host cells. It should be noted that, even if a DNA molecule contains an origin of replication, this may not function in a foreign host cell. There is an additional, subsequent problem. If the early experiments were to proceed, a method was required for assessing the fate of the donor DNA. In particular, in circumstances where the foreign DNA was

maintained because it had become integrated in the host DNA, a method was required for mapping the foreign DNA and the surrounding host sequences.

8.2.1.3 The solutions: Basic techniques:

If fragments of DNA are not replicated, the obvious solution is to attach them to a suitable replicon. Such replicons are known as *vectors* or *cloning vehicles*. Small plasmids and bacteriophages are the most suitable vectors for they are replicons in their own right, their maintenance does not necessarily require integration into the host genome and their DNA can be readily isolated in an intact form. Suffice it to say at this point that initially plasmids and phages, suitable as vectors were only found in *E.coli*. An important consequence follows from the use of a vector to carry the foreign DNA: simple methods become available for purifying the vector molecule, complete with its foreign DNA insert, from transformed host cells. Thus not only does the vector provide the replicon function, but it also permits the easy bulk preparation of the foreign DNA sequence, free from host-cell DNA. Composite molecules in which foreign DNA has been inserted into a vector molecule are sometimes called DNA *chimeras* because of their analogy with the Chimaera of mythology – a creature with the head of a lion, body of a goat and tail of a serpent. The construction of

such composite or *artificial recombinant* molecules has also been termed *genetic engineering* or *gene manipulation* because of the potential for creating novel genetic combinations by biochemical means. The process has also been termed *molecular cloning* or *gene cloning* because a line of genetically identical organisms, all of which contain the composite molecule, can be propagated and grown in bulk, hence *amplifying* the composite molecule and *any gene product whose synthesis it directs*. Although conceptually very simple, cloning of a fragment of foreign, or *passenger*, or *target* DNA in a vector demands that the following can be accomplished.

• The vector DNA must be purified and cut open.

• The passenger DNA must be inserted into the vector molecule to create the artificial recombinant DNA joining reactions must therefore be performed. Methods for cutting and joining DNA molecules are now so sophisticated.

• The cutting and joining reactions must be readily monitored. This is achieved by the use of gel electrophoresis.

• Finally, the artificial recombinant must be transformed into *E. coli* or another host cell.

As we have noted, the necessary techniques became available at about the same time and quickly led to many cloning experiments, the first of which were reported in 1972 (Jackson *et al.* 1972, Lobban & Kaiser 1973).

8.2.1.3 Basic techniques:

8.2.1.3.1 Agarose gel electrophoresis

The progress of the first experiments on cutting and joining of DNA molecules was monitored by velocity sedimentation in sucrose gradients. However, this has been entirely superseded by gel electrophoresis. Gel electrophoresis is not only used as an analytical method, it is routinely used preparatively for the purification of specific DNA fragments. The gel is composed of polyacrylamide or agarose. Agarose is convenient for separating DNA fragments ranging in size from a few hundred base pairs to about 20 kb (Fig.8. 2.1).

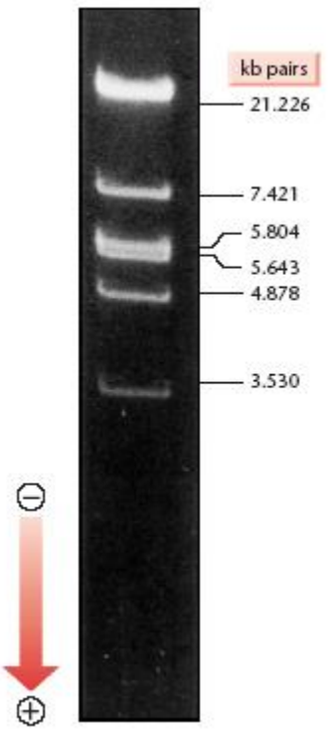


Figure 8.2.1

Electrophoresis of DNA in agarose gels. The direction of migration is indicated by the arrow. DNA bands have been visualized by soaking the gel in a solution of ethidium bromide (see Fig. 2.3), which complexes with DNA by intercalating between stacked base-pairs, and photographing the orange fluorescence which results upon ultraviolet irradiation.

Polyacrylamide is preferred for smaller DNA fragments.

The mechanism responsible for the separation of DNA molecules by molecular weight during gel electrophoresis is not well understood. The migration of the DNA molecules through the pores of the matrix must play an important role in molecular-weight separations since the electrophoretic mobility of DNA in free solution is independent of molecular weight. An agarose gel is a complex network of polymeric molecules whose average pore size

depends on the buffer composition and the type and concentration of agarose used. DNA movement through the gel was originally thought to resemble the motion of a snake (reptation). However, real-time fluorescence microscopy of stained molecules undergoing electrophoresis has revealed more subtle dynamics . DNA molecules display elastic behavior by stretching in the direction of the applied field and then contracting into dense balls. The larger the pore size of the gel, the greater the ball of DNA which can pass through and hence the larger the molecules which can be separated. Once the globular volume of the DNA molecule exceeds the pore size, the DNA molecule can only pass through by reptation. This occurs with molecules about 20 kb in size and it is difficult to separate molecules larger than this without recourse to pulsed electrical fields.

In pulsed-field gel electrophoresis (PFGE) (Schwartz & Cantor 1984) molecules as large as 10 Mb can be separated in agarose gels. This is achieved by causing the DNA to periodically alter its direction of migration by regular changes in the orientation of the electric field with respect to the gel. With each change in the electric-field orientation, the DNA must realign its axis prior to migrating in the new direction. Electric-field parameters, such as the direction, intensity and duration of the electric field, are set independently for each of the different fields and are chosen so that

the net migration of the DNA is down the gel. The difference between the direction of migration induced by each of the electric fields is the *reorientation angle* and corresponds to the angle that the DNA must turn as it changes its direction of migration each time the fields are switched.

A major disadvantage of PFGE, as originally described, is that the samples do not run in straight lines. This makes subsequent analysis difficult. This problem has been overcome by the development of improved methods for alternating the electrical field. The most popular of these is contour-clamped homogeneous electrical-field electrophoresis (CHEF . In early CHEF-type systems (Figure 8.2. 2)

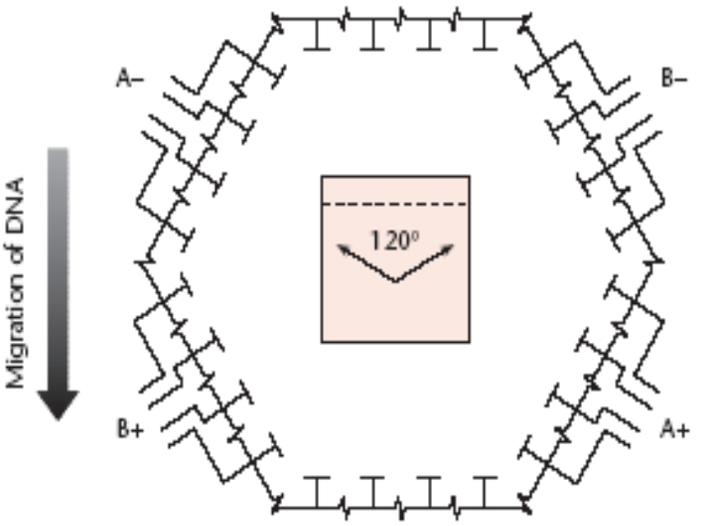


Figure 8.2.2: Schematic representation of CHEF (contour-clamped homogeneous electrical field) pulsed-field gel electrophoresis.

the reorientation angle was fixed at 120°. However, in newer systems, the reorientation angle can be varied and it has been found that for whole-yeast chromosomes the migration rate is much faster with an angle of 106° . Fragments of DNA as large as 200–300 kb are routinely handled in genomics work and these can be separated in a matter of hours using CHEF systems with a reorientation angle of 90° or less.

A particular advantage of gel electrophoresis is that the DNA bands can be readily detected at high sensitivity. The bands of DNA in the gel are stained with the intercalating dye ethidium bromide (Figure 8.2.3).

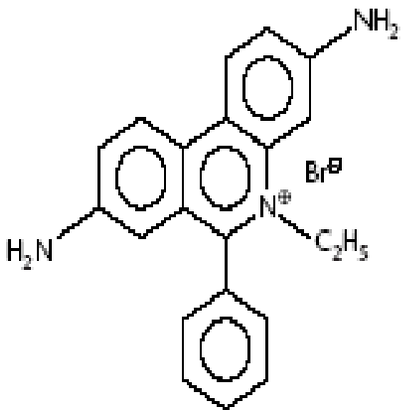


Figure 8.2.3 Ethidium bromide.

and as little as 0.05 µg of DNA in one band can be detected as visible fluorescence when the gel isilluminated with ultraviolet light.

In addition to resolving DNA fragments of different lengths, gel electrophoresis can be used to separate different molecular configurations of a DNA molecule .

8.2.1.3.2 Nucleic acid blotting:

Nucleic acid labelling and hybridization on membranes have formed the basis for a range of experimental techniques central to recent advances in our understanding of the organization and expression of the genetic material. These techniques may be applied in the isolation and quantification of specific nucleic acid sequences and in the study of their organization, intracellular localization, expression and regulation. A variety of specific applications includes the diagnosis of infectious and inherited disease. An overview of the steps involved in nucleic acid blotting and membrane hybridization procedures is shown in Fig. 8.3.4.

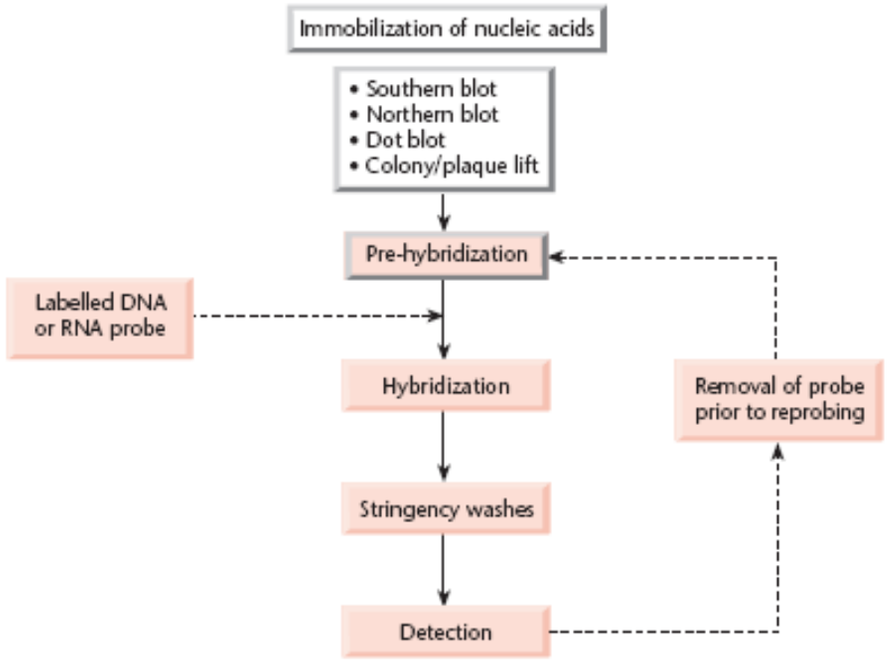


Figure 8.3.4 Overview of nucleic acid blotting and hybridization (reproduced courtesy of Amersham Pharmacia Biotech).

Blotting describes the immobilization of sample nucleic acids on to a solid support, generally nylon or nitrocellulose membranes. The blotted nucleic acids are then used as 'targets' in subsequent hybridization experiments. The main blotting procedures are:

- blotting of nucleic acids from gels;
- dot and slot blotting;
- colony and plaque blotting.

Colony and plaque blotting are described in detail on

(a) Southern blotting

The original method of blotting was developed by Southern (1975) for detecting fragments in an agarose gel that are complementary to a given RNA or DNA sequence. In this procedure, referred to as Southern blotting, the agarose gel is mounted on a filter-paper wick which dips into a reservoir containing transfer buffer (Fig. 8.3.5).

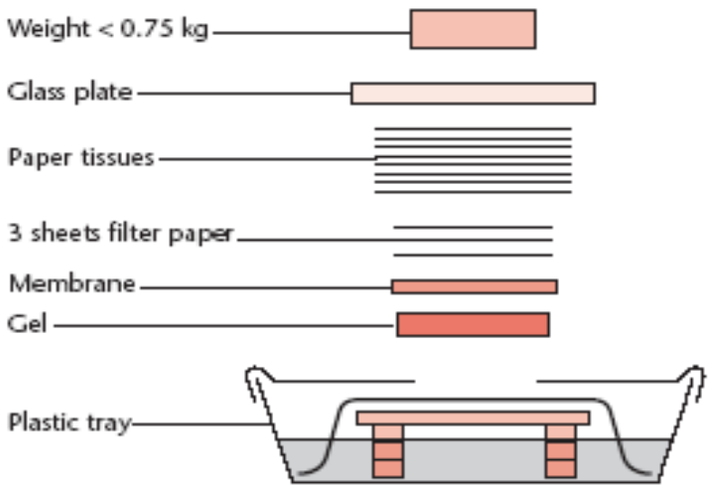


Figure 8.3.5 A typical capillary blotting apparatus.

The hybridization membrane is sandwiched between the gel and a stack of paper towels (or other absorbent material), which serves to draw the transfer buffer through the gel by capillary action. The DNA molecules are carried out of the gel by the buffer flow and immobilized on the membrane. Initially, the membrane material used was nitrocellulose. The main drawback with this

membrane is its fragile nature. Supported nylon membranes have since been developed which have greater binding capacity for nucleic acids in addition to high tensile strength. For efficient Southern blotting, gel pretreatment is important. Large DNA fragments (>10 kb) require a longer transfer time than short fragments. To allow uniform transfer of a wide range of DNA fragment sizes, the electrophoresed DNA is exposed to a short depurination treatment (0.25 mol/1 HCl) followed by alkali. This shortens the DNA fragments by alkaline hydrolysis at depurinated sites. It also denatures the fragments prior to transfer, ensuring that they are in the single-stranded state and accessible for probing. Finally, the gel is equilibrated in neutralizing solution prior to blotting. An alternative method uses positively charged nylon membranes, which remove the need for extended gel pretreatment. With them the DNA is transferred in native (non-denatured) form and then alkali-denatured *in situ* on the membrane. After transfer, the nucleic acid needs to be fixed to the membrane and a number of methods are available. Oven baking at 80°C is the recommended method for nitrocellulose membranes and this can also be used with nylon membranes. Due to the flammable nature of nitrocellulose, it is important that it is baked in a vacuum oven. An alternative fixation method utilizes ultraviolet cross-linking. It is based on the formation of cross-links between a small fraction of the thymine residues in the DNA and positively

charged amino groups on the surface of nylon membranes. A calibration experiment must be performed to determine the optimal fixation period. Following the fixation step, the membrane is placed in a solution of labelled (radioactive or non-radioactive) RNA, single-stranded DNA or oligo deoxynucleotide which is complementary in sequence to the blot transferred DNA band or bands to be detected. Conditions are chosen so that the labelled nucleic acid hybridizes with the DNA on the membrane. Since this labelled nucleic acid is used to detect and locate the complementary sequence, it is called the *probe*. Conditions are chosen which maximize the rate of hybridization, compatible with a low background of non-specific binding on the membrane. After the hybridization reaction has been carried out, the membrane is washed to remove unbound radioactivity and regions of hybridization are detected autoradiographically by placing the membrane in contact with X-ray film

(b) Northern blotting

Southern's technique has been of enormous value, but it was thought that it could not be applied directly to the blot-transfer of RNAs separated by gel electrophoresis, since RNA was found not to bind to nitrocellulose. Alwine *et al.* (1979) therefore devised a procedure in which RNA bands are blot-transferred from the gel on to chemically reactive paper, where they are bound covalently. The

reactive paper is prepared by diazotization of aminobenzyloxymethyl paper (creating diazobenzyloxymethyl (DBM) paper), which itself can be prepared from Whatman 540 paper by a series of uncomplicated reactions. Once covalently bound, the RNA is available for hybridization with radiolabelled DNA probes. As before, hybridizing bands are located by autoradiography. Alwine *et al.*'s method thus extends that of Southern and for this reason it has acquired the jargon term *northern* blotting. Subsequently it was found that RNA bands can indeed be blotted on to nitrocellulose membranes under appropriate conditions (Thomas 1980) and suitable nylon membranes have been developed. Because of the convenience of these more recent methods, which do not require freshly activated paper, the use of DBM paper has been superseded.

(c) Western blotting

The term 'western' blotting (Burnette 1981) refers to a procedure which does not directly involve nucleic acids, but which is of importance in gene manipulation. It involves the transfer of electrophoresed protein bands from a polyacrylamide gel on to a membrane of nitrocellulose or nylon, to which they bind strongly (Gershoni & Palade 1982, Renart &Sandoval 1984). The bound proteins are then avail able for analysis by a variety of specific protein–ligand interactions. Most commonly, antibodies are used to

detect specific antigens. Lectins have been used to identify glycoproteins. In these cases the probe may itself be labelled with radioactivity, or some other 'tag' may be employed. Often, however, the probe is unlabelled and is itself detected in a 'sandwich' reaction, using a second molecule which is labelled, for instance a species-specific second antibody, or protein A of *Staphylococcus aureus* (which binds to certain subclasses of IgG antibodies), or streptavidin (which binds to antibody probes that have been biotinylated). These second molecules may be labelled in a variety of ways with radioactive, enzyme or fluorescent tags. An advantage of the sandwich approach is that a single preparation of labelled second molecule can be employed as a general detector for different probes. For example, an antiserum may be raised in rabbits which reacts with a range of mouse immunoglobins. Such a rabbit anti-mouse (RAM) antiserum may be radiolabelled and used in a number of different applications to identify polypeptide bands probed with different, specific, monoclonal antibodies, each monoclonal antibody being of mouse origin. The sandwich method may also give a substantial increase in sensitivity, owing to the multivalent binding of antibody molecules.

8.2.1.4 Transformation of *E. coli*

Early attempts to achieve transformation of *E. coli* were unsuccessful and it was generally believed that *E. coli* was refractory to transformation. However, it was found that treatment with CaC12 allowed *E. coli* cells to take up DNA from bacteriophage λ. It was also showed that CaC12-treated *E. coli* cells are also effective recipients for plasmid DNA. Almost any strain of *E. coli* can be transformed with plasmid DNA, albeit with varying efficiency, many bacteria contain restriction systems which can influence the efficiency of transformation. Although the complete function of these restriction systems is not yet known, one role they do play is the recognition and degradation of foreign DNA. For this reason it is usual to use a restriction-deficient strain of *E. coli* as a transformable host. Since transformation of *E. coli* is an essential step in many cloning experiments, it is desirable that it be as efficient as possible. Several groups of workers have examined the factors affecting the efficiency of transformation. It has been found that *E. coli* cells and plasmid DNA interact productively in an environment of calcium ions and low temperature (0–5°C), and that a subsequent heat shock (37–45°C) is important, but not strictly required. Several other factors, especially the inclusion of metal ions in addition to calcium, have been shown to stimulate the process.

A very simple, moderately efficient transformation procedure for use with *E. coli* involves re suspending log-phase cells in ice-

cold 50 mmol/l calcium chloride at about 1010 cells/ml and keeping them on ice for about 30 min. Plasmid DNA (0. 1 μg) is then added to a small aliquot (0.2 ml) of these now *competent* (i.e. competent for transformation) cells, and the incubation on ice continued for a further 30 min, followed by a heat shock of 2 min at 42°C.The cells are then usually transferred to nutrient medium and incubated for some time (30 min to 1 h) to allow phenotypic properties conferred by the plasmid to be expressed, e.g. antibiotic resistance commonly used as a selectable marker for plasmid containing cells. (This so-called *phenotypic lag* may not need to be taken into consideration with high-level ampicillin resistance. With this marker, significant resistance builds up very rapidly, and ampicillin exerts its effect on cell-wall biosynthesis only in cells which have progressed into active growth.) Finally the cells are plated out on selective medium. Just why such a transformation procedure is effective is not fully understood. The calcium chloride affects the cell wall and may also be responsible for binding DNA to the cell surface. The actual uptake of DNA is stimulated by the brief heat

(a) Infection by vectors packaged as virions

Those vectors that have the λ phage `cos' sequences (cosmids, phasmids & λ vectors) are generally packaged in vitro into specially produced empty λ phage heads and complete λ particles are

constituted. These phage particles are used to infect E.coli cells. These vectors can also be used to transform E.coli cells directly as naked DNA using the $CaCl_2$ technique. Generally, infection by phage particles containing DNA insert is far more efficient than direct transformation. For Eg: the frequency of infection by recombinant λ phage vectors packaged in phage particles is upto 10^8 plaques / μg of DNA, while it is less than 10^3 plques / μg DNA by transformation by $CaCl_2$ technique.

(b) Transfection

Transfection denotes inherited changes in animal cells that are due to the addition of exogenous DNA. It is equivalent to the transformation of prokaryotes. There are three techniques that are commonly used to transform yeasts. Exogenous DNA can be added to yeast cells (1) that have had their cell walls removed either chemically or enzymatically (2) that have been treated with lithium acetate (or) (3) are subsequently subjected to electroporation.

(c) Calcium phosphate precipitation

The DNA preparation to be used for transfection is first dissolved in a phosphate buffer. Calcium chloride solution is then added to the DNA solution; this leads to the formation of insoluble calcium phosphate which co-precipitates with the DNA. The calcium phosphate-DNA precipitate is added to the cells to be

transfected. The precipitate particles are taken in by the cells by phagocytosis. In a small proportion of the transfected cells, the DNA becomes integrated into the cell genome producing stable or permanent transfection. This general approach can be applied to virtually all mammalian cells, and a very large no. of cells can be treated with little effort.

(d) Electroporation

A rapid and simple technique for introducing cloned genes into a wide variety of microbial, plant and animal cells, including *E. coli*, is electroporation. This technique depends on that high-voltage electric pulses can induce cell plasma membranes to fuse. Subsequently it was found that, when subjected to electric shock, the cells take up exogenous DNA from the suspending solution. A proportion of these cells become stably transformed and can be selected if a suitable marker gene is carried on the transforming DNA. Many different factors affect the efficiency of electroporation, including temperature, various electric-field parameters (voltage, resistance and capacitance), topological form of the DNA, and various host-cell factors (genetic background, growth conditions and post-pulse treatment. With *E. coli*, electroporation has been found to give plasmid transformation efficiencies (109 cfu/µg DNA) comparable with the best CaC12 methods. With conventional CaCl2- mediated transformation, the efficiency falls off rapidly as

the size of the DNA molecule increases and is almost negligible when the size exceeds 50 kb. While size also affects the efficiency of electroporation , it is possible to get transformation efficiencies of 106 cfu/µg DNA with molecules as big as 240 kb .

8.2.1.5 Tools for recombinant DNA technology

8.2.1.5.1 Plasmids

Plasmids are molecules of DNA that are found in bacteria separate from the bacterial chromosome. They:

- are small (a few thousand base pairs)
- usually carry only one or a few genes
- are circular
- have a single origin of replication

Plasmids are replicated by the same machinery that replicates the bacterial chromosome. Some plasmids are copied at about the same rate as the chromosome, so a single cell is apt to have only a single copy of the plasmid. Other plasmids are copied at a high rate and a single cell may have 50 or more of them.

Genes on plasmids with high numbers of copies are usually expressed at high levels. In nature, these genes often encode

proteins (e.g., enzymes) that protect the bacterium from one or more antibiotics.

Plasmids enter the bacterial cell with relative ease. This occurs in nature and may account for the rapid spread of antibiotic resistance in hospitals and elsewhere. Plasmids can be deliberately introduced into bacteria in the laboratory transforming the cell with the incoming genes.

8.2.1.5.2 Restriction endonucleases

One critically important advance that has greatly stimulated the rapid progress in molecular biology and genetic engineering was the discovery of a set of enzymes that are capable of cutting DNA at defined sequences. These enzymes are found in a variety of microorganisms and are called **restriction endonucleases**, or more simply, restriction enzymes. The first specific restriction enzyme was discovered by Hamilton Smith in 1970, an accomplishment for which he (and two others) were awarded the Nobel Prize. Since then, nearly 3,000 similar enzymes have been reported, many of which have different specificities

Nomenclature

Smith and another Nobel laureate, Daniel Nathans, devised a nomenclature for these enzymes. In brief, the name of each

restriction enzyme derives from the organism from which it is isolated. The first letter of the genus name plus the first two letters of the species name form the first three letters of the restriction enzyme's name. If necessary, a letter indicating strain designation is added, and finally a number is appended that stands for the order in which the enzyme was discovered in each organism. For example, BamHI is the name of an enzyme that is isolated from the bacterium *Bacillus amyloliquifaciens,* strain H, and it was, presumably, the first restriction endonuclease identified from that source. The first three letters of the name should be italicized, but because italicized letters are often hard to read on a computer, I have left them in plain text.

What they do

The restriction enzymes owe their usefulness to the fact that they bind to DNA at specific DNA sequences, four to eight nucleotides in size, called **recognition sites**. Once bound, restriction enzymes cut the DNA at or near this site. With a little thought, it should be clear that an enzyme that has a six base pair recognition site will, on the average, produce larger pieces of DNA than one that recognizes a four base site. Expressed quantitatively, the approximate size of the fragments produced by a particular enzyme, given that it is cutting a DNA containing an equal

proportion of all four nucleotides, can be calculated from the formula:

Average size of fragment = 4^N

where N is the number of bases that the enzyme recognizes. Hence, a four-cutter (the shorthand name for an enzyme that recognizes a site containing four base pairs) is expected to cleave random DNA into fragments of about 4^4 (256) base pairs while an enzyme with a recognition site of six bases will produce pieces (on the average) of about 4^6 (4096) base pairs

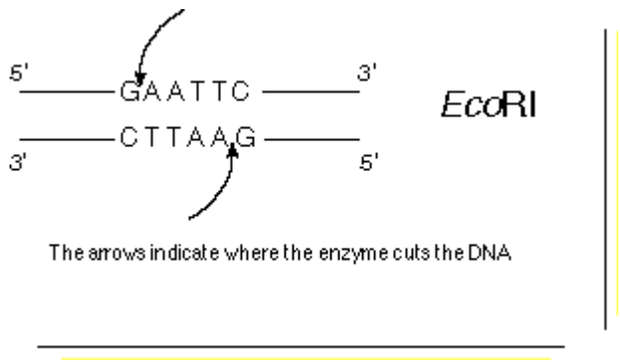
Properties of restriction enzymes

Ends

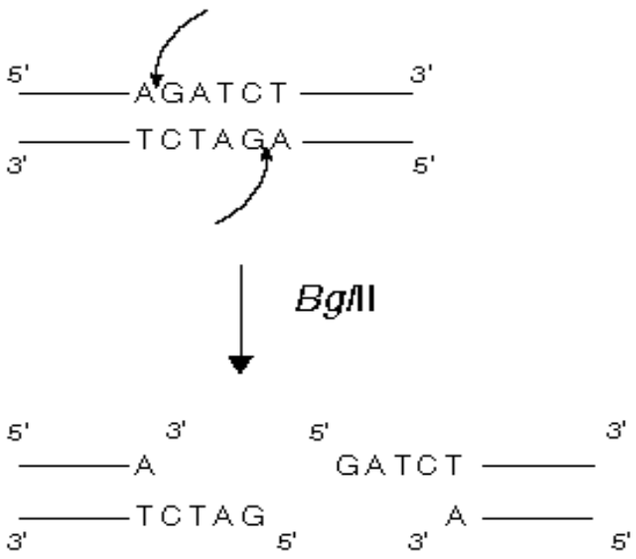
As illustrated, restriction enzymes invariably cut DNA in such a way as to leave a 3' hydroxyl on one end, and a 5' phosphate on the other In addition, most (but not all) enzymes recognize a symmetrical site.

Another interesting property of restriction enzymes is that while they often recognize a symmetrical site, they do not always cut at the axis of symmetry. For instance, the enzyme EcoRI (from *Escherichia coli* strain RY13 (I guess they didn't want to put all those letters in the name of the enzyme) and pronounced "echo are one"), recognizes the site GAATTC and cuts the DNA between the G's and the A's in a manner depicted below Note that the cut

produces an overhanging 5' single-stranded end of four nucleotides on each of the two pieces that are newly liberated (this is clearer if you look at the next two illustrations

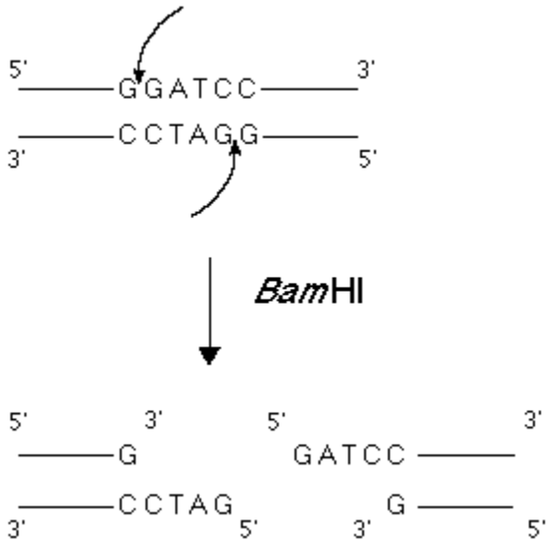


Similarly, the enzyme BglII (from the microorganisms, *Bacillus globiggi* and universally and irreverently pronounced BAGEL TWO) recognizes the sequence AGATCT and cuts between the first A and G residues.



Notice that BglII also yields a single-stranded 5' end

A third enzyme, BamHI (from the bacterium *Bacillus amyloliquifaciens*) cuts similarly



In fact, the four nucleotide single-stranded ends are the same for both BglII and BamHI. Moreover, there are at least two other six cutting enzymes that have been discovered that leave the same four nucleotide overhang: BclI and XhoII. These overhanging ends are very useful because -- under the proper conditions -- they may base pair with each other. In fact, because of their affinity for one

another, they are often called **cohesive** or **sticky ends**. Moreover, if molecules with these ends are treated with the appropriate enzyme -- **DNA ligase** -- their phosphodiester bonds may be rejoined (ligated). When two ends that originate from digestion by a single enzyme are ligated, the resulting molecule can be cut by the same enzyme again. But if the ends of a DNA molecule that originated with a BamHI cut and a BglII cut are joined together, the new sequence will not be cut with either enzyme (Why?).

Note that all restriction endonucleases do not generate 5' single-strand overhangs. In fact, some don't even produce an overhang at all. Several enzymes -- like SacI -- produce 3' single-stranded sticky ends. And some enzymes -- like PvuII -- cut at the axis of symmetry, leaving perfectly aligned ends. DNA molecules without overhangs are said to have **blunt ends**.

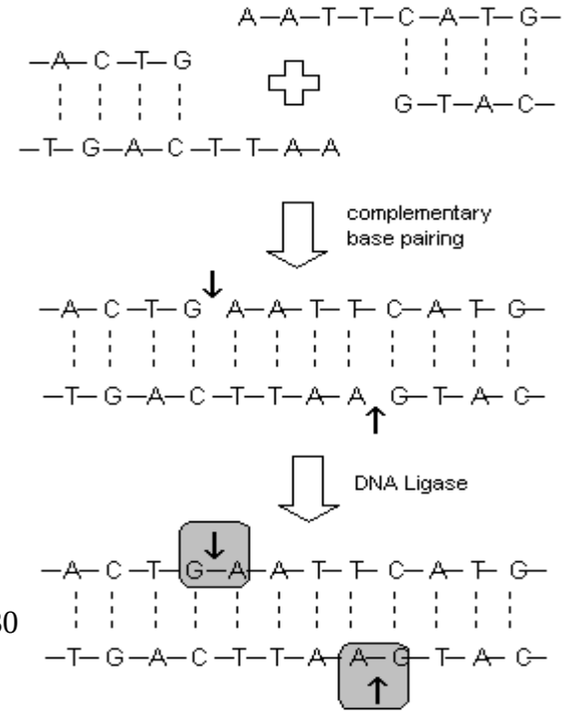
In addition there are restriction enzymes that cleave DNA some distance away from the sequence that they recognize. For example the enzyme HgaI makes staggered cuts that lie 5 and 10 nucleotides away from a 5 base pair sequence, GACGC. This leaves 5' overhanging ends, but, in contrast to the enzymes described above, these will be different almost every time the enzyme cuts (Why?).

The utility of the restriction enzymes

The discovery of these many restriction endonucleases have allowed genetic engineers to cut pieces of DNA at specific sites and into defined sizes. The result has been that a scientist can work with a collection of molecules all of the same size and with ends of known sequence. Restriction enzymes have proved to be valuable analytical and diagnostic tools as well

DNA Ligase

This enzyme repairs broken DNA by joining two nucleotides in a DNA strand. It is commonly used in genetic engineering to do the reverse of a restriction enzyme, i.e. to join together complementary restriction fragments The sticky ends allow two complementary restriction fragments to anneal, but only by weak hydrogen bonds, which can quite easily be broken, say by gentle heating. The backbone is still incomplete



8.2.1.5.3 Alkaline phosphatases

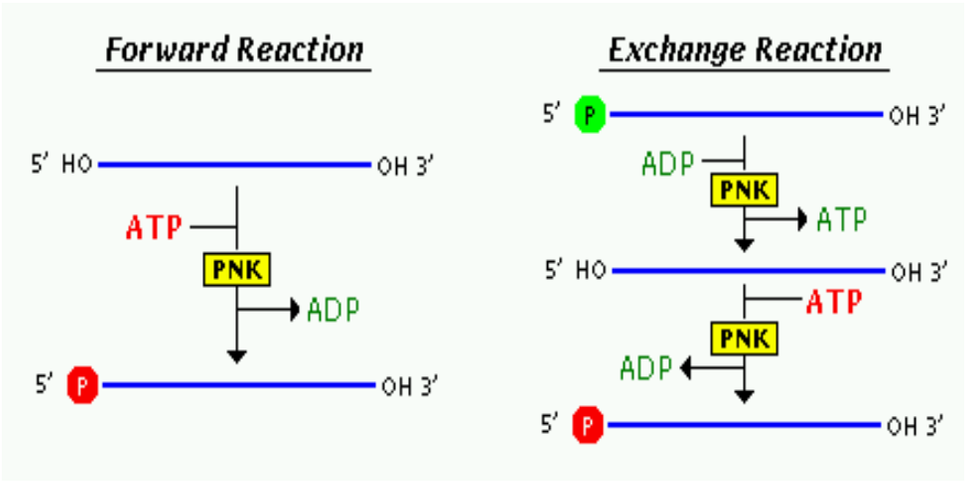
Alkaline phosphatases (AP) catalyze the removal of 5' phosphate groups from DNA, RNA, ribo- and deoxyribonucleoside triphosphates. Also they can remove phosphate groups from proteins. These enzymes are most active at alkaline pH - hence the name. There are several sources of alkaline phosphatase that differ in how easily they can be inactivated, such as bacterial alkaline phosphatase (BAP), calf intestinal alkaline phosphatase (CIP) and shrimp alkaline phosphatase. CIP is the most widely used AP and can be effectively destroyed by heat. There are two main applications for alkaline phosphatase in nucleic acid manipulations: 1)Removing 5' phosphates from plasmid and bacteriophage vectors that have been cut with a restriction enzyme. In subsequent ligation reactions, this treatment prevents self-ligation of the vector and thereby greatly facilitates ligation of other DNA fragments into the vector; 2) Removing 5' phosphates from DNA fragments prior to labeling with radioactive phosphate.

8.2.1.5.4 Polynucleotide Kinase

Polynucleotide kinase (PNK) is an enzyme that catalyzes the transfer of a phosphate from ATP to the 5' end of either DNA or RNA. It is a product of the T4 bacteriophage, and commercial preparations are usually products of the cloned phage gene expressed in E. coliThe enzymatic activity of PNK is utilized in two types of reactions:

- In the "forward reaction", PNK transfers the gamma phosphate from ATP to the 5' end of a polynucleotide (DNA or RNA). The target nucleotide is lacking a 5' phosphate either because it has been dephorphorylated or has been synthesized chemically.

- In the "exchange reaction", target DNA or RNA that has a 5' phosphate is incubated with an excess of ADP - in this setting, PNK will first transfer the phosphate from the nucleic acid onto an ADP, forming ATP and leaving a dephosphorylated target. PNK will then perform a forward reaction and transfer a phosphate from ATP onto the target nucleic acid.



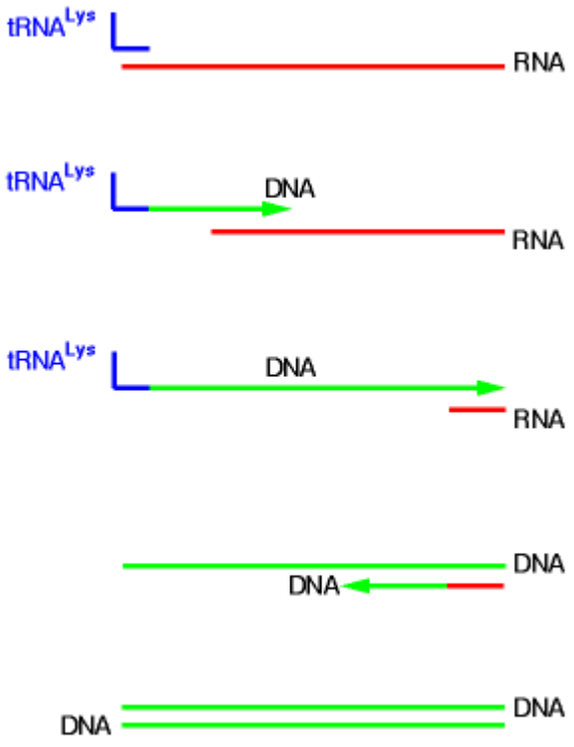
T4 Polynucleotide Kinase

T4 Polynucleotide Kinase (T4 PNK) is a polynucleotide 5'-hydroxyl kinase that catalyzes the transfer of the gama-phosphate from ATP to the 5'-OH group of single- and double-stranded DNAs and RNAs, oligonucleotides or nucleoside 3'-monophosphates (forward reaction). The reaction is reversible. In the presence of ADP, T4 PNK exhibits 5'-phosphatase activity and catalyzes the exchange of terminal 5'-phosphate groups (exchange reaction) The enzyme is also a 3'-phosphatase

8.2.1.5.5 Reverse Transcriptase

Reverse Transcriptase (RT) is a DNA polymerase that will either use an RNA or DNA strand as a primer. It is responsible for the production of a double stranded DNA copy of the single stranded RNA genome that is contained in the HIV-1 virus particle. Production of double stranded DNA is primed by the host cell lysine-tRNA Production of double stranded DNA is primed by the

host cell lysine-tRNA which partly unfolds and anneals to the 5' end of the viral genomic RNA. This is extended by the polymerase function of RT to give a DNA-RNA hybrid. The RNA component of this hybrid is degraded by the RNaseH function of RT once it has been copied. The polymerase function of RT is then able to synthesise the second strand of DNA



8.2.1.5.6 Exonucleases

These enzymes act upon genome and digest the base pairs of on 5^1 (or) 3^1 ends of a single stranded DNA or at single strand nicks or gaps in double stranded DNA. The major difference between

enxonucleases and endonucleases. Exonucleases cut the DNA at the ends endonucleases cleaves the double stranded DNA at any point except at the ends their action involves only one strand of the douplex.

8.2.1.5.7 S_2 nucleases

It degrades the single stranded DNA (or) single strand of double stranded DNA with cohesive ends. As a result of action of nuclease cohesive ends are converted in to blunt ends

8.2.1.5.8 DNA Polymerase

DNA polymerase plays the central role in the processes of life. It carries the weighty responsibility of duplicating our genetic information. Each time a cell divides, DNA polymerase duplicates all of its DNA, and the cell passes one copy to each daughter cell. In this way, genetic information is passed from generation to generation. Our inheritance of DNA creates a living link from each our own cells back through trillions of generations to the first primordial cells on Earth. The information contained in our DNA, modified and improved over millennia, is our most precious possession, given to us by our parents at birth and passed to our children.

8.2.1.6 summary

The recombinant DNA revolution in biology is rooted in the repertoire of enzymes that act on nucleic acids. Restriction enzymes

are a key group among them. These endonucleases recognize specific base sequences in double-helical DNA and cleave both strands of the duplex, forming specific fragments of DNA. These restriction fragments can be separated and displayed by gel electrophoresis. The pattern that they form on the gel is a fingerprint of a DNA molecule. A DNA fragment containing a particular sequence can be identified by hybridizing it with a labeled single-stranded DNA probe (Southern blotting).

8.2.1.7 Model questions:

1. what are the different kinds of enzymes used as tool in recombinant techonology?

2. what is southern blotting ? Explain

3. explain Agarose gel Electrophoresis?

8.2.1.8 Reference books

1. Bernard R. Glick and Jack J, Pasternak (1998), Molecular Biotechnology, 2nd edition, ASM press.

2. Old and Primrose (1994) Principles of gene manipulation 5th edition, Blackwell Scientific Publications Oxford, United Kingdom.

3. PK Gupta (2000) Elements of biotechnology, 1st edition, Rastogi publications.

4. H.D. Kumar (2001) A text book on Biotechnology, 2nd edition replica press Pvt. Ltd.

5. R.C. Dubey (2001) A text book of biotechnology, third edition, Rajendra, Ravindra Printers.

6. Sambrook J, E.F. Fritsch and T.Manialis 1989, Molecular cloning: a laboratory manbial, 2nd edition. Cold spring harbon laboratory, New York.

Lesson 8.2.2

ENZYMES USED IN GENE CLONING

8.2.2.1 Objective :

This lesson describe some important enzymes involved in Gene cloning and their importance. and their activities.

8.2.2.2 Introduction :

Before 1970 there was simply no method available for cutting a duplex DNA molecule in to discrete fragments. It became apparent that the related phenomenon of host controlled restriction and modification. Present day DNA technology is totally dependant upon our ability to cut

DNA molecules at specific sites with restriction endonuclease. The methods available for cutting DNA molecules we must consider the ways in which DNA fragments can be ligated to create recombinant molecules. The DNA ligase join covalently the annealed cohensive ende produced by certain restriction enzymes. The ability of DNA ligase from phage T4 – infected Ecoli to catalize the formation of phosphodiester bond between blunt – ended fragments. The enzyme terminal deoxy nucleotidyl transferase synthesizes homopolymer 3' single stranded tails at the ends of DNA fragments.

The fundamental unit of a genome that controls heredity is known as gene. Genetic engineering has been employed for the production of voluble polypeptides, insulin, interferons, growth hormones. Gene cloning alters the original sequence of the genes of an organism in a desired direction. Hence the process of gene cloning is also called genetic engineering. A new combination of genes is formed at the end of gene cloning. Thus the gene cloning results in the formation of a recombinant organism. The recombination has an altered sequen**ce** of genes in its genome. Hence this technology is often named Recombinant DNA technology. There are various biological tools which are used to carry out manipulation of genetic material and cells. These biological tools includes the enzymes.

8.2.2.3 Host controlled restriction and modification

Restriction enzymes allow to monitor the origin of incoming DNA and to destroy if it is recognized as foreign.

Restriction endonucleases recognize specific sequences in the incoming DNA and cleave the DNA in to fragments either at specific sites or more randomly. Modification involves methylation of certain bases at a very limited number of sequences with in DNA which constitute the recognition sequences for the restriction endonuclease. This explains the phage that survive one cycle of growth upon the restrictive host can subsequently re infect that host efficiently, their DNA has been replicating in the presence of the modifying methylase and so it likey that host DNA becomes methylated and protected from the restriction system restriction and modification of these processes can occur when DNA is transferred from one bacterial strain to another.

Restriction

A particular virus that plates effectively on one strain of host fails to plate effectively on another host strain its host range is said to be restricted.

Ex: If a λ phage is grown on E.coli strain C and this stock is titred upon E.coli C and E.coli K respectively then the efficiency of plating of λ is observed on these two strains will differ by several orders of magnitude. The titre on E.coli K is several fold lower. Thus the λ phage is said to be restricted by E.coli K.

Mechanism of restriction

Though the restricted phages absorb to restrictive hosts and inject their DNA normally their DNA is degraded soon after injection.

The restriction endonucleare are a batch of enzymes that cut the foreign DNA and degrade it thus preventing the phage development.

Restriction is a host controlled phenomenon.

Modification

The process by which the restrictive host protects its own DNA from the potentially lethal effects of the restriction endonuclease is called modification.

Mechanism of Modification

Modification occurs in the host DNA by methylation of the base pairs which resemble the cutting sites or recognition sites of restriction endonucleases, sequence specific methylation specific for same sequence, as host methylated sequences cannot be cut by restriction enzymes of host.

8.2.2.4 Restriction endonucleases

One critically important advance that has greatly stimulated the rapid progress in molecular biology and genetic engineering was the discovery of a set of enzymes that are capable of cutting DNA at defined sequences. These enzymes are found in a variety of microorganisms and are called **restriction endonucleases**, or more simply, restriction enzymes. The first specific restriction enzyme was discovered by Hamilton Smith in 1970, an accomplishment for which he (and two

others) were awarded the Nobel Prize. Since then, nearly 3,000 similar enzymes have been reported, many of which have different specificities.

(a) General characteristics and classification

The restriction endonucleaes were isolated from E.coli K by an experiment in which they incubated the fractionated cell extract with a mixture of unmodified and modified λ DNA'S which were differentially labeled with ^{3}H and ^{32}P. After incubation and sucrose gradiant centrifugation showed degraded unmodified DNA and un degraded modified DNA in separate bands since then hundred's of restriction enconucleases were isolated and characterized.

(b) General characters of restriction enzymes

They are encoded by the restrictive host (host controlled).

They are coded by the genes located on the chromosome or the genes located on the plasmid.

They perform the function of restriction and modification.

They recognize the foreign DNA and degrade it, they cannot cut the hosts own DNA because it is modified.

(c) Classification of restriction enzymes

Depending upon the number of substrates, cofactor requirement, and symmetry of cutting the cleavage sites and coupling of restriction

and modification functions. The restriction enzymes are classified in to three types. Type I, II and III.

Type I

- ➔ It is a single multifunctional enzyme. Same enzyme acts as endonuclease and methylase.(modification)
- ➔ It has three different subunit.S,Rand M, S for specificity to bind to the DNA, R for restriction activity and M for modification.
- ➔ IT requires ATP Mg^{2+} for its activity.
- ➔ Recognizes a sequence of 8-10 base pairs and cuts randomly at least 1,000 base pairs away from recognition/host specificity site.
- ➔ If the restriction site in the DNA is hemimethylated, the enzyme binds to the site and modify the site by adding the methyl group to other strand , if it unmethylated it cuts the DNA
- ➔ It methylases host DNA at host specific sites.
- ➔ It cannot be used in recombinant DNA technology because of non specificity cutting.

Type II

The organisms containing the type II restriction endonucleases, has two separate enzymes for restriction and modification.

- ➔ It is composed of two homo dimers
- ➔ It requires only Mg^{2+} for nuclease function.
- ➔ These enzymes cut the restriction sites near or within the site they recognize
- ➔ Most widely used enzymes in recombinant DNA technology. Most abundant in microorganisms
- ➔ There are many kind of type II restriction endonuclease identified so far

Type III

A rare kind of restriction endonuclease which occurs as complex of two subunits. M and R.

- ➔ Subunit M is responsible for recognition and modification of specificity sites.
- ➔ Subunit R is responsible for nuclease action.
- ➔ Nuclease subunit requires Mg^{2+} and ATP are cofactor.

- ➔ Recognition sites are asymmetric and cleavage occurs by nicking one strand at a measured distance to one side of recognition site, 24-26 base pairs to 3' end of specificity site.

- ➔ So two target site in opposite orientation are necessary to break the DNA strand

- ➔ Because of this reason the Type III restriction endonucleases are not applicable in recombinant techonology

Mcr Systems

There are some restriction systems called Mcr. modified cytosine restriction. The modified methyl cytosine of some phages may escape the normal endonuclease action (Type I, II and III). But Mcr systems recognizes the modified or methyl cytosine sites also and restricts such phages.

- ➔ The genes for restriction enzymes are located in the main chromosome (or) in the plasmid. Some times they are also encoded by the Prophages. Like elements.

(d) Nomenclature of restriction enzymes

Smith and another Nobel laureate, Daniel Nathans, devised a nomenclature for these enzymes

The discovery of large number of restriction enzymes in the recent part called for a uniform nomenclature. The following rules are observed in nomenclature

1. The specific name of the host organism is represented by three letter abbreviation in Italics, the first letter being of generic name (in capital) and the second and letters of specific in small letters.

Ex: E.coli – *Eco*

Haemophilus influenca – *Hin*

2. Strain/type identification is represented by a subscript.

Ex: Eco_k Hin_d

3.If a particular strain has several different restriction modification systems they are identified each by a Roman number not to be confused with type of restriction endonuclease.

Hsn_dI Hin_dII Hin_dIII

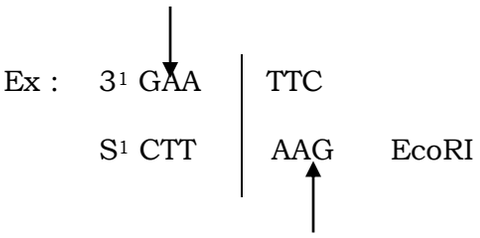
4.In case where restriction and modification enzymes are separate the restriction enzyme is given a general name R followed by system name and the methylase is given a general name M follows by system name.

Ex: *R* Hin_dIII, *M* Hin_dIII

(f) Target sites and cutting pattern

Rotational symmetry

Particular sequences of DNA containing tetra penta hexa or hepta nucleotides which have two fold axis of rotational symmetry are recognized by vast majority of Type II restriction endonuclease.



These sequences resemble palindromes, that is words that read alike either backwards and forward.

Sticky and cutting

Some restriction endonucleases makes single strands breaks four nucleotides apart in the opposite strands of its target sequence so generally fragments with protruding 5' termini which can associate by hydrogen bonds and by over lapping 5¹ termini circularize by intermolecular ends. Hence the name sticky (or) cohesive ends. These cohesive ends in principle can offer possibly to join fragments from diverse source to be joined and by seaking the nicks.

Ex: *Pst I*

Providentia stuartii : S¹ T | C G A 3¹
3¹ A G C | T S¹

Blunt and cutting

Some restriction enzymes cut the DNA at the target sequences giving rise to blunt ends.

Ex*: Hae III Haemophilus aegyptius*

G G | C C
C C | G G

Some restriction endonuclease can produce even 3'protruding ends

Some restriction enzymes and their cleavage sites

Microorganism	Enzyme abbreviation	Sequence	Note
Haemophilus aegyptius	Hae III	S^1 G G | C C 3^1 3^1 C C | G G S^1	1
Staphylococcus aureus 3A	Sau 3AI	S^1 | G A T C 3^1 3^1 C T A G | S^1	2
Bacillus amyloliquefaciens H	Bam HI	S^1 G G A | T C C 3^1 3^1 C C T A G | G S^1	2
Thermus aquaticus	Taq I	S^1 T | C G A 3^1 3^1 A G C | T S^1	2

Escherichia coli RY 13	Ecor I	S^1 G | A A T T C 3^1 3^1 C T T A A | G S^1	2
Haemophalus influenia	*Hind II*	S^1 G T Py / Pu A C 3^1 3^1 C A Pu / Py T G S^1	1,5
	Hind III	S^1 A | A G C T T 3^1 3^1 G A C G A | S^1	2
Providentia stuarti	*Pst I*	S^1 C T G C A | G 3^1 3^1 G | A C G T C S^1	3
Haemophilus haemolyticus	Hha I	S^1 G C C | G 3^1 3^1 C | G G C S^1	3
Serratia marcescens	Sma I	C C C | G G G G G G | C C C	1
Xanthomane malvacearum	X maI	C | C C G G G G G G C C | C	2
Moranella bovis	Mbo II	G A A G A N8 | C T T C T N7 |	4

Note :

1. Produces blunt ends

2. Produces cohesive end with 5^1 single stranded over hang.

3. Produces cohesive end with 3^1 single stranded over hang.

4. This is a type II S enzyme. It does not cut with in the recognition sequence but at whatever sequence lies to the right as shown N is any necleotide.

5. Pu is any purine [A (or) G]

   Py is any pyramidine [C (or) T]

(d) Role of restrictive systems invivo

Restriction system is analogous to immune system in higher organisms to distinguish between self and non self DNA. However it is moderately effective in providing immunity to phage infections. This is because bacteriophage evolve some mechanisms to evade the action of restriction endonuclease system.

8.2.2.5 DNA ligase

Mertz and Davis (1972) for the first time demonstrated that cohesive termini of cleaved DNA molecules could be covalently sealed with E.coli DNA ligase and were able to produce recombinant DNA molecules. DNA ligase seals single strand ricks in DNA which has 5'phosphate and 3' hydroxyl termini. There are two enzymes which are

extensively used in covalently joining restriction fragments. The ligase from E.coli and that encoded by T_4 phage. The main source of DNA ligase is T_4 phage, hence the enzyme is known as T_4 DNA ligase.

For the joining reactions the E.coli DNA ligase uses NADP as a cofactor while T_4 DNA ligase requires ATP. In both cases cofactor breaks in to AMP which in turn adenylate the enzyme to form Enzyme AMP complex. This complex binds to nick containing 3^1 – OH and 5^1 – phosphate ends on a double stranded DNA molecule. The 5^1 phosphory terminus of the nick is adenylated by the Enzyme AMP complex with 3^1-0H terminus resulting in formation of phosphodiester and liberation of AMP. After the formation of phosphodiester nick is sealed. T_4 enzyme has the ability to join the blunt ends of DNA fragments. Where as E-coli DNA ligase join the cohesive ends produced by restriction enzymes. Additional advantage with T_4 enzymes is that it can quickly join and produce the full base pairs. Cohesive end ligation proceede about 100 times faster then the blunt end ligation. Action of DNA ligase shown in Fig.3.1.

Fig. 3.1 Action of DNA ligase.

8.2.2.6 Alkaline phosphotase

When plasmid vector, for joining a foreign DNA fragment is treatment with restriction enzymes the major difficulty arises at the same time. Because the cohesive ends of broken plasmids instead of joining with foreign DNA join the cohesive end of the some DNA molecules and get recircularized. To overcome this problem the restricted plasmid is treated with an enzyme alkaline phosphotase, that digests the terminal 5^1 phosphoryl group. The restriction fragments of the foreign DNA to be cloned are not treated with alkaline phosphotase. Therefore the 5^1 end of foreign DNA fragment can covalently join to 3^1 end of the plasmid. The hybrid (or) recombinant DNA obtained has a nick with 3^1 OH group and 5^1 OH ends. Ligase will only join 3^1 and 5^1 ends of the recombinant DNA to gether if the 5^1 end is phosphorylated, thus alkaline phosphotase and ligase prevent recircularization of the vector and increase the frequency of production of recombinant DNA molecules. The nicks between two 3^1 ends of DNA fragment and 5' vector DNA are repaired inside the bacterial host cells during the transformation.

Mechanism of alkaline phosphotase as shown in Fig.3.2.

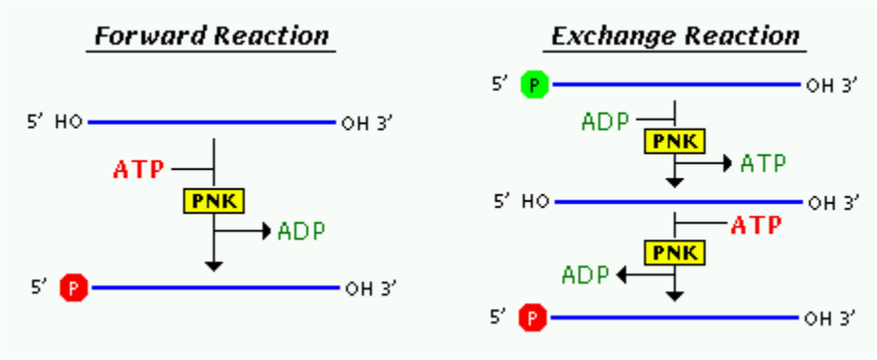


Fig.3.2 Mechanism of Alkaline phosphotase.

8.2.2.7 Reverse transcriptase

In addition to these enzymes reverse transcriptase is used to synthesize the copy DNA or complementary DNA (cDNA) by using m-RNA as a template. Reverse transcriptase is very useful in the synthesis of cDNA and construction of cDNA clone bank. It was known that the genetic information of DNA pass to protein through mRNA. During 1960's Teman and coworkers postulated that in certain cancer causing animal viruses which contain RNA as genetic material transcription of

cancerous genes takes place most probably by DNA polymerase directed by virus RNA. Then DNA is used as template for synthesis of many copies of viral RNA in a cell. Reverse transcriptase, which is produced by certain RNA viruses uses an RNA strand as a template while directing deoxy ribo nucletides in to the growing chain. Thus when an A,G,C or u residue of the templet RNA strand is encountered, the complementary deoxy ribo nucleotide is incorporated in to the growing DNA strand. The synthesis of DNA strand by reverse transcriptase in vitro is often incomplete, however before synthesis ceases the DNA stand usually turned back on it self for a few nucleotides.

The second DNA strand is synthesized by the addition of the klenow fragment of E.coli DNA polymerase which uses the first DNA strand as a template and adds deoxy ribo nucleotides to the growing strand starting from the end of the hair pin loop. After the reaction is complete the sample is treated with the enzyme RNAase which degrade the mRNA molecules and with S_1 nucleases which opens the hair pin loops and degrade single stranded DNA extensions. At the end of this procedure the sample contains a minute of partial and complete double stranded complementary DNA copies of the more prevalent mRNAs in the original sample. The mechanism of reverse transcriptase as shown in Fig.3.3.

Fig. 3.3 Mechanism of reverse transcriptase.

8.2.2.8 DNA Polymerase

This enzyme polymerizes the DNA synthesis on DNA template and also catalyses a 5^1 - 3^1 and 3^1 – 5^1 exonuclease degradation of DNA. The DNA polymerase investigated by A.Kornberg and coworkers in E.coli in 1956 is now known as DNA polymerase I. The other two enzymes are DNA polymerase II and DNA polymerase III. These have almost similar catalytic activity. DNA polymerase I mol. Wt 1,09,000 has a single polypeptide chain of about 1,000 aminoacid residues. The addition of mononucleotide to the free hydroxy end of a DNA chain is catalyzed by this enzyme. It catalyses the reactions that is 3^1 – 5^1 exonuclease activity and 5^1 – 3^1 exonuclease activity. DNA polymerase useful for the growing of DNA strand. The incoming nucleotides is enzymatically joined by phosphodiater linkages to the 3^1 – 0H group of the last nucleotide that was incorporated in the growing strand. The action of DNA polymerase show in Fig.3.4 & 3.5.

Fig.3.4 The incoming nucleotide is a deoxy ribo nucleotide triphosphate that is directed by DNA polymerase to pair with the complementary strand.

Fig.3.5 The α phosphate of the incoming nucleotide forms a phosphodiester bond with the 3^1 hydroxyl group of the growing strand.

DNA polymerase have mol.wt 120,000. It catalyses 3^1 – 5^1 enonuclease activity. DNA poly III have mol.wt 140,000 is about several times more active than the other two polymerase. It is a dimmer of DNA polymerase III core enzymes it requires an auxillary protein called as loader protein and after combination yield a DNA polymerase III halo enzyme complex. It produces a parallel strands in the presence of ATP.

8.2.2.9 Exonucleases

These enzymes act upon genome and digest the base pairs of on 5^1 (or) 3^1 ends of a single stranded DNA or at single strand nicks or gaps in double stranded DNA. The major difference between enxonucleases and endonucleases. Exonucleases cut the DNA at the ends endonucleases cleaves the double stranded DNA at any point except at the ends their action involves only one strand of the douplex. The difference of action accure both exonuclease and endonucease are shown in Fig.3.6.

Fig.3.6 Action of restriction endonuclease.

8.2.2.10 S_2 nucleases

It degrades the single stranded DNA (or) single strand of double stranded DNA with cohesive ends. As a result of action of nuclease cohesive ends are converted in to blunt ends.

8.2.11 Polynucleotide kinase

Polynucleotide kinase plays an very important role for the phosphorylotion. That means addition of phosphate group to bases. This phospharylation plays an very important role for the formation of recombinat DNA molecule.,During ligation the 5’ ends converted ti 5’ OH ends , to prevent vector recolourse, after the foreign DNA is inserted the 5’ OH ends of the DNA is converted back to 5’ phosphate ends by the enzyme polynucleotide kinase.

The enzymatic activity of PNK is utilized in two types of reactions:

- In the "forward reaction", PNK transfers the gamma phosphate from ATP to the 5' end of a polynucleotide (DNA or RNA). The target

nucleotide is lacking a 5' phosphate either because it has been dephorphorylated or has been synthesized chemically.

- In the "exchange reaction", target DNA or RNA that has a 5' phosphate is incubated with an excess of ADP - in this setting, PNK will first transfer the phosphate from the nucleic acid onto an ADP, forming ATP and leaving a dephosphorylated target. PNK will then perform a forward reaction and transfer a phosphate from ATP onto the target nucleic acid.

8.2.2.12 Terminal trnasferase

An enzyme purified from calf thymus, provides the means by which homopolymeric extensions can be synthesized. It will repeatedly add a polulation of nucleotides to the 3^1 – OH termini of the DNA

8.2.2.13 Summary

Recombinant DNA technology comprises a battery of experimental procedures used to isolate clone pieces of DNA that contain specific genes. The success of gene cloning depends on being able to cut DNA molecules reproducibly in to fragments of discrete sizes. This precise cleaving of DNA is achieved by using type II restriction endonucleases. These enzymes bind to specific sequences with in a DNA molecule and symmetrically cut phosphodiester bond of each strand at the recognition site.

DNA ligase plays an important role for the joining of DNA molecules. Alkaline phosphatases plays an important role for the removal of the phosphate groups of the terminal ends. This prevents the direct ligation of DNA molecules.

Reverse transcriptase plays an important role for the formation DNA from m-RNA molecule. DNA polymerase plays an important role for the synthesis of DNA.

8.2.2.14 Model Questions

1. What is the uses of restriction endonucleases in gene cloning.
2. Write about nomenclature and types of restriction endonucleases.
3. Explain different types of enzymes used in genetic engineering and their importance.

8.2.2.15 Reference Books

1. Bernard R. Glick and Jack J, Pasternak (1998), Molecular Biotechnology, 2nd edition, ASM press.
2. Old and Primrose (1994) Principles of gene manipulation 5th edition, Blackwell Scientific Publications Oxford, United Kingdom.
3. PK Gupta (2000) Elements of biotechnology, 1st edition, Rastogi publications.
4. H.D. Kumar (2001) A text book on Biotechnology, 2nd edition replica press Pvt. Ltd.

5. R.C. Dubey (2001) A text book of biotechnology, third edition, Rajendra, Ravindra Printers.

6. Sambrook J, E.F. Fritsch and T.Manialis 1989, Molecular cloning: a laboratory manbial, 2nd edition. Cold spring harbon laboratory, New York.

Lesson 8.2.3

VECTORS

8.2.3.1 Objective

8.2.3.2 Introduction

8.2.3.3 Plasmids
- (a) Salient features and properties of plasmid DNA
- (b) Classification
- (c) Purification of plasmid DNA
- (d) Plasmid as a vector
- (e) PBR 322
- (f) PBR 327
- (g) PUC Vector
- (h) PACYC 184 plasmid
- (i) PVN 121
- (j) Yeast Plasmid vector
- (k) Ti and Ri plasmid

8.2.3.4 Bacteriophages
- (a) Salient features of λ DNA
- (b) Life cycle of λ phage
- (c) Lambda phage vector
  - (i) λgt 10, λgt11
  - (ii) EMBLS, EMPLY
  - (iii) Charon 34 and charon 35
  - (iv) M_{13} phage

8.2.3.5 Cosmid
- (a) Characteristic features of cosmid

8.2.3.6 Phasmids
8.2.3.7 PI cloning vectors for cloning large DNA segments
8.2.3.8 F-factor based vector
8.2.3.9 Plant and Animal Viruses as vector
8.2.3.10 Transposone as vector
8.2.3.11 Binary (or) shuttle vectors

8.2.3.12 Summary

8.2.3.13 Model Questions

8.2.3.14 Reference Books

8.2.3.1 Objective

The lesson explains the different kinds of vectors and their application is recombinant DNA technology.

8.2.3.2 Introduction

A variety of vectors have been developed which not only allow multiplication but also may be manipulated in such a way that the inserted gene may be express in the host The cloning of DNA is possible only with the help DNA molecule which is a vector, which is capable of replicating in a host. This other DNA molecule often used as a vector could be plasmid, bacteriophage, a derived cosmid, phagemid, a transposon or even a virus. The desired feature of any cloning vector is that it should possess a site at which foreign DNA can be inserted without disrupting any essential function. Some times vectors are modified by inserting a DNA segment to create unique sites for one (or) more enzymes to facilitate its use in gene cloning. These vector's act as a cloning vehicles because they carry the foreign DNA to be expressed in the host cells in to which they are transformed.

8.2.3.3 Plasmids

Plasmids are the extra chromosomal circular DNA molecules found in bacterial cells. They are inherited sharply without the influence of chromosomal DNA. They replicate independently from the replication of chromosomal DNA. Bacterial plasmid DNA's are double stranded in nature. The bacterial plasmid DNA's exist in three forms namely covalently closed circular DNA, linear DNA and as super coiled DNA.

(a) Sailent features of plasmid DNA (or) Basic Properties of Plasmid DNA

The plasmid DNA is a circular double stranded DNA molecule found in the protoplasm of bacterial cells. It is inherited from organism to organism without the influence of chromosomal DNA. The replication of the plasmid DNA is independent from the replication of chromosomal DNA. In the bacterial cells plasmid DNA's exist in three forms. They are covalently closed circular DNA, linear DNA and super coiled DNA. The twisting and super coiling of DNA are caused by the enzyme topoisomerase. Types of plasmid are shown in Fig.3.2.

The number of copies of plasmid DNA'S present in each bacterial cell is constant for many generations. Some plasmid DNA'S get integrated with the chromosomal DNA, these integrations results in the formation of episomes. The replication of these episomes depends on the replication of chromosomal DNA. The number of copies of plasmid present in a bacterial cell varies. Some cell contain high copy number plasmids or relaxed plasmid which the number of plasmids per cell is between 10 to 100 and some cell contain low copy number plasmid called as stringent plasmid , the no of stringent plasmid ranges between 1 to 10.The plasmid DNA'S replicate either through the rolling circle model (or) through the D-loop formation. Plasmids are widely distributed throughout the prokaryotes. These plasmids vary in size from less than 1 x 10^6 Daltons to greater than 200 x 10^6 Daltons. Plasmids can be categorized into one of two major types conjugative (or) non conjugative depending upon whether (or) not they carry a set of transfer genes called the "tra" genes that promotes bacterial conjugation. Plasmids can also be categorized on the basis of their being maintained as multiple copies per cell. (relaxed plasmids) (or) as a limited number of copies per cell (stringent plasmids).

The plasmids used for recombinant DNA technology should be ideal for cloning they should posses certain characteristics

→ Should contain origin of replecation

→ Multiple cloning site

→ Low M.wt.

→ Small in size

→ Genetic markers such as antibiotic resistance genes

Fig.3.7 Types of plasmids.

(b) Classification

On the basis of the function of plasmids they are classified.

R-plasmids

These plasmids give resistance to the organism against one (or) a few antibiotics. They transfer their resistance from one organism to another organism during conjugation. They are important in field of medicine and Agriculture.

Virulance plasmid

These plasmids encode for the production of some specific to proteins which increases the virulence of pathogens for example end plasmid is a virulence plasmid which encode for the production of endotoxins. The endotoxins increases the pathogenicity of pathogens..

Beneficial plasmids

These plasmide carry some genes along with their original sequence and integrate them with the transfer of genes from one organism to another organism. Ex: Ti plasmid.

- ➔ Some plasmids give resistance to the organism against heavy metals example P 1258, and R6. Some other plasmids provide resistance to the organism against U.V. rays. They protect the cells against the action of U.V. rays.

Some phenotypic traits exhibited by plasmid carrier genes :

Antibiotic resistance

Antibiotic Production

Degradation of aromatic compounds

Haemolysin production

Sugar fermentation

Heavy metal resistance

Induction of plant tumors

(C) Purification of plasmid DNA

A obvious prerequisite for cloning is the purification of the plasmid DNA. The isolation and separation of plasmid are mainly based on the shape and size of plasmid.

In general plasmids are isolated by breaking the cells with a detergent and the cellular contents is centrifuged with Cscl. But biotechnological works need cent percent pure plasmid DNA. So proper care must be taken during the purification and separation of plasmid DNA'S to avoid the unwanted chromosomal DNA fragments and proteins. Some times large plasmid DNA'S also undergo breakdown and go along with the chromosomal DNA'S during their isolation. So the breakage of plasmid must be avoided.

Pre treatment of the plasmid

The number of copies of plasmids in bacterial cells must be increased before the isolation of plasmid DNA. This process helps to isolate a large amount of plasmid DNA'S from the bacterial culture. For this purpose the bacterial culture is treated with chloramphenicol which inhabit the protein synthetic machinery of

bacterial cells. The blocking of protein synthesis results in a rapid increase in the number of plasmid DNA'S in the cells.

Isolation procedure

The cultured bacterial cells are treated with EDTA and lysozyme. The EDTA and lysozyme degrade the complex polysaccharide of bacterial cell wall, some amount of sucrose solution is added to the culture to equalize the osmotic potential of the culture solution to that of the bacterial cells. Thus sucrose prevents the lysis of the wall less bacterial cells.

The wall less cells are then lysed by treating them with sodium lauryl sulphate (SLS) solution. The cell lysate is centrifuged to remove the larger and smaller debris from the cell lysate. During the centrifugation of cell lysate, the chromosomal proteins and the larger RNA molecules rapidly reach the bottom of the centrifuge tube and are removed carefully from the tube. The supernatant solution contains plasmid DNA'S along with some fragments of chromosomal DNA.

This supernatant solution is again centrifuged with cscl. (Cesium chloride) to remove the chromosomal DNA fragments from the plasmid DNA.

The removal of chromosomal DNA fragments from the plasmid DNA'S is due to the difference in the number of CG base

pairs found in the plasmid DNAS and in the chromosomal DNAS. Hence two clear bands are formed each band is separately collected from the centrifuge tube. The plasmid DNA thus obtained are purified for further use.

In some cases there is no difference in the number of C-G bases pairs in between the chromosomal DNA and plasmid DNA. Hence a red dye ethidium bromide is used for the separation of plasmid from chromosomal DNA.

The dye gets incorporated with in the gaps between the adjacent base pairs of large linear chromosomal DNA. But the circular plasmid DNA'S do not allow the dye to bind with them. The dye creates a difference in the molecular weight between the plasmid DNA and the chromosomal DNA fragment. Then they are separated by centrifuging the solution in the presence of cesium chloride density gradient.

Separation of different forms of plasmid DNA'S from the mixture of plasmids. There are three forms of plasmid DNAS, covalently closed circular plasmids, linear plasmids and super coiled plasmids. In these three types of plasmids differ from each other in their sedimentation coefficient. This property is used for the separation of one type of plasmids from other types of plasmids. The plasmid mixture is centrifuged with the help of sucrose density

gradient which separates all the three forms of plasmids according to their specific density.

During the centrifugation the covalently closed circular DNA'S sediment more rapidly with great velocity and reach the bottom of the centrifuge tube the super coiled plasmids move and form a layer just above the covalently closed circular DNA'S and the linear plasmids form an upper layer in the centrifuge tube. These different forms of plasmids are carefully removed from the centrifuge tube and are used as vectors in gene cloning. The Se value of a linear plasmid DNA is ISS, the S value of a super coiled plasmid is 17S and that of covalently closed circular plasmid is 23S. The brief explanation of plasmid isolation as shown in Fig.3.8.

Figure 3.8 Plasmid isolation procedure.

Gel electrophoresis

Gel electrophoresis is also used in the separation of plasmid DNA's. The mixture of plasmid DNAS is electrophorsed through an agarose gel which separates the plasmids from one another due to the difference in their size and molecular weight. The smaller plasmids move rapidly along the gel while the larger plasmids move slowly. Thus clear bands are formed in the agarose gel. The agarose gel is treated with fluorescent dye and exposed to auto radiographic film. Dark bands appear on the autoradiogram. The corresponding regions in the agarose gel is removed and the plasmid DNA'S are separated from it for further use.

(d) Plasmid is a vector

Chang and Cohen proved that the plasmid act as vectors which participates in the transfer of certain genes from one organism to another organism. Gene cloning alters the original sequence of the gene of an organism in a desired direction. Hence the process of gene cloning is also called genetic engineering.

All the plasmids present in the cells are not useful for the genetic engineering works because some plasmids lack the property of carrying the foreign gene along with their own sequences. Some plasmids successfully replicate only in the cells from which they were isolated but fail to replicate in the recipient cell.

Desirable properties of plasmid cloning vehicle

An ideal plasmid vector must have the following characteristic features for the successful gene cloning they are :

1. They should be high copy no plasmids
2. They should be of low molecular weight and smaller in size
3. The plasmid should contain a redly scorable phenotypic character
4. They should contain a multiple cloning site so the foreign DNA fragment generated by any restriction endonuclease would be able to be inserted in to the vector
5. The plasmid must have the capability to transfer the foreign gene in to the recipient cell.
6. The plasmid must be a shuttle vector which replicates both in the donor cell and in the recipient cell.
7. The plasmid should not have any pathogenic property.
8. The plasmid must have its own regulatory genes for the successful replication.

Usefulness of natural plasmids as cloning vehicles

The term nature is used to describe the plasmids which were not constructed in vitro for the sole purpose of cloning.

Example include,

Col E1, a naturally occurring plasmid, PSC 101, RSF 2124.

Col E1 plasmid size is 4.2 k.d restriction site EcoRI, immunity to colicin E1

Use – colicin E1 production

RSF 2124 – size 7.4 kd resriction sites – EcoRI, BamHI

Use – colicin E1 production

PSC – 101 plasmid size 5.8 kd restriction sites EcoRI, Hind III Bam HI.

(e) Construction and characterization of a new cloning vehicle : PBR 322

PSC 101, Col EI and RSF 2124 can be used to clone DNA. They suffer from a number of disadvantages. For this reason considerable effort has been expended on constructing invitro superior cloning vehicles.

The most versatile and widely used of these artificial plasmid vector is PBR 322 plasmid PBR 322 contains the AMP^R and TC^R genes, genes of RSF 2124 and PSC 101 respectively.

PBR 322 palsmid vector as shown in Fig.3.9.

Plasmid PBR 322 is small in size it is made up of 4362 base pairs long. The sequence was revised by the inclusion of an additional CG base pair at position 526, thus increasing the size of the plasmid to 4363 base pairs. The most useful aspect of the DNA sequence is that it totally characterizes PBR 322 in terms of its restriction sites such that the exact length of every fragment can be calculated.

The plasmid has a number of restriction sites for the action of different types of restriction enzymes. The restriction enzymes break the DNA at their restriction sites in the plasmid. As a result the linear plasmid DNA with cohesive tail is formed. The foreign gene can be inserted at the restriction site. There are over 20 enzymes with unique cleavage sites on the PBR 322 genome.

The target sites of seven of these enzymes (ECOR I, Bam HI, Sai I, Xma III, Nhe I, Nru I Sph I) lie with the Tc^R gene and there are

sites for a further two (Cla I and Hind III) with in the promoter of that gene.

There are unique sites for three enzymes (Pst I, Pvu I and Sca I) within the AP^R gene. Thus cloning in PBR 322 with the aid of any one of those 12 enzymes will result in insertional inactivation of either the AP^R or the TC^R markers. However cloning in the other unique sites does not permit the easy selection of recombinants because neither of the antibiotic resistance determinants is inactivated.

Plasmid PBR 322 is the most widely used cloning vehicle. In addition it has been widely used as a model system for the study of prokaryotic transcription and translation as well as investigation of the effects of topological changes on DNA confirmation.

Figure 3.9 PBR 322 plasmid vector.

(f) PBR 327

Another vector derived from PBR 322 by deletion of nucleotides between 1,427 to 2,516. These nucleotides are deleted to reduce the size of the vector and to eliminate sequences that were known to interfere with the expression of the cloned DNA in eukaryotic cells. PBR 327 still contains genes for resistance against two antibiotics (Tetracycline and Ampencillin). Both PBR 322 and PBR 327 are very common plasmid vectors.

(g) PVC vectors

Another series of plasmids that are used as cloning vectors belong to puc series. These plasmids are 2,700 base pairs long and possess Ampencillin resistance gene and the origin of replication derived from PBR 322. The Lac Z gene derived from E.coli with in the Lac region is also found. A poly linker sequence having unique restriction sites when DNA fragments are cloned in this region of PUC the Lac gene is inactivated. These plasmids when transformed in to an appropriate E.coli strain which is a Lac mutent and grown in the presence of IPTG (Isopropyl thiogalactoside) which behaves

like lactose and induces the synthesis of β-galactosidase enzyme and x-gal substrate for the enzyme will give rise to white (or) blue colored clear colonies on the other hand PUC having no inserts and transformed in to bacteria will have an active Lac Z gene and therefore will produce blue colonies thus permitting identification of colonies having PUC vector with cloned DNA segments. The cloning vectors belonging to PUC family are available in pairs with reversed orders of restriction sites relative to Lac Z promoter. PUC 8 and PUC 9 other similar pairs include PUC 12 and PUC 13 (or) PUC 18 & PUC 19 PUC vector structure as shown in Figure 3.10.

Figure 3.10 Plasmid PUC vector.

(h) PACYC 184 plasmid

Plasmid contains two sequences for giving existence to the organism against drugs, one sequence gives it resistance against

Tetracycline while the other sequence gives it existence against Chloramphenicol. If the gene is cloned at EcoRI restriction site, the plasmid fails to give the organism resistance against Chloramphenicol.

Sal I, Bam HI cut the plasmid at a sequence which gives the organism resistance against Tetracycline. So it the gene is cloned at the restriction sites, the plasmid fails to give resistance to the organism against Tetracycline. Hind III cuts the plasmid at a sequence which is free from those sequences giving resistance to the organism against Tetracycline and Chloramphenicol so if the gene is cloned at Hind III restriction site, the transformed organisms grow successfully in the medium containing both of these drugs.

(i) PVN – 121 Plasmid

Contains two sequences for giving resistance to the organism against the drugs. One sequence gives it resistance against Ampencillin and the other sequence gives it resistance against Tetracycline.

(j) Yeast plasmid vectors

Special vectors are also known for introducing DNA segments in yeast cells, a Eukaryotic system that has been used extensively for developing genetically engineered yeast cells. Although E.coli

plasmids or phages can be used for transfer of genes to yeast cells. The possible examples of the yeast plasmid vectors are YIP (or) Yest integrative plasmids, YEP (or) yeast episomal plasmids.

(k) Ti and Ri plasmids as vector for higher plants

In higher plants Ti plasmid of Agrobacterium tumefaciens (or) Ri plasmid of Agrobacterium rhizogenes is the best known vector. T-DNA from Ti (or) Ri plasmid of Agrobacterium is considered to be a very potential vector for cloning Experiments with higher plants. The foreign DNA has to be first cloned in to T-DNA of Ti (or) Ri plasmid modified hybrid T-DNA can be transferred to the genome of plant cells by Agrobacterium infection. These recombinant Ti plasmids can then be used for transformation of higher plants. This system can be widely used. Agrobacterium infects nearly all dicotyledonous plants. Such cloning in plants bas proved to be of immense use to modify agricultural plants to increase their productivity.

8.2.3.4 Bacteriophage λ

Bacteriophage provide another source of cloning vectors since usually a phage has a linear DNA molecule, a single break will generate two fragments which are later joined to with foreign DNA to generate a chimeric phage particle. This explain in Figure 3.11.

Figure 3.11 Cloning of foreign DNA in a phage vector.

Coli phage λ is one of the temperate phages, it infects the E.coli cells. It was first isolated by Lederberg in 1951. The life cycle of λ phage is a lysogenic cycle in which the host bacterium is not broken. This is because λ DNA does not interfere with the synthetic machinery of the host cells. The viral genes get integrated with the chromosomal DNA of bacteria and replicate along with the chromosomal DNA of the host cell. In this condition the phage DNA is called prophage and the bacterium containing the prophage is called lysogenic bacterium. The λ DNA is expressed in the host bacterium. The λ phage has the molecular wt of 57×10^6 Daltons but its DNA is 32×10^6 Daltons in its molecular weight.

(a) Sailent features of λ DNA

It is a double stranded DNA which may exist in linear form or circular form. The linear λ DNA has cohesive ends, each of the ends is complementary to another end. The cohensive end or the single stranded tail ends with free 5^1 phosphate group.

The circular form of DNA is formed due to the base pairing between the complementary bases found in its cohensive ends. Some times a number of λ DNA'S join together end to end and form a large sized nucleic acid multimer called concatmer.

The base composition of λ DNA differs The λ DNA contains about 40 genes of which only 18 genes are essential for lytic (or) lysogenic cycle of phages. The remaining genes may precipitate in some other cellular functions. The λ DNA contains two sequences for viral assembly of which one sequence participates in the synthesis of head protein while the other participates in the synthesis of tail proteins.

The λ DNA has three regulator genes, three promoter genes and a few termination sites for the regulation of its structural and functional genes.

(b) Life cycle of λ phage

In general both lytic life cycle and lysogenic life cycle occur in λ phages. But the frequency of lytic life cycle is very low when

compared with that of lysogenic life cycle. The frequency of lytic life cycle is increased by exposing the phage culture towards U.V. rays or by treating the culture with mitomycin. The different stages of λ phage life cycle are explained below.

The phage attaches it self to the cell wall of E.coli. cells with the help of tail fibers. The phage injects its DNA in to the bacterial cells. The λ DNA is a double stranded DNA containing the complementary single stranded tails. Inside the bacterial cell, the two ends of λ DNA get joined together and form a circular DNA. It is carried by complementary base pairing between the sticky ends of λ DNA.

The circular λ DNA gets integrated with the chromosomal DNA of the bacterial cell. Thus a new combination of genes is formed. The host DNA containing the λ DNA is named prophage DNA. The bacterium containing the prophage DNA is called lysogenic cell. Thus the λ DNA gets integrated with the chromosomal DNA of the bacterial cell. Thus a new combination of genes is formed. The host DNA containing the λ DNA is named prophage DNA. The bacterium containing the prophage DNA is called lysogenic cell. Thus the λ DNA multiplies rapidly and survives with in the bacterial cells for a number of generations

along with the bacterial DNA. This process is called lysogenic life cycle.

Rarely the prophage DNA gets disintegrated from the chromosomal DNA. As a result, the λ phage enters the lytic life cycle. This phage DNA synthesizes its head and tail proteins more rapidly and the phage multiplies rapidly in the host cells finally the virus particles break the host cell and they are then released in to the culture. Here the λ DNA interferes with the host's synthetic machinery. This method of multiplication of bacteriophage is called lytic life cycle.

(c) Lambda phage vectore

λ gt 10, λ gt 11, EMBL 3 EMBL 4, charon.

(i) λ gt 10 & it 11

λ gt 10 and λ gt 11 are modified lambda phages designated to clone cDNA fragments. The major difference between these two vectors is that λ g11 is an expression vector where inserted DNA is expressed as β-galactosidase fusion protein.

λgt 10 is a 43 kb double stranded DNA for cloning fragments that are only 7 kb in length. λ g11 is a 43.7 kb double stranded λ

phase for cloning DNA fragments which are less than 6 kb in length.

(ii) EMBL3, EMBL4

EMBL3, EMBL4 are two vectors that are designed so that a central non essential part of 44 kb long phage can be replaced by a foreign DNA. Cleavage of the phage with an appropriate enzyme generates three fragments (left arm, right arm and a central fragment called stuffer). The central fragment representing 40% of the phage genome is non essential for propagation of the phage and can be replaced by foreign DNA that may be as long as 20 – 23 kb. EMBL3, EMBL4 are two such replacement vectors used for preparing genomic libraries in eukaryotes with cloned fragments 15-25 kb in size. The two vectors have poly linkers with reverse orders of restriction sites with respect to each other.

(iii) Charon 34 and charon 35

Charon 34 and charon 35 differ from each other only in their central fragment and will accept fragments 9-20 kb long.

(iv) M 13 phage as cloning vector for DNA sequencing

M 13 is a filamentous bacteriophage of E.coli and contains a 7.2 kb long single stranded circular DNA. M 13 phage has been variously modified to give rise to a M 13 modified phage series of

cloning vectors which can be used for the purpose of sequencing through sanger's metod of dideoxy chain termination.

8.2.3.5 Cosmids

Cosmids are plasmid particles into which certain specific DNA sequences namely those for cos sites are inserted. Cosmids are the constructed vector DNA's. The cos sites is excited from the DNA of λ phage and cloned in the plasmid DNA. Thus the resulting DNA shares the properties of plasmid DNA and λ phage DNA such a modified plasmid DNA is called cosmid DNA.

This type of DNA is not naturally found in living cells.

(a) Characteristics feature of cosmid DNA

Cosmids are the constructed vectors of DNA's the characteristic features of an ideal cosmid DNA are.

The cosmid is a circular double stranded DNA. The cos site consists of two complementary single strands held to gether by complementary base pairing between these two strands.

At the cos site 3^1 end of each of the DNA strand does not establish covalent bond with the 5^1 end of the same chain. That is a definite nick is present in each of the two strands.

The nicks are retained in the cosmid for a number of generations.

The cosmid does not participate in the multiplication of phage particles.

The cosmid DNA packed with in the protein coat of bacteriophage thus the transformed virus particle is formed.

The phage particles thus formed is transferred to another organism through the induction of the phage particle to infect the organism. This method of gene transfer from one organism to another organism is often named transduction.

8.2.3.6 Phasmids

Plasmids are prepared artificially by combining features of phages with plasmids as the name suggests one such phasmids which is commonly used in molecular biology laboratories is P Blue Script II KS which is derived from PVC 19 and is 2961 base pairs long. The KS designation indicates the orientation of polylinker

such that the transcription of Lac Z gene proceeds from the restriction site for *Kpn I.*

The following features of P Blue script II KS. It has a multiple cloning sites flanked by T_3 and T_7 promoters to be read in opposite direction on the two strands.

1.An origin of replication (col E1 Dri) derived from plasmid.

2.A gene for amphicillin resistance for antibiotic selection of

chimeric phasmid vector

3.Phasmid has been developed that consists of hybrid between plasmid and filamentous phage cloning vehicle. There are generally smaller than the M 13 vectors and were formed by combining sequences from PBR 322 with the M 13 origin of replication. These vectors can be propagated normally as double stranded plasmids with in the cell.

8.2.3.7 PI cloning vectors for cloning large DNA segments

The bacteriophage PI cloning vectors can allow cloning of 100 kbp long DNA segments with an efficiency of 10^5 clone per ug of insert DNA. Therefore in their capability, the fall between YAS and cosmids. The vector with insert DNA is amplified in E.coli and

several microorganisms with cloned DNA can be recovered from 5-10ml of exponential phase of E.coli cells.

8.2.3.8 F-factor based vectors

F-factor based vectors have recently been developed for cloning large DNA segments in E.coli. The cloning of large DNA segments is achieved by a method called chromosomal building in which through repeated recombination size of cloned segment can be increased. These bacterial vectors will complement the YAC vectors for cloning segments larger than 100 kbp in length and offers some advantages over YAC system. These vectors have already been used for cloning large DNA segments from the bithron gene of drosophila.

8.2.3.8 Plant and animal viruses as vectors

A number of plant and animal viruses have also been used as vectors both for introducing foreign genes into cells and for gene amplification and expression in host cells.

8.2.3.9 Plant and Animal vires as vectors:

(a): Plant viruses

Cauliflower mosaic virus (CaMV) tobacco mosaic virus (TMV) and Gemini viruses are three groups of viruses that have been used as vectors for cloning of DNA segments.

CaMV infects particularly the members of crucifierace and has a double stranded DNA molecule. Following infection the virus spreads simultaneously through out the plant in a very high copy number reaching up to 105 virus particles/cell. These features make cauliflower mosaic virus a suitable vector for transformation of higher plants and produce transgenic plants.

Gemini viruses comprise a group of single stranded DNA. Plant viruses causing important diseases in maize and other cereals.

(b) Animal viruses

A number of animal viruses are also used as vectors either for the delivery of nucleic acids in to cultured cells followed by its integration with host genome or for the amplification and high level expression of foreign gene using the promoters from virus genes. These cloned genes can have a variety of uses including gene therapy in mammalian cells and synthesis of important proteins by cloned genes in cell cultures.

8.2.3.10 Transposons are vectors

Transposons of higher plants. Ac and DS are popular transposons in corn and were earlier known to represent activator – dissociation system. Each represents a transposon with short terminal repeats enclosing a long DNA segment which measures

more than 4,500 bp in AC and 400 base pairs in DS. Each possesses genes including the gene for transposase enzyme responsible for transposition. Part of this region can be deleted and the transposon can be used for cloning of foreign DNA segment in the same way as in other cases.

8.2.3.11 Binary and shuttle vectors

For the purpose of gene transfer in higher plants a number of binary vectors were developed. These were based on the PCV (plant cloning vectors) series of plasmids these vectors contain a conditional mini – RK 2 replicon which is maintained and mobilized by trans – acting functions derived from the plasmid RK 2 replicon. The plasmid RK 2 was introduced in to both E.coli as well as Agrobacterium to facilitate replication of binary vectors in both these hosts so that the vector can be maintained and shuttle between both – hence the name shuttle vector also. Similarly binary (or) shuttle vectors for maintenance and transfer between E.coli and yeast cells have also been designed.

Properties of shuttle vectors

1. The vector must replicate in many organisms to facilitate the isolation and characterization of genes.

2. The vector must be easily recognized by selectable markers.

3. The vector should be small in size to accommodate DNA inserts.

4. Cloned genes should be easily detected.

5. The vector must be stable, non pathogenic, and non stress inducing.

6. The vector must effectively deliver genetic information for stable maintenance in alternate derived recipients.

7. The introduced genetic information should be stable maintained as a new heritable determinant

8.2.3.12 Summary

Vectors plays an very important role in gene cloning. Plasmid is nothing but an extra chromosomal circular DNA. It contains resistance markers and origin of replication. Small DNA fragment is inserted into plasmids. Bacteriophage is another important vector. It contains cos sites in between the two cos sites packaging of large sized DNA. Cosmid having the both characteristics of plasmid and bacteriophage. Ti plasmids are useful for transfer of genes to plants. Shuttle vectors having the characteristics of generate the genes in different organisms. Animal viruses and plant viruses are also act as vectors.

8.2.3.13 Model Questions

1. What is plasmid & explain its importance in gene cloning?
2. Why cosmid is more advantages than plasmids.
3. What is bacteriophage and its importance.
4. Explain the different types of vectors used in gene cloning.

8.2.3.14 Reference Books

1. H.D. Kumar (2001), A text book of Biotechnology, 2nd edition replied Press Pvt. Ltd.
2. P.K. Gupta (2000), Elements of biotechnology 1st edition Rastogi Publications.
3. Old and Primrose (1994), Principles of gene manipulation 5th edition, Blackwell Scientific Publications Oxford, United Kingdom.
4. Bernard R. Glick and Jack J. Pasternak (1993), Molecular Biotechnology, 2nd edition, ASM Press.

Lesson 8.2.4

Application of Recombinant DNA Technology

8.2.4.10 Reference Text Books

8.2.4.1 Objective

The main objective of the lesson is to describe the application of Recombinant DNA technology.

8.2.4.2 Introduction

As DNA technology allows isolation and manipulation of DNA sequence in vitro, it is possible to produce organism capable of synthesizing or modifying any number of useful proteins. The practical reach of rDNA technology has enlarged considerably due to the possibilities to express virtually any kind of coding sequence from any possible source. Sequences from mammals or any other animals, plants, fungi, bacteria or even sequences synthesized in vitro can be introduced into and expressed in almost any other organisms. These spectacular advances made in the area of rDNA technology are being successfully used m various sectors such as agriculture, health care, process industry and environment management. An overview of applications of rDNA technology is presented below

There are four primary areas in healthcare in which rDNA technology is being used i.e. production of medicines or therapeutics, vaccines, diagnostics and gene therapy.

(i) Therapeutics: rDNA technology enables modifying micro-organisms, animals and plants so that they yield medically useful substances, particularly scarce human proteins. rDNA technology compliments industrial fermentation in production of therapeutics. The first recombinant therapeutic product has been made globally to utilize rDNA technology to:

- Create new therapeutic molecules,
- Create safer and/or more effective versions of conventionally produced therapeutics, and
- make conventional therapeutics more cost-effective.

Recombinant therapeutics developed/being developed include proteins that help the body to fight infection or to carryout specific functions and can be categorised into blood factors, hormones, growth factors, interferons, interkukins, etc. These therapeutic proteins are preferred to conventional drugs because of higher specificity and absence of side effects. Also proteins can perform both complex and specific biological functions, thereby offering human healthcare in ways not possible with small drug molecules. Therapeutic proteins are less toxic than chemical drugs and are by their nature neither carcinogenic nor

teratogenic. Further, once the biologically active principle of a protein has been identified, for medical application, its further development into a medicinal product involves fewer risks titan chemical drugs.

Traditional agriculture includes improvement of crops by selecting seeds from plants showing higher yields, better nutrition and resistance to diseases. Plant breeders thus took advantage of the genetics of those plants long before the science of genetics was understood. rDNA technology however, allows selection of genes that produce desired traits and move them from not only one plant to another but from other to another but from other organisms also. The process is far more precise and selective than traditional breeding.

Several commercially important transgenic crops such as maize, soyabean, tomato, cotton, potato, mustard, rice, etc. have been cultivated and the following traits have been imparted

The enzymes with industrially useful special functions can be located in the natural environment. To improve the productivity-to-cost ratio, it is now possible to modifying genes to increase enzyme productivity in microorganisms commonly used in manufacturing thereby making it possible to manufacture the desired enzyme in commercial quantities.

An off shoot of rDNA technology, protein engineering and directed protein evolution has been used to modify the substrate specificity of enzymes, improve catalytic properties or broaden the spectrum of reaction conditions under which enzymes can function so that they are more compatible with existing industrial processes.

8.2.4.3 Recombinant DNA Technology in the Synthesis of Human Insulin

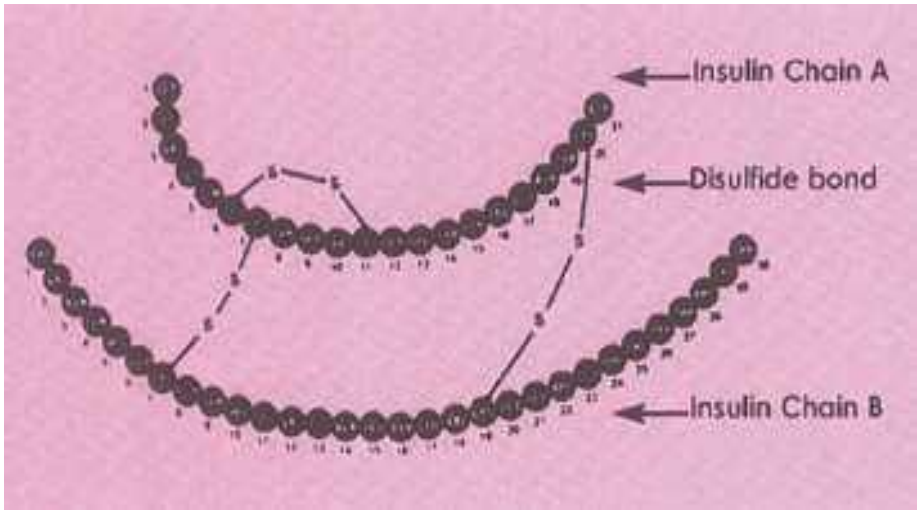
The nature and purpose of synthesising human insulin

Since Banting and Best discovered the hormone, insulin in (1921). diabetic patients, whose elevated sugar levels are due to impaired insulin production, have been treated with insulin derived from the pancreas glands of abattoir animals. The hormone, produced and secreted by the beta cells of the pancreas' islets of Langerhans, regulates the use and storage of food, particularly carbohydrates. Although bovine and porcine insulin are similar to human insulin, their composition is slightly different. Consequently, a number of patients' immune systems produce antibodies against it, neutralising its actions and resulting in inflammatory responses at injection sites. Added to these adverse effects of bovine and porcine insulin, were fears of long term complications ensuring from the regular injection of a foreign substance, as well as a

projected decline in the production of animal derived insulin. These factors led researchers to consider synthesising *Humulin* by inserting the insulin gene into a suitable vector, the E. coli bacterial cell, to produce an insulin that is chemically identical to its naturally produced counterpart. This has been achieved using Recombinant DNA technology. This method is a more reliable and sustainable method than extracting and purifying the abattoir by-product

The structure of insulin

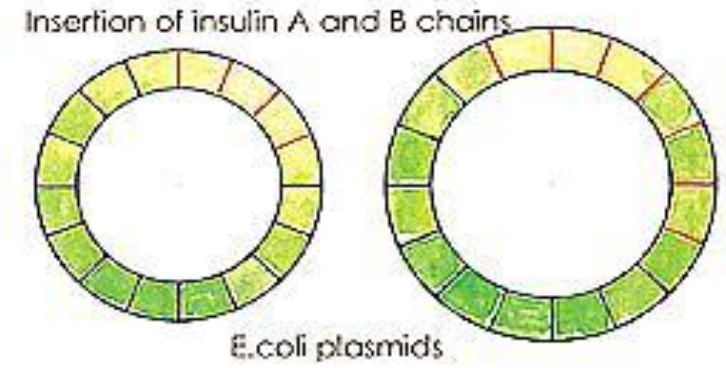
Chemically, insulin is a small, simple protein. It consists of 51 amino acid, 30 of which constitute one polypeptide chain, and 21 of which comprise a second chain. The two chains are linked by a disulfide bond.



Manufacturing Humulin

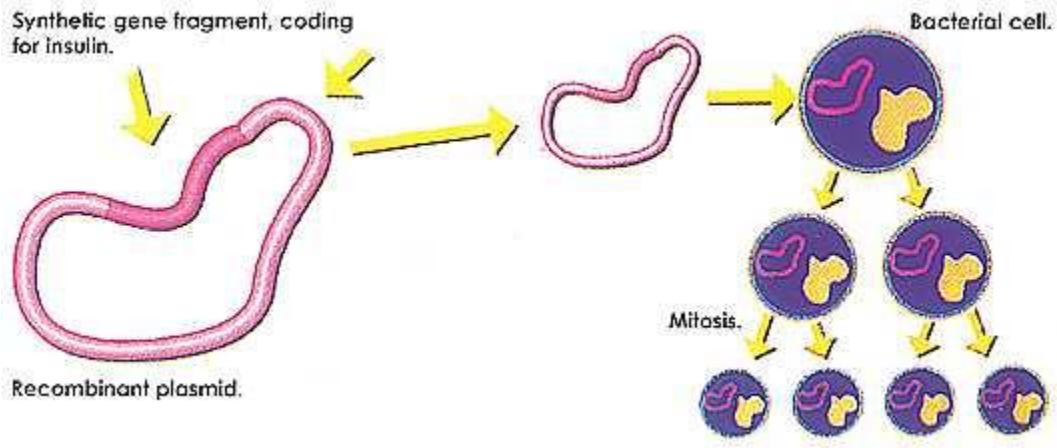
The first step is to chemically synthesis the DNA chains that carry the specific nucleotide sequences characterising the A and B polypeptide chains of insulin.

The required DNA sequence can be determined because the amino acid compositions of both chains have been charted. Sixty three nucleotides are required for synthesising the A chain and ninety for the B chain, plus a codon at the end of each chain, signalling the termination of protein synthesis. An anti-codon, incorporating the amino acid, methionine, is then placed at the beginning of each chain which allows the removal of the insulin protein from the bacterial cell's amino acids. The synthetic A and B chain 'genes' are then separately inserted into the gene for a bacterial enzyme, B-galactosidase, which is carried in the vector's plasmid. At this stage, it is crucial to ensure that the codons of the synthetic gene are compatible with those of the B-galactosidase.



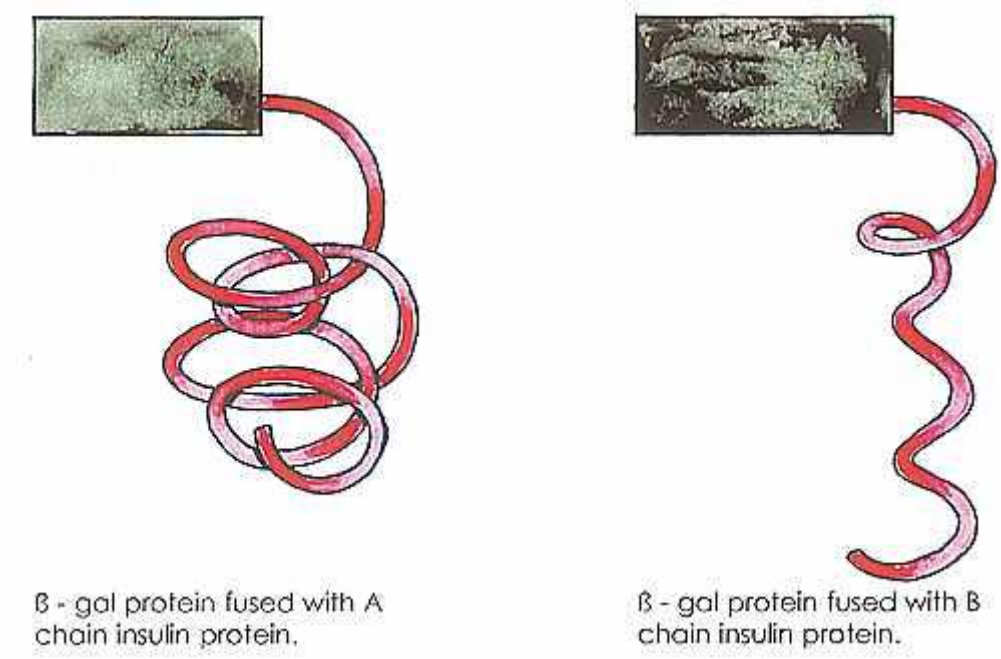
Source: Watson, J.D., Gilman, M.,Witkovski., Zoller, M. - Recombinant DNA, pg 456.

The recombinant plasmids are then introduced into E. coli cells. Practical use of Recombinant DNA technology in the synthesis of human insulin requires millions of copies of the bacteria whose plasmid has been combined with the insulin gene in order to yield insulin. The insulin gene is expressed as it replicates with the B-galactosidase in the cell undergoing mitosis.



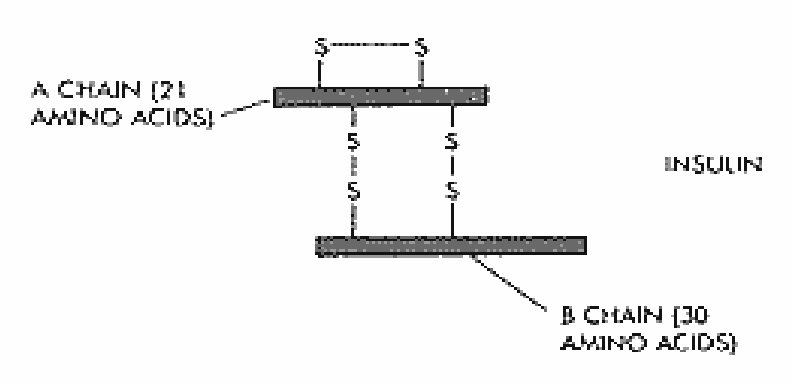
The process of mitosis. Source: Novo-Nordisk promotional brochure, pg 11.

The protein which is formed, consists partly of B-galactosidase, joined to either the A or B chain of insulin. The A and B chains are then extracted from the B-galactosidase fragment and purified.



Source: Watson, J.D., Gilman, M., Witkovski, J., Zoller, M. - Recombinant DNA, pg 456.

The two chains are mixed and reconnected in a reaction that forms the disulfide cross bridges, resulting in pure *Humulin* - synthetic human insulin.



Human insulin molecule. Source: Source: Watson, J.D., Gilman, M., Witkovski, J., Zoller, M. - Recombinant DNA, pg 456.

Biological implications of genetically engineered Recombinant human insulin.

Human insulin is the only animal protein to have been made in bacteria in such a way that its structure is absolutely identical to that of the natural molecule. This reduces the possibility of complications resulting from antibody production. In chemical and pharmacological studies, commercially available Recombinant DNA human insulin has proven indistinguishable from pancreatic human insulin. Initially the major difficulty encountered was the contamination of the final product by the host cells, increasing the risk of contamination in the fermentation broth. This danger was eradicated by the introduction of purification processes. When the final insulin product is subjected to a battery of tests, including the finest radio-immuno assay techniques,- no impurities can be detected.- The entire procedure is now performed using yeast cells as a growth medium, as they secrete an almost complete

human insulin molecule with perfect three dimensional structure. This minimises the need for complex and costly purification procedures.

8.2.4.4 Production of somatotrophin (human growth hormone

Somatrotrophin is required for the growth of the skeleton, and is secreted by the anterior lobe of the pituitary. Only the Growth hormone is effective for treatment of growth disorders. The extraction of hGH by classical methods is very time consuming and expensive. It required the pituitary glands from about 80 human bodies to produce a supply of hGH for one year's treatment. Long-term treatment for dwarfism lasts for a minimum of 8-10 years.

Synthesis of hGH by genetic engineering is by a combination of artificial gene synthesis & cDNA cloning. The mRNA for hGH present in the pituitary consists of 26 codons specifying a signal peptide and 191 codons for the aminoacids of the hormone. If the hGH gene is inserted into in bacterial cells, it codes for the entire polypeptide, including the 26 aminoacid signal region. Such a protein is non-functional if used for treatment.

If the signal sequence is cut off, the rest of the DNA sequence would code for the active homone polypeptide. However, no restriction enzyme is known that can exactly cutoff the DNA

between signal peptide and the active hormone coding region. The restriction enzyme Hae III cuts off 50 bases from cDNA. This region includes the coding region for the unwanted 26 aminoacids of the hormone. The missing 24 aminoacid coding region is artificially synthesized and joined to the traucated hGH gene.

To this complete gene is added a bacterial control gene and the resulting sequence is inserted into a plasmid. The recombinant plasmid is cloned into bacteria, which can synthesize the complete hGH without the signal sequence. The first 24 of 191 aminoacids of the expressed gene are synthesized chemically, while aminoacids 25-191 are derived from a CDNA copy of the hGH mRNA.

Fig.

8.2.4.5 Production of vaccines

8.2.4.5.1 Recombinant subunit vaccines

Recent advances in immunology and protein engineering have allowed the design and production of recombinant subunit vaccines [3,5–8]. The epitopes recognized by neutralizing antibodies are usually found in just one or a few proteins present on the surface of the pathogenic organism.

Isolation of the genes encoding such epitope-carrying protein immunogens and their expression in heterologous hosts form the basis of recombinant-subunit-vaccine development. The main advantage of using single proteins displaying immunodominant epitopes as vaccines is the possibility of inducing protective immunity without having side effects and immune reactions caused by other parts of the pathogenic organism. Potential challenges in the development of subunit vaccines are that they often are poorly immunogenic and have short *in vivo* half-lifes. Another difficulty with subunit vaccines is that they often elicit only strain-specific protection, so, to evoke full protection to a disease caused by several related strains, combinations of immunogens from the different strains might be needed.

8.2.4.5.2 Nucleic acid vaccines

Nucleic acid vaccines constitute a new class of recombinant subunit vaccine, consisting of, for example, plasmid DNA containing the gene encoding the antigen of interest under the control of a strong mammalian viral promoter. The antigen-encoding gene will be expressed by the vaccine upon delivery of the plasmid DNA. DNA vaccines have been shown to generate both humoral and cellular immune responses and the first report of protective efficacy of DNA immunization was against influenza. Immunization of BALB}c mice with plasmid DNA encoding influenza A nucleoprotein resulted in the induction of nucleoprotein

specific antibodies, and protection from a subsequent challenge with a heterologous strain of influenza A virus. In addition to the advantage of a subunit vaccine including only the antigen (or antigens) required for protective immunization, nucleic acid vaccines present several other advantages. Nucleic acid vaccines are relatively easy to construct and produce, and considering the post-trans lational modifications and the presentation of the antigen to the host's immune system, they provide antigen synthesis in the host in a similar way to that which occurs during a natural infection.

8.2.4.6 Transgenic Plants

Genetically modified plants are created by the process of genetic engineering that allows scientists to move genetic material between organisms with the aim of changing their characteristics. All organisms are composed of cells that contain DNA (deoxyribonucleic acid) molecules. Molecules of DNA form units of genetic information known as genes. Each organism has a genetic blueprint made up of DNA that determines the regulatory functions of its cells and thus the characteristics that make it unique.

Prior to genetic engineering, the exchange of DNA material was possible only between individual organisms of the same species. With the advent of genetic engineering in 1972, scientists have been able to

identify specific genes associated with desirable traits in one organism, and transfer those genes beyond the boundaries of species into another organism. For example, genes from bacteria, viruses, or animals may be transferred into plants to produce genetically modified plants having changed characteristics. This method, therefore, allows mixing of genetic material among species that cannot otherwise breed naturally.

Some current applications of plant biotechnology include:

- Developing plants that are resistant to diseases, pests, and stress
- Keeping fruits and vegetables fresh for longer periods of time which is extremely important in tropical countries
- Producing plants that possess healthy fats and oils
- Producing plants that have increased nutritive value
- Producing soybeans with a higher expression of the anti-cancer proteins naturally found in soybeans
- Developing a whole range of higher value added feed
- Other applications such as lignin modification in trees that will make possible much higher fiber extraction rates in the paper and pulp industry

- Producing new substances in plants, including biodegradable plastics, and small proteins or peptides such as prophylactic and therapeutic vaccines

The development of transgenic plants involves the following steps:

- Identification of a gene that would impart a useful character to the target crop plant, and cloning the gene. The work, inter alia, involves constructing and screening a genomic library, and identifying the gene with a probe/marker DNA using standard protocols.

- Modification of the target gene for expression in crop plants. This operation involves selecting and cloning a promoter, a coding sequence for facilitating efficient binding of ribosomes to the constituted messenger RNA (ribonucleic acid), and selecting the appropriate gene terminator sequences. Incorporating all these sequences into the target plasmid, and marker genes, along with the right positioning of the promoter and the terminator sequences are steps in the modification of the target gene.
- Incorporation of the modified gene construct into the target plant genome. This involves transferring the modified gene into the plant through a suitable method: *Agrobacterium*, *Particle gun* or *direct gene uptake through protoplasts*

- Regeneration of whole plants capable of transmitting the incorporated target gene to the next generation. This process uses a

selection agent, usually an antibiotic-resistant marker gene, incorporated initially into the modified gene construct to facilitate the selection process. Cells not containing the resistant marker gene would be destroyed under the selection pressure deliberately built into the experimental design for the selection of target plants.

- Molecular analysis such as PCR, Southern, Western, etc. for confirming the integration of the gene of interest.
- Inheritance pattern of gene of interest in the transgenic progeny.
- Performance of transgenic plants under containment.
- Field trial of transgenic plants.

8.2.4.6.1 Production herbicide resistant plants

Herbicides typically act by blocking metabolic pathways leading to the synthesis of enzymes involved in vital functions, such as those in involved in photosynthesis or biosynthesis of essential aminoacids. Broad spectrum herbicides such as Glyphosate are also toxic to crop plants. Therefore it has become essential to identify and transfer herbicide resistance genes into crop plants. This can be achieved by the following three methods.

a. Overproduction of target protein

The target protein of the herbicide is stimulated for over production, so that sufficient amount escapes the action of herbicide.

b. Insertion of the herbicide disabling enzyme

This inserts an enzyme that disables the herbicides. Glyphosate tolerance genes were isolated and attached to kanamycin resistance genes engineered into agrobacterium.

c. Genetically altered target protein

The genetically altered from of a target protein is (insensitive) less sensitive to the herbicide.

- The enzyme 5-enolpyruvyl shikimate – 3-phosphate (EPSP) is important for the synthesis of aromatic aminoacids in bacteria and in plant chloroplasts.
- The enzyme is inhibited by the herbicide Glyphosate which is widely used in commercial weed-killer.
- A chimeric gene was constructed by fusing the glyphosate –resistant EPSP gene from bacteria to a targeting sequence consisting of the gene for a 72-

aminoacid transit peptide from petunia EPSP. The chimeric gene was cloned into a T-DNA expression vector and controlled by a cauliflower mosaic virus (CaMV) promoter.

- The expression vectors were introduced into tabacco plants by Agrobacterium and made their way into the cell cytoplasm.

- The EPSP gene was expressed in the cytoplasm of the transgenic plants, producing both tobacco EPSP & bacterial glyphosate-insensitive EPSP. The transit peptide targets both EPSP enzymes to the chloroplasts.

- When plants were sprayed with weed killer containing the herbicide glyphosate, the bacterial EPSP remained active, while the endogenous EPSP (tobacco EPSP) was inhibited by glyphosate.

- The sprayed wild type plants were killed but transgenic plants survived.

Fig.

Fig.

8.2.4.6.2 Production of pest resistant plants

The bacterium Bacillus thuringiensis produces an endotoxin, Bt toxin. The toxin is environmentally safle insecticide which is active against a number of caterpillars, and tobacco tromworm. The Bt gene linked to a constitutive promoter has been inserted in the T-DNA of Ti plasmid and integrated into tobacco & tomato

plants. Here it has expressed itself to kill a large number of caterpillars.

Fig.

8.2.4.7 Transgenic Animals

A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The foreign gene is constructed using recombinant DNA methodology. In addition to a **structural gene**, the DNA usually includes other sequences to enable it

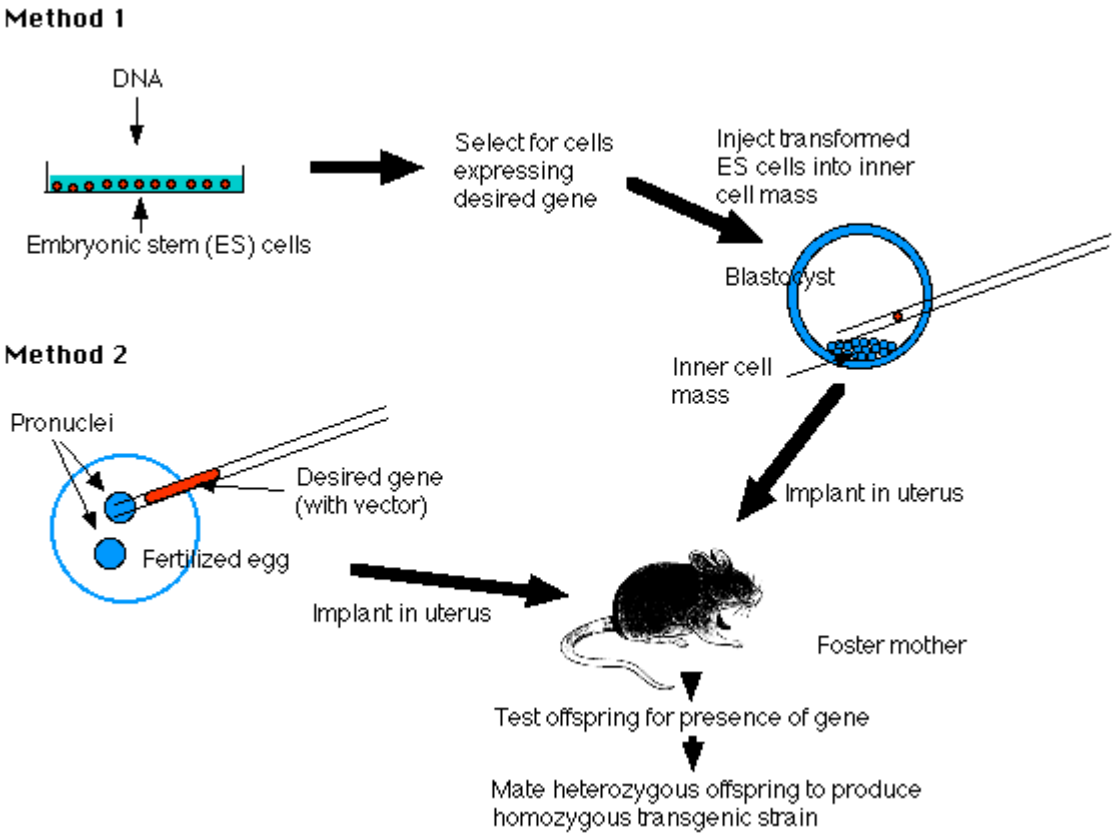
- to be incorporated into the DNA of the host and
- to be expressed correctly by the cells of the host.

- Transgenic **sheep** and goats have been produced that express foreign proteins in their milk.
- Transgenic chickens are now able to synthesize human proteins in the "white" of the eggs.

The method of producing transgenic mice are widely used:

- transforming embryonic stem cells (ES cells) growing in tissue culture with the desired DNA;

The Embryonic Stem Cell Method Embryonic stem cells (ES cells) are harvested from the inner cell mass (ICM) of mouse blastocysts. They can be grown in culture and retain their full potential to produce all the cells of the mature animal, including its gametes.



Using recombinant DNA methods, build molecules of DNA containing

- the structural gene you desire (e.g., the insulin gene)
- vector DNA to enable the molecules to be inserted into host DNA molecules
- promoter and enhancer sequences to enable the gene to be expressed by host cells

2. Transform ES cells in culture

Expose the cultured cells to the DNA so that some will incorporate it.

3. Select for successfully transformed cells.

4. Inject these cells into the inner cell mass (ICM) of mouse blastocysts.

5. Embryo transfer

- Prepare a pseudopregnant mouse (by mating a female mouse with a vasectomized male). The stimulus of mating elicits the hormonal changes needed to make her uterus receptive.
- Transfer the embryos into her uterus.
- Hope that they implant successfully and develop into healthy pups (no more than one-third will).

6. Test her offspring

- Remove a small piece of tissue from the tail and examine its DNA for the desired gene. No more than 10-20% will have it, and they will be heterozygous for the gene.

7. Establish a transgenic strain

- Mate two heterozygous mice and screen their offspring for the 1:4 that will be homozygous for the transgene.
- Mating these will found the transgenic strain.

8.2.4.8 Summary

Recombinant DNA (rDNA) technology or Genetic Engineering is an umbrella term for a set of experimental techniques that enable individual genes and DNA sequences to be manipulated resulting in genetically modified organisms (GMO) and products.

There have been many potential applications of rDNA in medicine, agriculture and industry. Conventionally proteins and other biological products, processed from human or animal serum or tissues, often are of low purity. Production of therapeutic products by the rDNA technology has several advantages such as provision of drugs that could not be produced by conventional methods, manufacture of sufficient quantities of drugs and provision for manufacture of safe drugs.

8.2.4.9 Model Questions

1.What are transgenic plants ?

2.explain in brief the application of recombinant technology?

3.What is the importance of insulin and how is recombinant insulin produced?

8.2.4.10 Reference text books:

1. H.D. Kumar (2001), A text book of Biotechnology, 2nd edition replied Press Pvt. Ltd.

2. P.K. Gupta (2000), Elements of biotechnology 1st edition Rastogi Publications.

3. Old and Primrose (1994), Principles of gene manipulation 5th edition, Blackwell Scientific Publications Oxford, nited Kingdom.

Lesson 8.3.1

MEDIA PREPARATION

Objective

8.3.1.1 Introduction

8.3.1.2 Composition of plant tissue – culture media

8.3.1.3 Media preparation

1. Preparation of stock solutions
2. Procedure for the preparation of culture medium

8.3.1.4 Selection of a suitable medium

8.3.1.5 Summary

8.3.1.6 Key words

8.3.1.7 Self assessment test

8.3.1.8 References

Objective

The excised plant tissues and organs can grow only on artificially prepared nutrient medium which is known as culture medium. In this chapter discussions relating to the composition of nutrient medium, preparation of a nutrient medium and selection of nutrient medium for a particular type of explant are made.

8.3.1.1 Introduction

Excised plant tissues and organs require a proper nutrient medium for their growth and development, which is known as culture medium. Many workers have proposed the composition of a nutrient medium for the growth of plant tissue. But no single medium is capable of maintaining optimum growth of all plant tissues. Therefore the most suitable medium for a particular tissue must be determined by trial and error. The proposed composition of a culture medium will be often modified to stimulate the growth of a particular plant material.

Mostly all tissue culture media are synthetic. A synthetic medium consists of only chemically defined compounds. The medium must have a carbon source, a nitrogen source, inorganic salts, organic supplements, vitamins and growth regulators.

Different types of media have been used by different workers for culturing different types of explants. The different types of

tissue – culture media are, M.S medium, B5 medium, whites medium, S.H. medium, L.S. medium etc. The M.S. medium was formulated by Murashige and Skoog in 1962. B5 medium was formulated by Gamborg et al in 1968. S.H. medium was formulated by Schenk and Hilderbrandt in 1972. The L.S. medium was formulated by Linsmaier and Skoog in 1965.

The M.S. medium and B5 medium are generally used in plant cell and tissue culture. The composition of M.S. and B5 media are listed in the table.

8.3.1.2 Composition of plant tissue – culture media

Inorganic Nutrients

All nutrient media provide the 15 essential elements necessary for plant growth. They are categorized into macronutrients and micronutrients.

Macro Nutrients

These are needed in concentration >0.5 m mol.

They include C, H, O, N, P, K, Ca, S, Mg

Micro Nutrients

There are required in concentrations <0.5 m mol.

The different tissue culture media provide different concentrations of the inorganic nutrients. Iron is provided as iron. EDTA complex to keep it available at higher pH i.e. above 5.8. MS and B5 are the most commonly used media.

Carbon Source

Sucrose 20 – 50 gms/lit is the most commonly used carbon source for all cultured plant materials. Autoclaving hydrolyses sucrose and enhances its availability to plant cells. Plant tissue can utilize other sugars like maltose, galactose, lactose, mannose and starch. But these are rarely used.

Vitamins

For optimum callus growth vitamins are essential. Inositol, thiamine, pyridoxine and nicotinic acid are the vitamins used it plant tissue culture of these thiamine is essential and the rest are promontory.

Growth Regulators

Auxins, cytokinins and Gibberellins are the growth regulators commonly used in tissue culture.

Auxins

They are commonly used to support cell division and callus growth, somatic embryo induction and rooting.

Eg:- IAA - Indole – 3 – acetic acid

IBA - Indole – 3 – butyric acid

NAA - Naphthalene acetic acid

NOA - Naphthoxy acetic acid

2,4-D- 2,4 – dichlorophenoxy acetic acid

Cytokinins

They are used to promote cell division, regeneration of shoots, somatic embryo induction and to enhance proliferation and growth of axillary buds.

Eg:- Kinetin (furfuryl aminopurine)

BAP (benzyl amino purine)

Zeatin

2-ip (Isopentenyl adenine)

TDZ – thidiazuron (a compound having cytokinin activity)

- Abscisic acid promotes somatic embryo and shoot bud regeneration in many species and also improves somatic embryo maturation.

- Among the 20 gibberellins known, GA_3 is used in plant tissue culture. It promotes shoot elongation and somatic embryo gemrination.

- The common range of concentrations used are,

Auxins - 0.1 – 3 mg/l

Cytokinins - 0.1 – 3 mg/l

ABA - 0.2 mg/l

GA_3 - 0.1 – 1 mg/l

Aminoacids

Cultured tissues are capable of synthesizing the aminoacids necessary for their metabolism. Inspite of this the addition of aminoacids to media is important for stimulating cell growth in protoplast culture and for establishing cell lines.

Eg:- L – glycine – 2 m mol/l

Casein hydrolysate – 0.05 – 0.1%

L – glutamine – 8 m mol/l

L – Cysteine – 10 m mol/l

L – asparagine – 100 m mol/l

L – arginine – 10 m mol/l

Tyrosine – 100 m mol/l

Aminoacids added singly are found to be inhibitory to cell growth and their mixtures are found to be beneficial.

Activated Charcoal

Addition of activated charcoal to the medium stimulates growth and differentiation in orchids, carrot, ivy and tomato. Its effect is found to be inhibitory in tobacco, soybean and camellia. Inhibition of growth is due to the absorption of phytohormes by activated charcoal. The stimulation may be due to the adsorption of inhibitory compounds to activated charcoal and darkening of the medium. It also absorbs toxic compounds produced during the culture and promotes cell growth. Activated charcoal is acid washed and neutralized before adding to the medium at a concentration of 0.5 – 3%.

Antibiotics

Addition of antibiotics to tissue culture media is generally avoided because their presence retards cell growth. But, some plant cells have a systemic infection of microorganisms. To prevent the growth of these microbes the media are to be enriched with antibiotics.

Streptomycin (or) Kanamycin at low concentration effectively controls systemic infection and the media supplemented with these antibiotics do not adversely inhibit the growth of cell cultures.

Complex Organic Additives

The organic extracts like yeast extract, coconut milk, case in hydrolysate, corn milk, malt extract and tomato juice were used to support plant tissue growth.

In tissue cultures the success achieved with the use of coconut milk and casein hydrolysate are significant. For another culture, potato extract has been found to be suitable.

In many cases their effects could be reproduced by a single amino acid. For example L-asparagine could replace yeast extract and tomato juice in the medium for callus culture of maize endosperm. L-glutamine alone has demonstrated favourable tissue responses in several species and enrichment by fruit extracts was found unnecessary. Such additives should be used only when synthetic media fail.

Solidifying Agents

Solidifying agents are commonly used for preparing semi solid or solid tissue culture media. The most commonly used solidifying agents are Agar, Gelatin, Alginate, Phytagel, Gelrite and Seaplaque (K) (highly purified agarose).

- Agar, a polysaccharide obtained from sea weeds, has several advantages over the other gelling agents. These gels donot react with media constituents, they are not digested by plant enzymes and remain stable at all incubation temperatures.

- Generally 0.5 – 1% agar is used in plant cell culture media preparation.

- In nutritional studies the use of commercially available agar should be avoided because it contains impurities in the form of Ca, Mg, K, Na and trace elements. These impurities can be removed by washing agar in double distilled water for 24 hours, then rinsing in ethanol and drying at 60^{O} C for 24 hours. This can be used for such critical experiments.

pH

Plant cells and tissue require optimum pH for growth and development in cultures. pH affects the uptake of ions. For most of the culture media pH 5 – 6 before sterilization is considered optimal. Higher pH gives a hard medium and a lower pH results in unsatisfactory solidification of the agar.

Table 1. Chemical composition of some plant tissue culture media.

8.3.1.3 Media Preparation

Nowadays the plant tissue culture media most commonly used are available in the market as dry powders. These powders contain inorganic and organic nutrients. The medium can be prepared by dissolving these powders in some quantity of distilled water. After mixing the contents thoroughly in water, sugar, agar (melted) and other organic supplements are added.

Finally, the volume is made up to one litre the pH is adjusted and the medium is autoclaved.

For the preparation of the culture medium by using conventional methods, we have to prepare the stock solutions, of (1) macro nutrients, (2) micronutrients, (3) iron and (4) organic nutrients except sucrose. For each growth regulator a separate

stock solution is prepared by dissolving it in a small quantity of suitable solvent and then adjusted with distilled water to the desired volume to get the final concentration of 1 m mol/l (or) 10 m mol/l. All the stock solutions are stored in proper plastic (or) glass containers and kept at low temperatures. Iron stocks should be stored in coloured bottles. The containers should be shaken well before use and if there is any detection of contaminant (or) precipitate the stocks must be discarded. In storing coconut milk we have to take extracare. The liquid extract from the fruit is boiled to deproteinise it, filtered and stored in plastic bottles in a deep – freezer at – 20^{O}C.

In experiments in which changes in the quantity and quality of media constituents is needed, it is better to weigh and dissolve each ingredient separately before mixing them together.

1. Preparation of Stock Solutions

It is not possible to weigh and mix all the constituents just before the preparation of medium, which is a time consuming and tedious job. If we want to prepare very little volume of the medium, then also it is very difficult to weigh some constituents that are used in very small quantity for one litre medium. So, it is convenient to prepare the concentrated stock solutions of macro – salts, micro-salts, vitamins, aminoacids, hormones etc. All stock

solutions should be stored in a refrigerator and should be checked visually for contamination with microorganisms (or) precipitation of ingredients. Stock solutions of vitamins, aminoacids and hormones should not be stored for indefinite period and should be kept in a deep freezer chamber.

The widely used culture medium was formulated by Murashige and Skoog (MS – medium). The procedure for the preparation of stock solutions of MS – medium is given as follows. Macro salts should be made at 10 or 20 times of their final concentration in the medium and the micronutrients can be prepared at 1000 times of their final concentration. While making the stock solutions, it is advisable to dissolve each constituent completely before adding another. Otherwise precipitation of salts may occur.

Stock solution of macro salts

To make 1000 ml of this stock solution, dissolve the salts one after another in 800 ml of double distilled water and then make up the volume to 1000 ml. The solution is filtered and can be stored in the refrigerator (10 – 16^{O}C) for a long period.

Stock Solution of KI (X 1000)

Dissolve 83 mg of KI in 100 ml of double distilled water and store in the refrigerator. (0.83 mg/litre present in the original medium).

Table of Macro Salts

Constituents	Amount (mg/l) present in the original medium	Amount (gm) to be taken for stock solution (X20)	Final volume of stock (ml)
$NH_4\ NO_3$	1650	33.0	
KNO_3	1900	38.0	
$Cacl_2,2H_2O$	440	8.8	1000
KH_2PO_4	170	3.4	
$MgSo_4,\ 7H_2O$	370	7.4	

Stock Solution of Micro Salts (X 1000)

To make 1000 ml of this stock solution dissolve the salts one after another in 800 ml of double distilled water and then make up the final volume.

Table of Micro Salts

Constituents	Amount (mg/l) present in the	Amount (X100) to be taken for stock	Final volume of stock (ml)

	original medium	solution (value expressed in mg)	
H_3BO_3	6.2	620	
$Na_2 MoO_4$, $2H_2O$	0.25	25	
$CoCL_2$, $6H_2O$	0.025	2.5	
$CuSO_4$, $5H_2O$	0.025	2.5	100
$NzSO_4$, $7H_2O$	8.6	860	
$MnSo_4$, $4H_2O$	22.3	2230	

Stock Solution of Iron (X200)

Dissolve 45 mg of Na_2EDTA (37.25 mg/lit in original medium) in 75 ml of boiling double distilled water. Then add gradually 557 mg of $FeSO_4$, $7H_2O$ (27.85 mg/lit in original medium). Keep the magnetic stimer on for at least 1 hour in hot condition until the colour of the solution changes to golden yellow. Finally make up the volume to 100ml and store in the refrigerator (5^oC). This solution must be kept in an amber coloured bottle.

To make 100 ml of this solution, dissolve the salts sequentially as mentioned above, one by one in 80 ml of double distilled water. Make up the final volume. Filter and store the solution at 5^oC in a refrigerator for long periods.

Stock Solution of Meso – Inositol (X500)

Dissolve 1 gm of Meso – Inositol in 20 ml of double distilled water. Store at O^{O}C for 15 days.

Stock Solution of Glycine (X1000)

Dissolve 40mg glycine in 20 ml of double distilled water and store at O^{O}C for 15 days.

Stock Solution of MS Vitamins, (X1000)

Table

Constituent	Amount (mg/l) present in the original medium	Amount (x50) to be taken for stock solution (values in mg)	Final volume (ml)	Storage tempera-ture (^{O}C)	Duration of storage (in days)
Thiamine HCL	0.1	5			
Nicotinic acid	0.5	25	50	0	15
Pyridoxine HCL	0.5	25			

Stock Solution of Hormones

Stock solutions of hormones are general and can be used for any medium at any combinations and concentrations.

Auxins and cytokinins are not directly dissolved in water. They are first made soluble in water miscible solvents and then water is added to get the final volume.

Table for stock solutions of hormones

Hormone	Required amount for stock solution	Amount of solvent required to dissolve	Amount of water to be added	Final concen-tration	Storage tempe-rature (^{O}C)	Duration of storage (days)
Auxins 2,4-D	10	1ml abs. Ethyl alcohol	9 ml	0.5 mg/ml	0	7
IAA	10	- do -	- do -	- do -	- do -	- do -
NAA	10	- do -	- do -	- do -	- do -	- do -

IBA	10	- do -	- do -	- do -	- do -	- do -
Cytokinins Kinetin (6-furfuryl amino purine)	10	1ml (1N) HCL	- do -	- do -	- do -	- do -
BAP (6-Benzyl aminopurine)	10	- do -	- do -	- do -	- do -	- do -
Zeatin	10	- do -	- do -	- do -	- do -	- do -
2iPA (2,iso-pentenyl-adenine)	10	- do -	- do -	- do -	- do -	- do -

2. Procedure for the preparation of culture medium

Media should be prepared with care. To make 1 litre of MS medium.

- 30 gms of cane sugar is dissolved in 200 ml of double distilled water.
- 1-2 gms of activated charcoal is added to this and filtered through a filter paper.
- Take double distilled water in another flask and add the appropriate amount of stock solutions in sequence as shown in the table.

Stock solution of macrosalts → 50 ml

Stock solution of microsalts	→ 1 ml
Stock solution of KI	→ 1 ml
Stock solution of Fe-EDTA	→ 5 ml
Stock solution of MS-vitamins	→ 1 ml
Stock solution of Glycine	→ 1 ml
Stock solution of Meso-inositol	→ 2 ml

- Desired concentration of auxin and cytokinin are added from stock solution according to the formula given below,

$$\text{Amount of stock solution to be taken in ml. For one litre medium} = \frac{\text{Desired concentration}}{\text{Stock concentration}}$$

- If the quantity of the medium is less than one litre, then the hormones are added using another formula,

$$\text{Amount of stock solution to be added in ml} = \frac{\text{Required concentration} \times \text{volume of medium}}{\text{Stock concentration} \times 1000}$$

- Pour the filtered sucrose solution and the solution mixture of salts, vitamins, aminoacids and hormones in to a one litre measuring cylinder. Make the final volume to one litre with double distilled water. Shake well to mix up uniformly.

- Adust the pH of the liquid medium in between 5.6 to 5.8 by using 0.1(N) HCl (or) 0.1(N) NaOH. This operation is done by using pH meter.

- 5 – 8% agar is added to the liquid medium to make the solid medium. Heat it to 60^{O}C to dissolve the agar completely.

- Distribute the culture medium in to culture tubes or conical flasks. Close their mouths with non-adsorbent cotton plug wrapped in a gauge cloth.

- Cover the plug with brown paper with the help of a rubberband.

- The medium is sterilized finally by autoclaving.

8.3.1.4 Selection of a suitable medium

A sutable medium may be deviced for a new system in several ways. All components of the medium are divided in to four

categories, viz. minerals, auxins, cytokinins and organic nutrients. Now each group of substances is prepared in 3 concentrations: low (L), medium (M) and High (H).

In 1974 De Fossard et al. have provided a broad spectrum experiment for this purpose. According to them the various combinations of these four categories of components of three different concentrations gives us an experiment with 81 treatments. The best of these treatments may be selected as a new medium suitable for an untested system.

8.3.1.5 Summary

Excised plant tissues and organs can only grow invitro on a suitable artificially prepared solid (or) liquid nutrient medium which is known as culture medium. The Murashige and Skoog (MS) based culture media are commonly used or plant tissue culture and have proven effective for growth promotion of both monocotyledons and dicotyledons.

A culture medium is composed of inorganic salts, an iron source, vitamins, aminoacids, plant hormones and a carbon source. The inorganic salts are supplied in two groups viz. macrosalts and microsalts. The most commonly used phytohormes are synthetic auxins and cytokinins. The auxin are 2,4-D, NAA, IBA etc. and the cytokinins are kinetin, 6-BAP, Zeatin, etc. The

concentration and ratio of hormones may vary from plant to plant and should be standardized for a particular plant tissue. Some plant tissues grow in the presence of complex natural additive such as coconut milk, casein hydrolysate, yeast extract, water melon extract, malt extract, potato extract, ripe tomato extract, orange juice extract etc. Diphenyl urea a growth factor found in coconut milk, exhibits cytokinin like responses.

Based on the constituents, there are two types of culture media Viz. chemically defined and chemically undefined media. In the former the composition and concentration of all constituents are known, whereas in the latter the exact composition and the concentration of all constituents are not known due to the addition of natural products like coconut milk.

It is not possible to weigh and mix all the constituents just before the preparation of the medium. It is convenient to prepare the concentrated stock solutions of macrosalts, microsalts, vitamins, aminoacids hormones etc. All stock solutions should be stored in a refrigerator for a limited period.

8.3.1.6 Key Words

Fe-EDTA – Ferric – sodium – ethylene – diamine – tetra acetate.

NAA - α - Nathphalene acetic acid.

6 – BAP – 6, Benzyl aminopurine.

8.3.1.7 Self assessment test

1. What is culture medium? Give the basic composition of a general plant tissue culture medium? How can you prepare and sterilize the culture medium?

8.3.1.8 References

1. An introduction to plant tissue culture Dr.M.K. Razdan, Oxford & IBH publishing Co. Pvt., Ltd.
2. Plant cell and tissue culture, Narayana Swamy, Tata McGraw-Hill.
3. An introduction to plant tissue culture, Dr.Kalyan Kumar De, New Central Book Agency.
4. Biotechnolgoy, B.D.Singh, Klayani Publishers.
5. Elements of Biotechnology, P.K. Gupta, Rastogi Publications.
6. Biotechnology, V.Kumaresan, Saras Publications.

Lesson 8.3.2

STERILIZATION

Objective

8.3.2.1 Introduction

8.3.2.2 Different sterilization techniques used in plant tissue culture

1. Dry heat
2. Flame Sterilization
3. Autoclaving
4. Filter Sterilization
5. Wiping with 70% ethanol
6. Surface sterilization

8.3.2.3 Summary

8.3.2.4 Key Words

8.3.2.5 Self-assessment test

8.3.2.6 References

Objective

All the materials that are used in tissue culture must be freed from microbes. The culture medium contains sugar, which supports the growth of microorganisms like bacteria, fungi etc. when they come in contact with the medium either in cellular form (or) in spore form, they grow faster than the higher plant tissue due to their brief life cycle and will kill the tissue. So, to avoid this situation maintainance of complete aseptic conditions inside the culture vial is essential. In this chapter, the different sterilization techniques used in plant tissue culture are discussed.

8.3.2.1 Introduction

The most important aspect of invitro techniques is the necessity to carry out various operations under aseptic conditions. Bacteria and fungi are the most common contaminants observed in cultures. When the microbes come in contact with the culture media from different sources viz. glassware, instruments, nutrient medium and also from the plant material itself, they grow faster than the cultured tissue. So, the tissue is killed due to contamination. The contaminants may also release metabolic wastes which are toxic to the plant tissues. So, to avoid this, the maintenance of complete aseptic environment inside the culture vial where the tissue grows is very much essential. Therefore, the

surface of plant tissue and all non-living articles including the nutrient medium should be sterilized.

8.3.2.2 Different sterilization techniques used in plant tissue culture

All the materials viz. vessels, instruments, medium, plant materials etc. that are used in culture work must be freed from microbes. This can be achieved by the following techniques of sterilization.

1. Dry heat
2. Flame sterilization
3. Autoclaving
4. Filter sterilization
5. Wiping with 70% ethanol
6. Surface sterilization

1. Dry heat

Glasswares and Teflon plastic wares, instruments and aluminium foil can be sterilized by exposure to hot dry air in an oven at 160^{O} – 180^{O}C for 2 – 4 hr. All the items should be properly sealed before sterilization. Sealing with paper is not advisable as it decomposes at high temperatures. The disadvantage of dry heat

sterilization is poor circulation of air and slow penetration of heat. So, sterilization of the glassware by autoclaving and flame sterilization of the instruments like forceps is recommended. More recently, glass bead sterilizers are being employed for the sterilization of forceps, scalpels etc. These devices use dry heat and the temperature is maintained around 300^{O}C.

2. Flame Sterilization

Instruments like forceps, scalpels, needles etc. are flame sterilized by dipping them in 95% alcohol followed by flaming. These instruments are repeatedly sterilized during the operation to avoid contamination. It is customary to flame the mouths of the culture vessels prior to inoculation/subculture. During flame sterilization the main problem is with alcohol, because it is inflammable and if spilled near a flame will cause an instant fire. Nowadays, in place of flame sterilization, dry sterilization of instruments using steripots is practiced in order to avoid instant fires.

3. Autoclaving

The culture vessels which may be empty (or) containing the medium are sterilized by heating in an autoclave (or) a pressure cooker to 121^{O+C} at a pressure of 15 pounds per square inch for 15 minutes for 20-50 ml medium and for 40 minutes for 2 l medium.

So the larger the volume of the medium the more will be the time of sterilization. The pressure should not exceed 20 PSI as higher pressure may lead to the decomposition of carbohydrates and other components of the medium. Care should be taken such that all the culture vessels are sealed properly with cotton plugs. The autoclave should be opened only when its pressure gauge indicates zero pressure.

4. Filter Sterilization

Some growth regulators, like GA_3, Zeatin, ABA, Urea, some vitamins and enzymes, thermolabile and may decompose during autoclaving. The solutions of these compounds are filter sterilized by passing through a membrane filter under positive pressure. A Millipore filter with a pore size of not more than 0.2 μm is generally used in filter sterilization. The membrane filter is held in a suitable assembly. The assembly together with the fitter is sterilized by autoclaving before use. The filter sterilized solutions are then combined with other nutrient substances which are sterilized in the autoclave and cooled, to give a complete medium. In the case of agar medium they are added when the medium has cooled to about $40^{O}C$.

Laminar air flow cabinets are used to create an aseptic working space by blowing filter sterilized air through an enclosed

space which is opened on one side. The air is first filtered through a coarse prefilter to remove large particles, it is then passed through a HEPA (high efficiency particulate air) filter which filters out all particles larger than 0.3 μm. This sterilized air blows through the cabinet at 1.8 mg/hr which is sufficient to keep the enclosed working area aseptic.

5. Wiping with 70% Ethanol

The surfaces that cannot be sterilized by other techniques, viz. culture room platform of the laminar air flow cabinet and the hands of the operator are sterilized by wiping them thoroughly with 70% alcohol and the alcohol is allowed to dry. The culture rooms are initially cleaned by gently washing the floor and walls with a detergent. This is followed by wiping with 95% ethanol.

6. Surface Sterilization

All plant materials that are to be used for culture are treated with an appropriate sterilizing agent to inactivate the microbes present on their surface. This is called surface sterilization. The excised piece of plant material that is used for culture is called explant. The surface sterilization protocol mainly depends on the source and the type of tissue of the explant which will determine the extent of contamination and tolerence to the sterilizing agent.

In the process of sterilization living matrials should not loose their biological activity and only the bacterial (or) fungal contaminants should be eliminated. So, the plant organs or tissues are surface – sterilized by treatment with a disinfectant solution at suitable concentrations for a specific period. The most widely used disinfectants and their concentrations in the solution are given in the table.

- Hard explants are treated directly with disinfectants for example in the culture of mature seeds (or) mature endasperm, the whole seeds (or) decoated seeds are surface sterilized.

- An explant that carries a heavy load of microorganisms is to be washed in running tap water for 1-2 hr prior to its treatment with disinfectant solution.

- In case of the delicate tissues such as shoot apices, pollen grains and shoot or flower buds, ethyl alcohol (or) isopropyl alcohol are used for surface sterilization. These explants are given a rinse in 70% ethanol for few seconds and then left exposed in the sterile hood until the alcohol evaporates.

- Usually shoot apices (or) pollen grains are free from micro contaminants and may be used for inoculation without surface sterilization.

Addition of a few drops of surfactants like Triton-X (or) tween – 80 to the solution (or) treating the plant material in a solution of cetavlon for 2 minutes before exposing to sterilant may enhance sterilization efficiency.

A general protocol for the sterilization of seeds will be as follows: Explants obtained from the field should be first thoroughly washed under running tap water to remove the dirt and epiphytotic microbes then they are treated with 1% cetarlon for 10 – 15 minutes to reduce bacterial contamination. Then they are rinsed with 70% ethyl alcohol for 30 sec. Then they are treated with 0.2% $HgCl_2$ for 10 – 15 minutes. This step is followed by a rinse with 70% ethanol and finally 4 – 6 rinseswith sterilized distilled water. The entire protocol is carriedout in an aseptic area provided by a laminar airflow cabinet.

Most of the explants are large and can be sterilized directly. But in the case of small explants like ovules, embryos, pollengrains etc., which are small, the organs containing them i.e. ovaries for ovules (or) developing seeds for embryos are surface sterilized

followed by which the desired explants are dissected out aseptically.

8.3.2.3 Summary

The various operations of tissue culture technique are to be carried out under aseptic conditions. Bacteria and fungi are the common contaminants that we will come across in invitrocultures. The contaminants may also release metabolic wastes which are toxic to plant tissues. So, to avoid this the maintainance of complete aseptic environment in the culture vessel is very much essential.

To achieve this objective all the materials that are used in culture work must be freed from microbes. This can be achieved by following different methods of sterilization viz. dry heat, flame – sterilization, filter – sterilization, autoclaving, wiping with 70% ethanol and surface sterilization. Glass wares, Teflon plastic wares and instruments are sterilized by dry heat in an oven at 160 – 180^{O}C for 3 hours. Instruments like forceps, scalpels, needles etc. are flame sterilized by dipping them in 95% alcohol followed by flaming.

Culture vessels, culture medium, micropipettes etc. are sterilized by autoclaving in an autoclave (or) pressure. Cooker at

121^{O}C temperature and 15 pounds per square inches pressure for 15 – 40 minutes basing on the volume of the medium.

Heat labile compounds such as growth regulators, urea, certain vitamin and enzymes are filter sterilized by passing their solution through a membrane filter of 0.45 μ (or) lower pore size.

Laminar flow cabinets are used to create an aseptic working space by blowing fitter sterilized air through an enclosed space.

The surfaces that cannot be sterilized by other techniques, viz. platform of laminar flow cabinet and the hands of the operator are sterilized by wiping them thoroughly with 70% alcohol.

All the plant materials that are to be used for culture are treated with an appropriate sterilizing agent to inactivate the microbes present on their surface. This is surface sterilization.

8.3.2.4 Key Words

Autoclave

Laminar air flow cabinet

Flame sterilization

Filter sterilization

Surface sterilization

Aseptic conditions

8.3.2.5 Self assessment test

1. What is sterilization? Discuss the different sterilization techniques used in plant tissue culture.

2. Write short notes on the following?

   (a) What is surface sterilization?

   (b) What is autoclaving?

   (c) What is filtersterilization?

8.3.2.6 References

1. An introduction to plant tissue culture Dr.M.K. Razdan, Oxford & IBH publishing Co. Pvt., Ltd.

2. Plant cell and tissue culture, Narayana Swamy, Tata Mcgrow-hill.

3. An introduction to plant tissue culture, Dr.Kalyan Kumar De, New Central Book Agency.

4. Biotechnolgoy, B.D.Singh, Klayani Publishers.

5. Elements of Biotechnology, P.K. Gupta, Rastogi Publications.

6. Biotechnology, V.Kumaresan, Saras Publications.

Lesson 8.3.3

CALLUS CULTURE

Objective

8.3.3.1 Introduction

8.3.3.2 Principles of callus culture

1. Introduction of the explant
2. Maintainance of culture flasks
3. Callus formation
4. Proliferation of callus tissue
5. Sub culture of callus

8.3.3.3 How the callus tissue is formed

8.3.3.4 Morphology, Anatomy and other characteristics of callus tissue

8.3.3.5 Habituation of callus

8.3.3.6 Chromosomal variation in callus tissue

8.3.3.7 Significance of callus culture

8.3.3.8 Summary

8.3.3.9 Key Words

8.3.3.10 Self Assessment Test

8.3.3.11 References

Objective

Callus is an undifferentiated mass of cells produced from isolated plant cells, tissues (or) organs when grown aseptically on the nutrient medium under controlled experimental conditions. In this chapter different aspects related to the callus viz. initiation, culture, maintenance, morphology, anatomy and other characteristics and its importance.

8.3.3.1 Introduction

Higher plant body is multi cellular and different tissue systems present in different organs function in a highly coordinated manner. In nature, sometimes callus or callus like tissue is found to be formed in various parts of the plant either due to deep would (or) due to some diseases. Deep wound in branches and trunks of the intact plants results in the formation of soft mass of unorganized parenchymatous tissue below the injured surface. Such callus tissue is known as wound callus. Wound callus is formed by the division of cambium tissue. The mainfunction of such callus heals up the injured part of the plant. In some higher plants, some times callus like tissue masses are formed on the stem, leaf, root by the stimulation of cell division due to some diseases caused by fungi, bacteria, viruses, insects or genetic factors. Such – callus like out growths are known as galls (or)

tumours. But the callus in tissue culture is produced from the excised plant tissues. The explants are cultured aseptically invitro under controlled condition on the nutrient medium. In culture, the excised plant tissue looses its structural integrity and changes completely to a rapidly proliferative unorganized mass of cells which is called the callus tissue. The development of callus tissue is first reported by a fresh scientist R.J.Gatheret (1934-1937) from the excised cambial tissue of salix capraea and other woody species.

8.3.3.2 Principles of Callus Culture

For inducing the callus production, the explant is first inoculated in a flask containing proper nutrient medium. Then the culture flask is maintained properly by keeping it in a room where the temperature is maintained constant. This process is called incubation. During incubation, the explant starts to produce callus tissue.

The important stages of cells induction are, inoculation of the explant, incubation of culture flasks, and formation of callus.

1. Inoculation of the explant

The culture room is sterilized properly before the worker enters the room. All the glassware are sterilized by autoclaving. The inoculation bench is sterilized by wiping it with ethylalcohol.

Thus the maintainance of aseptic condition inside the workroom is the first step in callus induction.

Then the sterilized medium is distributed in to the conical flasks and their mouths are plugged with sterilized cotton to prevent contamination. Then the surface sterilized explants are transferred to the semisolid medium in the flask and they are gently pressed in the medium. The pressing of the explants in the medium brings about good contact between the explant and the nutritional medium. The inoculation of the explants is done by keeping the mouths of the culture flasks near the flame of a spirit lamp. The mouths of the flasks are plugged with sterilized cotton.

2. Maintenance of Culture Flasks

The flasks inoculated with the explants are transferred in to a culture room where the temperature is maintained constant (or) an incubator. The temperature of the incubator should be adjusted to 25 $\pm$ 2^{O}C which is favourable for callus production. The culture room should be provided with proper light and air for the normal growth and development of explants. So, the culture rooms must be fitted with florescent light to supply sufficient light to the flasks. But some people are carrying out this callus culture evening darkness.

3. Callus Formation

During the incubation period, the cells of the explant undergo growth and division, resulting in callus with in 3-8 days after incubation. The callus is an undifferentiated mass of actively growing parenchymatous cells. The cells are soft and they are tightly held and it is a brittle mass of cells which are liable to breakdown due to mechanical disturbance. During culture the radicle tip emerges first if the explant is kept horizontally on the nutrient medium and the shoot tip emerges emerges first if the explant is placed vertically on the medium.

4. Proliferation of callus tissue

The well developed callus is cut in to small pieces with the help of a sterile knife. Then the callus pieces are transferred to another medium containing an altered composition of hormones. This medium induces the growth of some more callus tissues. This process of production of more callus tissues is called callus proliferation. The medium used for callus proliferation is known as proliferation medium. The callus tissues multiply more rapidly in this medium due to their fast growth and cell division. 2,4-D is a synthetic plant growth hormone which induces callus production in most of the tissues. 2,4-D is avoided in the proliferation medium.

The callus pieces are cultured in the medium containing growth hormones like IAA, NAA, Kinetin, 6-Benzyl aminopurine etc.

IAA induces callus proliferation in dicots, but high concentration of IAA reduces callus product in the monocots.

5. Sub-culture of Callus

After the proper growth of callus tissues, they are to be transferred to a fresh medium. At regular intervals. This transfer helps to maintain the cells in a viable condition. During subculturing the previously cultured tissues act as explant for establishing the secondary culture. This process of culture is known as subculture. Generally subcultures are practiced at a regular interval of every 4 weeks.

Figure Procedure for the callus culture from carrot.

8.3.3.3 How the callus tissue is formed

Formation of callus tissue is the out come of cell expansion and cell division of the cells of the explant. During the formation of callus tissue, the explant looses its original characterstic. Under the influence of exogenously supplied growth hormone, cell

enlargement and cell division will take place and forms an unorganized mass of cells. As a result, the explant undergoes an irreversible change in its shape, size, symmetry, structural organization and cellular integrity.

For the initiation of callus culture, tissues from young seedling (or) from juvenile part of the mature plant are generally taken. In such tissue a growth momentum is already present among the cells due to the presence of maximum number of physiologically active meristematic cells. There will be a nutrient gradient among the different cells of the explant basing on their position in the explant with reference to the nutrient medium. So, the cells divide asynchronously depending upon the availability of the nutrient and hormones and the callus tissue is formed. With in few days after inoculation either enlargement in size (or) the swelling followed by rupture of the tissue takes place, basing on the type of explant viz. Leaf, stemsegment, root segment, another etc. This change indicates the response of explant for callus formation, which will be followed by the appearance of little irregular cellular masses around the cut edges (or) from the ruptured surface. It is explained that the formation of cellular mass particularly at the cut end first may be due to injury during excision. Some endogenous growth substances oozes out through the injured tissue at the cut

end and stimulates the cell division which is simultaneously induced by the exogeneously supplied hormones.

There is another explanation that both endogenous product and exogenous hormones make a threshold level and their interaction results in the formation of an unorganized cell mass growth at the extend. It is generally accepted that exogenous hormones play the major role for the formation of callus in tissue culture. Both auxin and cytokinin are required for indefinite growth and cell division in callus culture.

When the explant are cultured on a suitable medium, many of its cells undergo division. Mature and differentiated cells like parenchyma and collenchyma cells undergo changes to become meristematic. This is called dedifferentiation. During the process of dedifferentiation there will be renewed and enhanced RNA and protein synthesis leading to the formation of new cellular components needed for meristematic activity.

Regarding the growth regulators, some times only 2,4-D (Auxin) alone is sufficient for callus formation. Auxins are required for growth and cytokinins are required for cell division. This concept is applicable for callus formation also.

After the initiation of visible unorganized mass of cells at the cut end, gradually the whole tissue is involved in callus formation

therefore, initially the cell divisions are confined to the cut ends, but gradually it covers the entire surface of the explant. The callus tissue gradually increases in mass as the new cells are added by mitosis. Estimation of callus growth can be made on the basis of changes in fresh weight (or) dry weight.

The type of tissue (or) cell present in the explant is an important factor for the rapid formation of callus tissue. If any meristematic tissue is present in the explant such as vascular cambium, the dividing cells of the vascular cambium continue the rapid growth under invitro condition, and proliferate into callus tissue. The vacuolated and highly differentiated cells of pith and cortex are also stimulated to divide and proliferate in the irregular mass of cells which spread over most of the surface and inner part of the explant.

8.3.3.4 Morphology, Anatomy and other characterstics of Callus Tissue

Morphology

Callus tissue proliferates as an irregular mass of cells. It is very difficult to describe its external morphology. The calli derived from different derived from different plants look alike. But they can be distinguished on the basis of other characteristics texture, colouration, hormone requirements etc. So the calli initiated from

explants of the some plant species may show considerable variation.

Anatomy

The anatomical structure of callus tissue is revealed by light microscopy and electron microscopy.

Microtome section (or) squash preparation of the callus tissue shows that the cellular composition of callus tissue is extremely heterogenous ranging from small cells with dense cytoplasm to large cells with vacuolated cytoplasm. The shape of the cells with in the callers tissue varies from spherical to markedly elongated. Large elongated cells are generally non-dividing cells having a large central vacuole where as the small actively dividing cells are with dense cytoplasm and small vacuoles. Elongated cells with in the callus tissue may be differentiated in to lignified xylen trachieds (or) phloem – like cells. Formation of xylem and phloem with in the callus tissue is known as cytodifferentiation. It is also observed that some localized groups of meristematic cells, constituting the active loci will arise in the callus tissue. These are called meristemoids. The meristemoids may differentiate in to either shoot (or) root primordial (or) embryoids.

Figure : Cells from a friable callus of carrot showing diversity in shape and size.

Electron microscope reveals that the cells at their non-dividing state have a large central vacuole and thin peripheral cytoplasm. The no. of organellae is also minimum in the cytoplasm. The synthesis of new cytoplasm and reduction in the size of the central vacuole takes place when the cells enter in to the dividing state. There is an increase in the no. of endoplasmic reticulum, mitochondria, golgibodies andribosomes. The endo plasmic reticulum occurs as sheets running parallel with the cytoplasm. Microtubules are associated with the walls of actively growing cells. Cells from the green coloured zone of the callus contains chloroplasts, but the grana are poorly developed. Mostly the plastids act as amyloplasts. Cultured cells contain membranous myelin like bodies and membrane bound groups of vesicles which are known as multivesicular bodies.

Other Characteristics

Texture

Callus tissue shows variability in its appearance and texture. Basing on its texture callus tissue can be divided in to two categories viz. soft callus and hard callus. Soft callus is friable in nature and is made up of heterogenous mass of cells having minimal contact. Hard callus consists of closely packed cells. It is compact in nature.

Figure : Sections of soft and friable callus tissue and compact callus tissue.

Colouration

Generally callus tissue is creamish yellow (or) white in colour. Sometimes it may be pigmented. Pigmentation may be uniform or patchy. It may be green in colour, sometimes white callus tissue grown under dark conditions turns green after transferring it to light. Green colour develops due to the development of chloroplast in the cells of callus tissue Eg:- Callus tissue from the cotyledons of Soyabean.

Callus may be yellow due to the synthesis of carotenoid pigments. Eg:- Callus tissue of Nigellasativa grown under dark condition.

Purple coloured callus is reported from cauliflower cultures due to the accumulation of anthocyanin in the vacuoles (or) due to the production of oxidized form the DOPA (3,4 – dihydroxy phenyl alanine).

Brown colour frequently develops in the explant and subsequently in the callus tissue. This is due to the excretionof phenolic substances. Plant tissues contain large no. of phenolic compounds and also contains polyphenol oxidase which is specially separated from the phenols. When the plant tissue is cut during explant preparation, the enzyme comes in contact with phenols which are then oxidized to quinines. These quinomes will subsequently polymerize to form brown products. The formation of quinines is responsible for the browning of the callus tissue. Excretion of phenols inhibit the growth of callus tissue.

8.3.3.5 Habituation of Callus

Generally callus tissue needs growth hormones in the nutrient medium in order to grow as long as they are maintained through serial sub-cultures. It has been observed that callus tissue in some plant species may become habituated after

prolonged culture. So, the callus tissue is able to grow on a standard maintainance medium or basal medium which is devoid of growth hormones. The property of the callus tissue is known as habituation and that callus tissue is known as habituated callus. The actual cause of this habituation is not clearly known. But, it is suggested that the cells in habituated callus tissue appear to have developed the capacity to synthesise adequate amounts of auxins and cytokinins which may accounts for their independence of exogenerasly supplied hormones, habituated callus tissue cannot be distinguished from the normal callus tissue except in their hormone independence.

The plant tumour tissue can be isolated from the plant and cultured aseptically. In culture, the tumour tissue is capable of growing on simple basal medium i.e. hormone free medium like the habituated callus. These tissues differ from the habituated callus in their mode of origin. In case of wound tumour, the virus remains with in the cells and multiplies and it may disappear after prolonged periods of culture.

In crowngall tumour tissue probably, the presence of bacterial DNA in the genome of the crown gall tumour cells may make them hormone – independent.

8.3.3.6 Chromosomal variation in callus tissue

Chromosomal variation in callus may occur genetically (or) epigenetically in the cells of the callus tissue.

1. Genetical basis of chromosomal variation

Callus tissue is raised from root, shoot, leaves and other organs. These explants are made up of numerous cells which remain in different states of differentiation. Normally, the meristematic diploid cells undergo selective division for the growth of an organ. Some times endoreduplication occurs frequently in the differentiated tissues of higher plants and the endoreduplicated cells remain in mitotically blocked condition. The degree of endoreduplication depends upon the degree of cellular differentiation. Therefore, the genomic constitution becomes heterogenous in the original explant. The callus tissue may get such genomic heterogeneity due to non-selective induction of asynchronous division of both diploid and endoreduplicated cells. So, the pe-existing genomic heterogeneity of the explant may be a source chromosomal variation in the callus tissue. This variation of chromosomal number ranges from aneuploidy to different levels of polyploidy viz. tetraploid, nexaploid and so on. Occurrence of both haploid and different level of polyploid cells in the some callus tissue is known as mixoploid cell population.

2. Epigenetic basis of Chromosomal Variation

As the early stage of callus growth the percentage of diploid cells is higher than that of the polyploid cells. The no. of polyploid cells may increase (or) decrease through serial subcultures. All the meristematic cells of the callus are expected to be diploid. But, the callus tissue derived from meristem may also show variation in chromosome number. The variation generated during tissue culture is called epigenetic variation. Eg:- In the plant crepis, cellular differentiation occurs without endoduplication. So, all the cells of the explants taken form that plant are diploid. But the cells of the callus tissue derived from such plant are not diploid. Thus it appears that the variation in chromosome number is not always derived from the original explant. But it may arise due to the interaction of both genome and cytoplasm. Their interaction may bring about mitotic disturbance. In peaplants it is found that kinetin encouraged the development of polyploid cells in culture. It is also observed that strong auxins like 2,4-D induces the polyploidy in callus culture. It is also found that prolonged sub-culture may lead to the establishment of one karyotype and others are gradually eliminated. In most of the cases polyploid cells are found.

Sometimes structural changes of chromosomes like deletion, translocation etc. may occur in culture. Cells of the callus tissue may be haploid if it is derived from microscope culture. An ideal

callus culture is characterized by the possession of numerical (or) structural stability in long term culture but it is very rare.

8.3.3.7 Significance of Callus Culture

1. The whole plant can be regenerated from callus tissue through the manipulation of nutrient and hormonal constituents. This phenomenon is known as plant regeneration (or) organogenesis (or) morphogenesis.

2. A large no. of embryos can be produced, from the somatic cells of the callus tissue by manipulating the nutrient and hormonal constituents.

3. Callus tissue is a good source of genetic variability. So, it may be possible to regenerate a plant from genetically variable cells of the callus tissue.

4. From the callus tissue we can initiate cell suspension culture.

5. Callus culture is very much useful to obtain commercially important secondary metabolites. Eg:- If an explant from a medicinally important plant is grown invitro, and the callus is produced, then the secondary metabolites (or) drug can be directly extracted from the callus tissue without sacrificing the whole plant. So, this technique is an alternative method for the conservation of medicinal plants in nature.

6. Many biochemical assays can be performed from callus culture.

8.3.3.8 Summary

Callus is an unorganized proliferative mass of cells without any differentiation. In nature, sometimes callus (or) callus like structure is found to be formed in various parts of intact plant either due to deep would (or) due to some diseases. Callus tissue developed from the injured part of the plant is known as would callas. It is formed by the division of either cortical cells (or) cambiat cells. Callus – like out growth caused by some diseases is commonly called gall (or) tumour. The callus tissue which is important to plant tissue culture, is produced through tissue culture, tehnique from a small explant of the plant. The explant is cultured aseptically on a solid (or) liquid nutrient medium under controlled conditions.

For successful initiation of callus tissue from the explants, three important criteria should be accomplished. 1. aseptic prepartion of the plant material. 2. Selection of suitable nutrient medium with growth regulators. 3. Incubation of culture under controlled physical conditions. The callus grows in presence of artificial light (2,000 – 3,000 lux for 16 hrs) or in complete dark conditions. The suitable temperature for the growth of callus

tissue is usually 25±2°C. Generally 55% to 60% relative humidity is maintained in the culture room. Callus culture can be maintained continuously by serial sub-cultures.

The callus formation is an outcome of cell expansion and cell division of the cells of the explant. Under the influence of exogenously supplied hormones, the explant is triggered off a growth sequence in which cell enlargement and cell division predominate toform an unorganized mass of cells. Both the endogenously produced and exogenously added hormones make a threshold level and their interaction results in the formation of an unorganized mass of cells from the cut end of the explant. There is a general concept that auxin is required for cell growth and cyto kinin for cell division. This is applicable for invitro callus formation callus tissue generally increases in mass as the new cells are added by mitosis.

Callus shows variation appearance and texture. The shape of individual cells of the callus mass ranges from nearly spherical to elongated. Sometime callus mass undergoes differentiation and forms phloem cell and lignified xylem cells. Generally the callus tissue is creamish yellow or white in colour. Sometimes callus tissue may be pigmented. Brown colour develops frequently in the explant and subsequently in the callus tissue due to the excretion of phenolic substances.

In some plants species the callus tissue may become habituated to grow on the culture medium which is devoid of hormones, after prolonged culture. Callus tissue derived from the plant tumour tissue (or) crown gall tissue is also capable of growing on growing on simple basal medium like the habituated callus.

Chromosomal variation may occur genetically (or) epigenetically in the cells of the callus tissue. Sometimes numerical (or) structural changes of chromosomes may occur in culture.

The whole plants can be regenerated from the callus tissue through the manipulation of nutrient and hormonal constituents in the culture medium embryoids can also be produced by the callus by the process of `somatic embryo genesis'.

Callus tissue is a good source of genetic variability, so it may be possible to regenerate the plant lets from the cells showing genetically variability. Callus culture is very important to obtain commertially important secondary metabolites.

8.3.3.9 Key Words

Cyto differentiation

Epigenetic Variation

Asynchrony

Crown gall tumour

8.3.3.10 Self Assessment Test

1. What is callus tissue? Describe the principle and discuss the significance of callus culture?

2. How the callus tissue is formed in vitro? Discuss the morphology, internal structure and other characterstics of callus tissue.

3. Write short notes on the following:

   (a) What is habituated callus tissue? How is it formed?

   (b) What are the main reasons of chromosomal variation in callus tissue? State its significance in callus culture?

   (c) Why do the explant and the callus tissue of some plants develop brown colour in culture?

4. Write short notes on the following :

   (a) Internal structure of callus tissue.

   (b) Significance of callus culture.

   (c) Difference between soft and compact callus.

8.3.3.11 References

1. An introduction to plant tissue culture Dr.M.K. Razdan, Oxford & IBH publishing Co. Pvt., Ltd.

2. Plant cell and tissue culture, Narayana Swamy, Tata Mcgrow-hill.

3. An introduction to plant tissue culture, Dr.Kalyan Kumar De, New Central Book Agency.

4. Biotechnolgoy, B.D.Singh, Klayani Publishers.

5. Elements of Biotechnology, P.K. Gupta, Rastogi Publications.

6. Biotechnology, V.Kumaresan, Saras Publications.

Lesson 8.3.4

CELL SUSPENSION AND SINGLE CELL CULTURES

Objective

8.3.4.1 Introduction

8.3.4.2 Suspension Cultures

1. Isolation of Single Cells
2. Types of Suspension Cultures
3. Culture Medium for Suspension Cultures
4. Conditioning of Medium
5. Agitation of Medium
6. Synchronisation of Suspension Cultures
7. Growth Patterns in Suspension Cultures
8. Measurement of Growth in Suspension Cultures
9. How to test the viability of Cultured Cells
10. Importance of Suspension Cultures

8.3.4.3 Single Cell Culture

1. Principle of Single Cell Culture
2. Methods of Single Cell Culture
3. Factors Effecting Single Cell Culture
4. Importance of Single Cell Culture

8.3.3.4 Summary

8.3.3.5 Key Words

8.3.3.6 Self Assessment Test

8.3.3.7 References

Objective

Cell suspension and single cell culture provides an opportunity to investigate the properties and potentialities of plant cells. In this chapter different aspects related to those cultures Viz. basic principles of culture, different types of cell suspension cultures, methods of single cell culture, growth pattern and estimation of growth in suspension cultures, test for cell viability, different factors effecting the cultures and the applications are discussed.

8.3.4.1 Introduction

Callus proliferates as an unorganized mass of cell. So it is very difficult to follow many cellular events during its growth and developmental phases. To overcome such limitations of callus culture, the cultivation of free cells and small cellular aggregates in a chemically defined liquid medium as a suspension was initiated to study the morphological and biochemical changes during their growth and developmental phases.

Cell suspension cultures were first reported by W.H. Muir (1953) from the callus of Tagetes erecta and Nicotiana tabacum. Later in 1956 F.C. Steward and E.M.Shantz reported the

suspension cultures from carrot root explant and obtained very large no. of plant lets from the culture.

Establishment of cell suspension and single cell cultures provide an opportunity to investigate the properties and potentialities of plant cells. Single cell systems have a great potential for crop improvement. Through the cell culture of an intact organ (or) whole plant we can synthesize natural products. Free cells in culture permit quick administration and withdrawal of diverse chemicals. So, the free cell cultures are the easy targets for mutant selection.

During cell culture variability arises. Such variability is called `special heterogeneity'. This is very important because the differences between the cells in their karyotype and the ability to accumulate secondary metabolites, would manifest during morphogenesis in the clones regenerated from single cells. Thus the cell line selection technique can be applied to produce high yielding cultures and also the plants with superior agronomic traits.

8.3.4.2 Isolation of Single Cells for the Initiation of cell suspension and single cell culture

For the initiation of suspension and single cell culture we should be provided with single cells, which can be obtained from

the preestablished callus (or) from the explant directly. Single cells can be isolated either from the cultured tissues (or) from the plant organs.

1. Isolation of single cells from plant organs

The most suitable material for the isolation of single cells is the leaf tissue. From the leaf the single cells can be isolated using mechanical (or) enzymatic method.

(a) Mechanical Method

The procedure for mechanical method involves mild mceration of 10 gms of leaves in 40 ml of the grinding medium (20 μ mol sucrose, 10 μ mol $MgCl_2$) and 20 μ mol tris –Hcl buffer pH 7.8) with a mortor and pestle. The homogenate is filtered through two layers of muslin cloth and the cells released thus are washed by centrifugation at low speed using the same medium. Pipette out the supernatant and suspend the cells in a volume of the medium sufficient to achieve the required cell density.

(b) Enzymatic Method

Takebe in 1968, treated the tobacco leaf with pectinase and obtained a large no. of metabolically active cells. Potassium dextran sulphate added to the enzyme mixture improved the yield of free cells. Isolation of single cells by the enzymatic method gives

high yields from the preparations of spongy parenchyma with minimum injury to the cells. This can be accomplished by providing osmotic protection to the cells while the enzyme macerozyme degrades the middle lamella and cell wall of parenchymatous tissue.

2. From Cultured Tissue

This is the most widely followed approach to obtain a single cell system from cultured tissues. Surface sterilized explants are placed on a nutrient medium consisting of a suitable proportion of auxins and cytokinins to initiate cultures. Then the explant starts callusing from its cut ends, which gradually extends to the entire surface of the tissue. The callus is separated from the explant and transferred to a fresh medium to get more amount of callus tissue. Repeated subculture on agar medium improves the friability of the callus. Friability is prerequisite for raising a fine cell suspension in a liquid medium. Pieces of undifferentiated and friable calli are transferred in to liquid medium dispensed in autoclaved flasks, which is continuously agitated in autoclaved flasks, which is continuously agitated to obtain a **Suspension Culture**. Agitation of pieces breaks them into free cells and small aggregates and also maintains uniform distribution of cells and cell clumps in the medium. Agitation also allows gaseous exchange between the culture medium and culture air.

8.3.4.3 Suspension Culture

It is a type of culture in which single cells (or) small aggregates of cells are suspended in agitated liquid medium and allowed to multiply.

Principle: It is very different to follow many cellular events during its growth and development through callus culture. So, the cultivation of free cells as well as small aggregates of cells in a chemically defined liquid medium as a suspension allows to study the morphological and biochemical changes during their growth and development.

Note: The suspension culture can be initiated from pre established callus (or) from the explant directly which is continuously agitated in a liquid medium on a rotary shaker. Agitation facilitates proper aeration and uniform distribution of nutrients in the medium and also allows the callus to break up.

An ideal cell suspension can be obtained by transferring a more friable callus to agitated liquid medium. It breaks up and disperses in to single cells and small cell aggregates. Eliminate the large callus pieces and transfer only single cells and small cell aggregates to fresh medium. After two (or) three weeks a suspension of actively growing cells is produced. The suspension

cultures are clonally maintained by the routine transfer of cells in the early stationary phase to a fresh medium.

An ideal culture should consist of only single cells which are physiologically and biochemically uniform. It can be achieve through synchronization of suspension culture. A synchronous culture is one in which the majority of cells proceed through each phase of the cell cycle simultaeneously (G_1, S, G_2, α M), More friable callus is an ideal material for dispersion of cells. The dispersion of less friable callus can be improved by increasing the concentration of auxins (or) by adding low concentrations of cellulase and pectinase enzymes to the liquid medium.

The time interval taken for the development of suspention culture from the callus tissue is called initiation passage. During this passage the callus tissue breaks up and the cells grow and divide until the depletion of some nutrient in the medium.

The suspension culture that is obtained at the end of the initiation passage is filtered through nylon mesh to remove larger pieces of callus tissue. Now the filtrate containing single cells and small aggregates is transferred in to a fresh liquid medium to initiate passage I suspension. In the subsequent passages the cell suspension is sub-cultured by pipetting aliquots of the suspension in to fresh culture medium.

8.3.4.2.1 Types of suspension culture

The susension cultures are broadly classified as, 1. Batch cultures 2. Continuous cultures and 3. Immobilized cultures.

8.3.4.2.2 Culture medium for suspension cultures

Generally, the media suitable for growing callus culture for a particular species is also suitable for growing suspension culture of that particular species provided such that the agar is omitted. In some cases the concentration of auxin and cytokinins is often Gitical for the growth of cell suspension culture and the concentration of auxins and cytokinins used for callus culture is generally reduced for suspension cultures. But in some cases it differs. The culture medium for tobacco cell suspension requires an increase in the concentration of 2,4-D from 0.3 mg/lit to 2 mg/lit, followed by supplementing the callus medium with additional vitamins and casein hydrolysate. The inorganic phosphate is rapidly utilized in the actively growing suspension cultures and consequently it becomes a limiting factor.

Many media have very little buffering capacity and the pH can change with an increase in the cell biomass. This necessitates monitoring and adjustment of pH in suspension culture. B5 and ER (Erikson – 1965) media re specially recommended for the suspension culture of higher plants. These media are used for

initial population density 5 x 10^4 cell/ml (or) more. With lower cell density, the medium requires to be conditioned (or) enriched with various other compounds.

8.3.4.3.2.2 Conditioning of Medium

A conditioned medium is a medium in which plant cells have been grown for about 48 hrs. and the cells are then filtered out. For the initiation of cell suspension cultures at a low inoculum density a conditioned medium is used. A conditioned medium can be dotained by filtering out the cells growing at high density from 4-6 week old liquid culture. This medium can be used in drops (or) as thin layer to culture cells at low population density/single cells. In 1989 – Torres gave the principle of conditioning. This principle involves the separationof a high – density cell – culture from a low density culture medium by a barrier that permits the diffusion of solutes and air. A high density cell suspension kept inside a dialysis tube is suspended by means of a thread in the flask containing the low cell density culture. The metabilites produced by the nurse culture (high density culture) diffuse in to the low – density medium and increases the growth of low cell density. This meets the conditions of growth for low cell density populations since the necessary substances that may not be found in the low density medium are released in to it by the biosynthetic activity of the nurse cells.

8.3.4.3.2.1 Agitation of the Medium

Suspension cultures require constant agitation of the medium for adequate aeration. This also facilitates dispersion of cells. This is done by using a shaker and suitable flaks. In 1953 – Muir introduced the orbital – platform shaker for growing suspension cultures of tobacco and Tagetes eracta. The platform of the shaker is fitted with interchangeable clips for holding the flaks. Generally a shaking speed of 30-150 tpm is optimum for most of the tissue. Rotary shakers are also used which can rotate at slow speed (1-2 tpm) and they will have a disc which can be rotated by a shaft. Both orbital and rotary shakers have a control for regulating the speed.

8.3.4.3.3 Synchronisation of Suspension Cultures

Cells in suspension cultures vary greatly in size, shape, DNA and nuclear content. The cell cycle time also varies with in individual cells. So, the cell cultures are mostly a synchronous. This variation complicates the study of biochemical, genetic physiological aspects of cell metabolism. So, it is essential to manipulate the growth conditions in an asynchronous culture to achieve high degree of synchronization. In a synchronous culture all the cells will be in the same phase of cell cycle (G_1, S, G_2 x M). Synchronisation is expressed as percentage synchrony of cells in

suspension cultures. Synchronisation can be achieved by physical and chemical methods.

8.3.4.3.3.1 Physical Methods

Physical properties of cells like the size of individual cells (or) growth environmental conditions like light and temperature are monitored to achieve synchronization. Some of them are,

A. Selection by Volume

Synchronisation may be achieved by selecting on the basis of size of cell aggregates. This method was proved successful for carrot suspension cultures, where 90% of cell aggregates that are isolated were in early embryonic stages.

B. Temperature Shock

Low temperature shocks combined with nutrient starvation are found to induce synchronization of suspension cultures.

8.3.4.3.3.2 Chemical Methods

Cell cultures are starved of a nutrient (or) supplied with a biochemical inhibitor to prevent the cells from completing a cell cycle. Through this approach the cells are first arrested at a particular stage of cell cycle and then they are allowed to undergo

division simultaeneously either by supplementing the starved chemical (or) by withdrawing the inhibition.

A. Starvation

This is based on depriving the suspension cultures from an essential growth compound leading to a stationary growth phase. Resupply of the missing compound induces the resumption of synchronous cell growth. This procedure has been found to be effective in acer pseudoplatanus suspension cultures.

In the cultures which are starved of nitrogen, phosphorus (or) carbonate, the arrest of cell growth during the G_1 (or) G_2 phase of cell cycle is observed. After a period of starvation, when these growth limiting compounds are supplied to the medium, the stationary cells undergo synchronous division. Growth hormone starvation is also reported to induce synchronization of cell cultures.

B. Inhibition

Synchronisation can be temperorily achieved by blocking the progression of events of cell cycle so that all the cells will be arrested in a specific phase, using a biochemical inhibitor. After releasing this biochemical block synchronous divisions can be achieved. This procedure is used in Tobacco, Tomato and Soyabean. Inhibitors of DNA synthesis like 5-aminouracil, FudR-

(Fluoro deoxy uracil) (or) 5-fluro deoxy purine, hydroxy urea, TDR (or) excess thymidine will bring the cells to G_1/S boundary. After the removal of the inhibitor synchronous divisions will take place.

C. Mitotic Arrest

Colchicine arrests the cell division at metaphase. Suspension cultures in exponential growth are supplied with 0.02% (W/V) colchicine for 4-8 hours. It inhibits spindleformation. Longer colchicines treatment may lead to an increased frequency of abnormal mitoses and chromosome stickiness. So, only shorter duration of treatment is recommended. This technique is used in case of zea mays. As colchicines can induce genomic changes, there is also possibility of obtaining asynchronous cultures.

8.3.4.3.5 Measurement of growth in suspension cultures

Growth assessment in cell suspension cultures can be accomplished by following selected parameters at regular intervals. These include; (a) cell counting, (b) packed cell volume and (c) Fresh/dry weight increase of cells and cell colonies.

1. Cell Counting

It is a more accurate measure to determine the growth of cultures. Increase in cell number depends on the rate of mitotic index (MI) of cells in suspension cultures. Determination of cell

number is a tedious procedure since suspension cultures carry cell aggregates. There are two methods generally employed for macerating the cell masses found in culture, They are 1. Chromic trioxide treatment, 2. Pectinase enzyme treatment.

1. Chromic Trioxide Treatment

1. A small volume of the sample suspension is treated with an aqueous chromic trioxide solution. (2 parts of the chromic trioxidet 1 part of sample).
2. The test solution is heated to about 70^{O}C for 2-15 mts.
3. Then the suspension is allowed to cool by keeping it in a water bath.
4. The cooled test suspension is staken well (or) agitated on a shaker to separate the individual cells from the mass of cells.
5. The suspension is now centrifuged, the chromic acid is poured off and the pellet is resuspended in 8% Nacl solution.
6. After 10-15 mts the free cells are counted on a haemocytometer.

2. Pectinase Enzyme Treatment

In this method the sample suspension is treated with 0.25% pectinase enzyme which dissolves the middle lamella present in

between the two adjacent cells. As a result, the individual cells are released from the cell mass. Then the suspension is used to count the cell number.

Packed Cell Volume

It is a more effective and the easiest method for the determination of cell growth in the suspension culture. For the determination of packed cell volume, 10 ml of culture suspension is transferred to a graduated measuring tube. This sample is centrifuged for favouring the deposition of cells at the bottom of the tube. After the settlement of all cells in the tube, the total volume of packed cells can be measured against the readings of the graduated measuring tube. The packed cell volume is expressed as ml of cell pellet per ml of culture.

Cell Fresh Weight

The total weight of cells in the suspension depends on the weight of all individual cells and the number of cells found in the suspension. The weight of cells in the sample can be measured by using the following procedure :

1. A desired volume of the sample is filtered through watman no.1 filter paper with the help of buchnar funnel under slight vacuum conditions. After the completion of filtration, only the cells will remain on the filter paper.

2. The cells that remain on the filter paper are washed properly with distilled water and again filtered through the filter paper and the excess water found along with the cells is allowed to drain-off.

3. The filter paper containing the cells is weighed properly with the help of a sensitive balance.

4. The weight of a wet filter paper alone is deducted from the weight of the filter paper with cells. The resulting value gives us the total weight of living cells found in the sample.

5. The weight of cells in the sample can be expressed in grams/litre. This weight is calculated with reference to the total volume of culture suspension.

Cell Dry Weight

A procedure similar to that for fresh weights is followed for determining cell dry weight except that the filter paper is dried in an oven for 12 hours at 60^{O}C. After cooling in a desiccator containing silica gel, the dried filter is reweighed the cell weight is expressed as gms/litre of culture.

8.3.4.3.6 How to test the viability of cultured cells

The growth of cultures is largely dependent on the viability of the cells. There are different methods which help to decide

whether the cultured plant cells in the medium are alive (or) not. This testing of viability is necessary to know the living cells for proceeding to further steps in plant tissue culture. The different methods used for testing cell viability are as follows :

1. Phase Contrast Microscopy

The cell viability can be determined by direct observation of cells under a phase – contrast microscope. Cytoplasmic streaming and the presence of healthy nucleus indicate that the cells are viable. Phase contrast microscopy is recommended as it is difficult to observe these aspects in unstained cells under a bright field.

2. Reduction of Tetrazolium Salts

This method is used to measure the respiratory efficiency of living cells. The cell masses are stained with 2,3,5-tri-phenyl tetrazolium chloride (TTC). Living cells reduces TTC to formazon which gives red colour. Formazon can be extracted and measured spectrophotometrically.

3. Fluorescein Diacetate (FDA) Method

This method offers quick visual assessment of the viability of cells. Stock solution of FDA (0.5% w/v) is prepared in acetone and stored at $O^{O}C$. The cells are treated with 0.01% solution of FDA. Live cells cleave FDA by esterase activity and produce fluorescein,

which cannot cross the plasma membrane and it accumulates mainly in the cytoplasm of the living cells. Under UV exposure, fluorescein gives green fluorescence so that the cells appear green. In dead cells the fluorescein is lost and they do not fluoresce.

4. Evan's Blue Staining

A dilute solution of (0.025%) Evan's blue dye stains the dead (or) damaged cells. It freely enters the damaged cells and it is not taken by live cells. So, the dead cells stain blue and the living cells remain unstained. Evan's blue is usually taken in conjunction with FDA.

8.3.4.3.3 Growth Patterns in Suspension Culture

Under appropriate light, temperature, aeration and nutrient medium the growth of suspension culture follows a growth curve. The growth of suspension culture can be monitored by counting the cell number per unit volume of culture in relation to days of culture. The growth curve for a typical higher plant suspension culture consists of lag phase, logarithamic (or) exponential phase, linear phase and stationary phase.

A lag phase is the period where the cells adjust them selves to the nutrient medium and prepares for cell division. This follow by very rapid cell division, causing a logarithmic increase in cell number. This phase is called logarithmic phase. It will be followed

by a further period of rapid cell division, which results in a linear increase in number. This phase is called linear phase. As the nutrients are depleted and some of the cells of the culture show scenescent characteristics, the rate of cell division with in the culture declines and it passes through the stationary phase. At this stage the growth curve forms a platean. If the cells are removed just before (or) just after the entry in to the stationary phase in each growth cycle and are subcultured to fresh medium, then identical patterns of growth of the cell line can be maintained in each culture passage. Dry weight, total protein, DNA synthesis etc. can also be considered as other parameters for the preparation of identical growth curves. The chemical composition of the cell changes throughout the growth cycle and such changes are closely coupled to the cell division in most of the plant material. However in some materials there is no correlation between the rate of cell division and the rate of dryweight accumulation. From these studies, it has been concluded that there are independent mechanisms and many biosynthetic pathways for controlling cell division. In synchronized cell populations, continuous changes in physiological property may also cause the divergence between the rate of cell division and the biochemical changes of the cell. It is also important to note that the degree of cellular aggregation is not constant but changes significantly during the growth cycle of the cell suspension culture. As the culture enters the period of most

active growth, the cell aggregation is maximum and during the stationary phase cell aggregation is minimum.

For experimental studies on the growth of cell suspension the inoculum cell density is an important factor. Very low density (or) highdensity of cells in liquid medium are unable to grow. So, to induce the growth an initial cell density of 2 x 10^6 to 2 x 10^8 cells/ml are inoculated in liquid medium. This initial cell density increases during the growth and attains a higher density at the stationary phase. The particular initial cell density that is able to grow in liquid medium is called critical initial density (CID). The CID may vary from plant to plant.

8.3.4.3.1 Types of Suspension Culture

The suspension cultures are broadly classified as, 1. Batch Cultures, 2. Continuous Cultures and 3. Immobilized Cultures.

1. Batch Culture

In the batch culture, the cells are nourished in a fixed volume of the medium and the amount of cell material increases until the nutrient elements are depleted and inhibitory substances get accumulated; as a result of which the growth ceases. In a batch culture the same medium and all the cells produced are retained in the culture vessel. The culture vessels may be the culture flasks (or) the fermenters. The cell number (or) biomass of a batch

culture exhibits a typical sigmoidal curve, having a lag phase during which the cell number remains unchanged, followed by a logarithmic phase (log phase) during these is a rapid increase in cell number and finally ending in a stationary phase during which cell number does not change. The log phase duration mainly depends on inoculum size and growth phase of the culture from which inoculum is taken. The log phase lasts or 3-4 cell generations. The duration of a cell generation may vary from 22-48 hours which depends upon the plant species. The stationary phase is forced on the culture by a depletion of the nutrients and also due to accumulation of cellular wastes. If the culture is kept in stationary phase for a prolonged period the cells may die.

Batch cultures are maintained by sub-culturing. They are used for the initiation of cell suspensions which may be used for cloning, cell selection (or) as seed cultures for scaling up (or) for continuous cultures. Batch cultures are chracterised by a constant change in the pattern of cell growth and metabolism and there will be a constant change in cell density α nutritional status of the medium. So, they are not ideal systems for studies on cell growth and metabolism. Batch suspension cultures are most commonly maintained in conical flask incubated on orbital platform shakers at the speed of 80-120 rpm.

1. Slowly Rotating Cultures

Single cells and cell aggregates are grown in a specially designed flask, the nipple flask. Each nipple flask possesses eight nipple – like projections. The capacity of each flask is 250 ml. There are 10 nipple flasks which are loaded in a circular manner on the large flat-disc of a vertical shaker. When the flat disc rotates at a speed of 1-2 rpm, the cells with in each nipple of the flask are bathed in culture medium and exposed to air alternately.

2. Shake Cultures

It is a simple and effective system of suspension culture. In this method, single cells and cell aggregates present in a fixed volume of liquid medium are taken in conical flasks. The conical flasks are mounted on a horizontal large square plate of an orbital platform shaker. The square plate moves by a circular motion at a speed of 60-180 rpm.

3. Spinning Cultures

Large volumes of cell suspension may be cultures in 10 litre bottles which are rotated in a culture spinner at 120 rpm at an angle of 45: Here the culture vessel is rotated.

4. Stirred Culture

This system is used for large scale batch culture of 1.5 to 10 litres. Here the large culture vessel is not rotated but the cell

suspension inside the vessel is kept dispersed continuously by bubbling sterile air through the culture medium. An internal magnetic stirrer is the most convenient way to agitate the culture medium safety. The magnetic stimer revolves at 200 – 600 rpm. The culture vessel is a 5-10 litres round – bottom flask.

8.3.4.3.1.2 Continuous Cultures

In continuous culture, the cell population is maintained in a steady state by regularly replacing a portion of the spent medium by fresh medium. There are two types of continuous culture systems. Viz. closed type and open type. In a closed continuous culture, the cells are separated from the used medium that is taken out for replacement and they are added back to the cultures so that the cell biomass keeps on increasing. In open continuous cultures, both cells and the used medium are taken out and replaced by an equal volume of fresh medium. The replacement volume is so adjusted that the cultures remain at submaximal growth indefinitely.

Note:- In continuous systems the nutrient depletion does not occur due to continuous replacement of nutrient medium and the cells always remain in the steady state of active growth phase.

There are two major types of open continuous cultures viz. chemostat and turbidostat.

Chemostat

In chemostats the cell growth is maintained steady by a constant inflow of fresh medium consisting of nutrients. A chosen nutrient is kept in a concentration so that it is depleted very rapidly to become growth limiting, while the other nutrients are still in concentrations higher than required. In such a situation, any addition of the growth limiting nutrient is reflected in cell growth. Chemostats are ideal for the determination of effects of individual nutrients on cell growth and metabolism.

Figure

Turbidostat

The turbidity of a suspension culture medium changes rapidly when the cells increase in number due to their steady state growth. The changes of turbidity of the culture medium can be measured by the changes in the optical density of medium. In a turbidostat, the cells are allowed to grow up to a preselected tubidity. PH of the medium also changes due to the increase in cell density. In the turbidostats, an automatic monitoring unit is

connected with the culture vessel and that unit adjusts the medium flow in such a way as to maintain the optical density (or) pH at a chosen, preset level.

Advantages of Continuous Cultures

1. Ease of maintaining sterility over a long period of time.
2. Less detrimental effects during mechanical failures.
3. A degree of automation.
4. Versatality with regard to growth conditions such as temperature, aeration, stirring speed, illumination, nutrient and growth regulator levels.

8.3.4.3.7 Importance of Suspension Culture

1. Important information about cell physiology, biochemistry, α metabolic events at the level of individual cells and small cellular aggregates can be obtained from suspension cultures.
2. It is also important to have an understanding of an organ formation or embryoid formation starting from single cell (or) small cell aggregates.
3. The technique of platingout cell suspension on agar plate is useful to obtain single cell clones.

4. Suspension culture derived from medicinally important plants can be studied for the production of secondary metabolites such as alkaloids.

5. Mutagenesis studies may be facilitated by the use of cell suspension cultures to produce mutant cell clones from which mutant plants can be raised. The cell population in a suspension culture can be subjected to a range of chemical mutagens, viz. Ethylmethane sulphonate (EMS), N-nitroso-N-methyl urea etc. The mutagens can be added directly in the liquid medium. After the mutagen treatment cells are plated on agar medium for the selection of mutant cell clones from which the plants can be raised.

8.3.4.4 Single Cell Culture

Single cell culture is a method of growing isolated single cell aseptically on a nutrient medium under controlled conditions. Free cells isolated either from plant organs (or) cell suspensions are grown as single cells under invitro conditions using a suitable medium. This process is called plating. The advantage of single cell culture over callus culture (or) cell suspension culture is that single cell culture is an ideal system fro studying cell metabolism, the effects of various substances on cellular responses and to obtain single cell clones.

In 1902, G. Haberlandt made an attempt to isolate and culture single cells from the leaves of flowering plants.

In 1934, W.H. Muir described paper raft nurse culture technique for the culture of single cells.

In 1968, L.Bergmann – Grew the single cells first using petridish plating technique.

8.3.4.4.1 Principle of Single Cell Culture

The basic principle of single cell culture is the isolation of large no. of intact living cells and culture them on a suitable nutrient medium for their requisite growth and development. Single cells can be isolated from a variety of tissue and organs of green plant as well as from callus tissue and cell suspension. Single cells from the intact plant tissue are isolated either mechanically (or) enzymatically. The single cells are traditionally isolated from the established friable callus tissue and cell suspension culture. The isolated single cells can be cultured either in the liquid medium or on solid medium. There are five different methods that are used for culturing single cells such as paper raft nurse technique, Bergmann petridish plating technique, The microchamber technique, The microdroplet technique, The plating with nurse tissue technique.

In culture, the single cells divide repeatedly to form a callus tissue. Such callus tissue also retains the capacity to regenerate the plant lets through organogenesis and embryogenesis.

8.3.4.4.2 Methods of Single Cell Culture

There are five different methods which are widely employed for culturing single cells. Isolated single cells are the essential prerequisite item for all there methods.

1. The Paper Raft Nurse Technique

Single cells are placed on small (8 x 8 mm) pieces of sterilized filter paper which are inturn placed on the top of an established callus cultures several days in advance. This allows the filter papers to be wetted by the exudates from callus tissues. The single cells placed on the filter papers derive their nutrition from the callus exudates diffusing through the filters. The cells divide and form macroscopic colonies on the filters. When the cell colony reaches a suitable size, it is transferred to fresh medium, where it gives rise to the callus tissue. A callus tissue originating from a single cell is known as a `single cell clone'.

2. Microchamber Technique

A microchamber can be created by using a microscope slide and cover slips. The coverslips are held in place by sterile mineral

oil. A cavity slide can also be used for creating a microchamber. Single cells are suspended in conditioned medium and a drop of medium having a single cell is placed in the microchamber which is coverd by a coverslip. In case of cavity slide, the drop is placed on to a coverslip which is then inverted in to the slide cavity. Microchambers allow microscopic observation and they can be kept in a petridish for incubation; under 16 hrs illumination (3000 lux) at 25^{O}C. When the cell colony becomes sufficiently large, the cover glass is removed and the tissue is transferred to fresh solid (or) semi solid medium.

3. Microdrop Method

A specially designed dish, called cuprak dish is used in this method. This dish is having a smaller outer chamber and a larger innerchamber. The outer chamber is filled with sterile distilled water to avoid desiccation of cells and the larger chamber is having many microwells.

Microdrops of 0.25 – 0.5 ml are distributed in the microwells and the dish is sealed with parafilm. The dish is incubated under 16hrs white cool light (3000 lux) at 25^{O}C. The cell colony derived from the single cell is transferred on to fresh solid (or) semisolid medium in a culture tube for further growth.

4. Nurse Callus Technique

This method is actually a combination of petridish plating method and paper raft nurse culture method. In this method the growth of single cell is induced by nurse callus. Here the single cells are plated on to agar medium in a petridish. Two or three callus masses (Nurse tissue) derived from the same plant tissue are also embedded along with the single cells in the same medium. The cells near the nurse callus will begin to divide first indicating that the single cells closer to the nurse callus in the solid medium gets essential growth factors that are liberated from the callus mass. The developing colonies growing near to the nurse callus also stimulate the division and colony formation of other cells.

Free cells isolated from mesophyll tissue (or) cell suspensions are grown as single cells under invitro conditions using a suitable medium.

5. Bergmann Plating Technique

This process is called plating technique. This is important to obtain single cell clones. This technique is developed by Bergmann in 1960. It is the most popular technique for plating single cells. In this technique the free cells are suspended in liquid medium at a density twice the finally desired plating cell density. Sterilized agar medium (0.6-1% w/v) is kept melted in a water bath at 35^{O}C. Equal volumes of the liquid and agar media are mixed thoroughly

and rapidly spreadout in petridishes, in such a manner that the cells become fixed in an evenly distributed thinlayer (Ca 1 mm thick) after the agar has cooled and solidified.

The dishes are sealed with parafilm and incubated in the dark (or) diffused light at 25^{O}C. The cells remain embedded in the soft agar medium are observable under a microscope. When microscopic colonies develop they are isolated and cultured separately on a fresh medium. Free cells can also be plated in the liquid medium but follow up of individual cells (or) their derivatives is difficult in this procedure because the cells do not remain in a fixed position. The plating efficiency (PE) can be calculated from the counting of cell colonies by the following formula.

$$PE = \frac{\text{Number of colonies per plate}}{\text{Number of total cells per plate}} \times 100$$

Figure

Factors affecting single cell culture

1. The composition of the medium for the growth of single cell culture is generally more complex than callus and cell suspension culture.

2. Paper raft nurse culture technique indicates that isolated cells get the exact essential nutrients from the cells mass for the induction of division of single cells.

3. In case of petridish plating technique the initial plating cell density is very critical.

8.3.4.4.3 Importance of single cell culture

1. Single cell culture could be used successfully to obtain single cell clones.

2. The occurrence of high degree of spontaneous variability in the cultured tissue and their exploitation through single cell culture is very important in relation to crop improvement programmes.

3. Single cells can be grown on a medium containing mutagenic compounds and the proliferating cell lines are isolated. The mutant nature of the selected cell line can be confirmed by regenerating the plants and comparing their phenotypes with a normal plant. Many cell lines resistant to amino acid

analogues, antibiotics, herbicides, fungal toxins etc. have been selected by this method.

4. Many plants synthesize the various natural compounds in the form of alkaloids, steroids etc. Some of these of medicinally important. It is reported that single cell cultures can synthesise several times higher amounts of alkaloid than the alkaloid content in the intact plant. So single cell culture could become a valuable technique for the industrial production of such important natural compounds.

5. Single cell culture is an ideal system for the study of biotransformation.

6. Induction of polyploidy has been found to be very much useful for plant breeding to overcome the problem of sterility associated with hybrids of unrelated plants. Polyploidy can be easily achieved by single cell culture.

8.3.4.4.4 Summary

Cell suspension culture is a type of culture in which cells (or) small aggregates of cells multiply while suspended in agitated liquid medium. To achieve an ideal cell suspension, a friable callus is transferred to agitated liquid medium where it breaks up and readily disperses. An ideal suspension culture should consist of only single cells which are physiologically and biochemically

uniform. Movement of cells in relation to nutrient medium facilitates gaseous exchange, removes any polarity of cells due to gravity and eliminates the nutrient gradient within the medium and at the surface of cells suspension culture can be initiated either from pre established friable callus (or) from the explant directly in to the liquid medium, which is continuously agitated on a moving rotary shaker. The concentration of auxins and cytokinins used for callus culture is generally reduced for suspension culture. To facilitate easy separation of cells the concentration of auxin is increased (or) enzymes such as cululase (or) pectinase are added to the medium. Critical initial density (or) the minimum effective density is the smallest inoculum from which a new suspension culture can be successfully grown.

There are two types of suspension cultures viz. 1. Batch cultures and 2. Continuous cultures. Batch culture is a type of suspension culture where the cell material (or) inoculum grows in a limited volume of agitated liquid medium. It is again divided in to, 1. Slowly rotating cultures 2. Shake culture 3. Spinning culture and 4. Stirred culture.

In continuous culture system the old liquid medium is continuously replaced by the fresh liquid medium to stabilize the physiological states of the growing cells. In this method nutrient depletion does not occur due to continuous flow of nutrient

medium and the cells always remain in the steady state of active growth phase. Continuous culture systems can be divided in to two categories Viz. open continuous culture and closed continuous culture. Chemostat and turbido stats represent open continuous systems. In a chemostat the growth rate and cell density are held constant by a fixed rate of input of growth – limiting nutrient. In a turbidostat the fresh medium flows in to it in response to an increase in the turbidity of the culture.

Under suitable conditions, the growth of suspension culture follows a predictable pattern (or) a growth curve. The growth curve for a typical suspension culture consists of lag phase, logarithmic phase (or) exponential phase, linear phase and stationary phase. In cell suspension culture the cells grow by cell division and the no. of cells increases. The cell growth can be measured by various ways viz. (1) by counting the cell number in haemocytometer under a microscope (2) detecting the changes of optical density of the cell suspension by a colorimeter (3) by measuring the packed cell volume (PCV) (4) by measuring the dryweight of cell mass per unit volume of culture at regular intervals (5) by measuring the fresh weight. For the studies on growth test for viability of cells is very important. It can be determine by using some stains like fluorescein diacetate, Evan's blue etc.

Suspension culture can be sued for obtaining single cell clones by plating cell suspension on agar plates. Plants can be regenerated from such clones by the process of embryo genesis. Cell suspention culture may be used for the synthesis of secondary plant products like alkaloids, Glycosides etc. Mutagenic studies may be facilitated by cell suspension cultures to produce mutant cell clones from which mutant plants can be raised.

Single cell culture is a method of growing isolated single cells aseptically on a nutrient medium under controlled conditions. Single cells can be obtained from the tissues of intact plants (or) from friable callus tissue. The isolated single cell can be cultured either in liquid medium (or) on solid medium. There are five important methods of single cell culture viz. 1. The paper raft nurse culture techique 2. Petridish plating technique 3. Nurse callus technique 4. Micro chamber technique 5. Micro droplet technique.

The composition of culture medium for the growth of single cell is generally more complex than callus and cell suspension culture. In case of Bergmann plating technique the initial plating cell density is very critical. Single cell culture technique is very important for the fundamental and mutation studies and it has wide industrial applications.

8.3.4.4.4 Key Words

Critical Initial Density (CID)

Optical Density (OD)

Packed Cell Volume (PCV)

Fluorescein Di Acetate (FDA)

Chemo stat

Turbido stat

Nurse tissue

Plating Efficiency (PE)

Biotransformation

8.3.4.4.5 Self Assessment Test

1. What is cell suspension culture? Give a general account of cell suspension culture.

2. Give an outline of the principle of cell suspension culture and its applications in biological studies.

3. Write short notes on the following :

    (a) CID
    (b) Turbido stat
    (c) Chemo stat
    (d) Batch Culture

(e) PCV

4. Give a general account of the principle and importance of single cell culture.

5. Write shorts notes on the following :

   (a) What is the advantage of a single cell culture over callus culture and cell suspension culture.

   (b) Importance of single cell culture in mutation breeding.

   (c) How are the single cells isolated from the infact plant tissue and cultured tissue.

   (d) Paper raft nurse culture technique.

   (e) Bergmann plating technique.

8.3.4.4.6 References

1. An introduction to plant tissue culture Dr.M.K. Razdan, Oxford & IBH publishing Co. Pvt., Ltd.

2. Plant cell and tissue culture, Narayana Swamy, Tata Mcgrow-hill.

3. An introduction to plant tissue culture, Dr.Kalyan Kumar De, New Central Book Agency.

4. Biotechnolgoy, B.D.Singh, Klayani Publishers.

5. Elements of Biotechnology, P.K. Gupta, Rastogi Publications.

6. Biotechnology, V.Kumaresan, Saras Publications.

Lesson 8.4.3

Animal Cell Culture Media (Serum & Serum free media)

Objective :

Growing of animal cells invitro is entirely different from culturing plant cells One should well known about the constituents of media, their properties and their role in animal cell growth. The present chapter includes different media components and their role in animal cell culture.

8.4.3.1 Culture media

8.4.3.2 Basal media

8.4.3.3 Serum media

8.4.3.4 Advantages of serum

8.4.3.5 Disadvantages of serum

8.4.3.6 Replacement of serum

8.4.3.7 Serum free media

8.4.3.8 Design of serum free media

8.4.3.9 Difficulties that may be encountered in using serum free media

8.4.3.1.Culture media

Even after several years of extensive research. media obtained from natural sources are still widely used for many purposes especially for growing tissue and cells, freshly isolated from the organism. However, they suffer from disadvantages like, their composition is unknown, variable time to time, virtually impossible to reproduce the same conditions exactly from one experiment to the other. To solve this problem, attempts were made to develop a media composed entirely of defined contents by two methods, analytical and synthetic approach.

In analytical method, attempts were made to analyze the medium totally and to know the essential components, but this has not given encouraging results because of the time constraint. In the synthetic method, the metabolites shown to be essential or valuable for the survival and development of the intact organism were combined to form as media, which is being tested for its ability to keep cells alive. However, it is apparent that both the methods of approach were dependent mutually.

Most media in general consists of mixture of substances, which were proved essential, generally beneficial and harmless.

To grow cells in vitro, culture conditions must mimic in vivo conditions with respect to temperature, oxygen and CO_2 concentration, pH, Osmolarity and nutrition. Most basal cell culture media can't, by themselves support the growth of the cells and it is common practice to supplement cell culture media with animal sera. Growing cells in serum free media has many advantages but the ideal general purpose of serum free media has not yet been developed. The main function of cell culture media are to maintain the pH and osmolality essential for cell viability and to provide the nutrients and energy needed for cell growth and multiplication. The temperature, oxygen and CO_2 concentration of the cell cultures must also be controlled.

A complete cell culture medium can be considered to be composed of two distict parts : (1)A basal medium that satisfies all cellular requirements for nutrients (2) A set of components that satisfy other types of cellular requirements and permit growth of cells in the basal medium.

8.4.3.2 Basal media

The culture media is by far the most important single factor in culturing animal cells. The function of this medium is to provide an environment for survival and also to provide substances required by the cells which they can't synthesize directly. The composition of early tissue culture media was based on biological fluids such as plasma lymph and serum and tissue extracts especially of embryonic origin. Basal tissue culture media were developed only to include nominal components which were essential for growth.

Constituents basal medium

The common constituents of basal medium may be considered as follows

Balanced salt solution

Balanced salt solution (BSSs) have been used since the early attempts at cell culture in vitro .A BSS is composed of a combination of inorganic salts that maintain physiological pH and osmotic pressure .In addition to these effects the inorganic ions used have other important physiological roles including the maintenance of membrane potential and as cofactors in enzyme reactions and in cell attachment. The inorganic ions employed are chiefly Na+,K+,Mg+2,Ca+2,Ci-,SO4-2,PO4-3 and HCO3-. When necessary osmolality may be adjusted by modifying the concentration of NaCl.Most BSSs do not contain the nutrients required by cell for long term maintenance and growth although glucose may be included. The four main categories of BSS are

1.Earles balanced salt solution (EBSS)

2.Dulbeccos phosphate –buffered saline (DPBS)

3.Hanks balanced salt solution (HBSS)

4.Eagles spinner salt solution (ESSS)

HBSS and DPBS are intended for use equilibrated with air while EBSS and ESSS requires equilibration with gas phase containing 5% CO_2 inorder to maintain correct pH

Buffering Systems

Culture media need to be buffered to compensate for evolution of CO_2 and production of lactic acid from the metabolism of glucose Media have traditionally been buffered with a bicarbonate buffer, often at a final concentration of 24mm. Bicorbonate forms buffer system with dissolved CO_2 produced by growing cells. However when cells are going at a low cell density or in a lag phase, insufficient CO_2 may be produced to maintain the required optimal pH For this reason the ,these cultures need to be maintained at atmosphere of 56-10% CO_2. Bicarbonate is both cheep and non toxic to the cells but its Pka (6.1)results in sub optimal buffering in the physiological range some media are designed to contain low HCO3-but high PO4-3 concentration and there fore do not require incubation in a CO_2 enriched atmosphere. Sodium –beta –glycerophosphte is also used as a buffer in some formulations.

For more effective buffering, with out the need for elevated levels CO2 a range of organic buffers can be employed. The mostly used of this

is Hepes (N-2- hydroxy ethyl piperazine –N1-2 ethane sulphonic acid)Hepes is very effective buffer in the pH range 7.2-7.6 and is most resistant to rapid pH changes than bicarbonate. Some media are buffered with both bicarbonate and Hepes. However Hepes is both expensive and toxic to the cells at concentrations above 100mm.

Energy sources

Carbohydrates are major source energy for cultured cells. Glucose is the most frequently used sugar. Other sugars, e.g. maltose, sucrose fructose, galactose and mannose may also be included. Glutamine can also supply a major proportion of the required energy in some cells .

Amino acids

Most animal cells have a requirement for essential amino acids i.e. those which are not synthesized by the body together with cysteine and tyrosine in addition although individual requirements for amino acids vary from one cell to another Other non essential requirements are often added as well, to compensate either a particular cell type s incapacity to make them or they made ,but lost by leakage in to the medium The concentration amino acids limits the maximum cell concentration attainable, and the balance may influence cell survival and growth rate .Glutamine is required by most cells although some cells utilize

glutamate, some evidences says that glutamine is also used by cultured cells as a source of energy and carbon .

Vitamins

Several vitamins of B group are necessary for cell growth and multiplication. Many vitamins are precursors for cofactors The vitamins most commonly added to basal media are Para –amino benzoic acid, biotin, choline, folic acid, nicotinic acid, pantothenic acid, pyridoxal, riboflavin, thiamine and inositol. The importance of other water soluble vitamins is less clear. Vitamin B12 has been reported to be essential for some cells and is included in F12 medium Few data are available on the role of fat soluble vitamins in medium.

Hormones and growth factors

Hormones and growth factors exhibit a variety of different effects on cells. These are included in some media (especially serum free media) at relatively low concentrations .Insulin and hydro cortisone are main examples but growth factors like NGF (nerve growth factors) and EGF

(epidermal growth factor)has also been used as well as certain interleukins, colony stimulating factors, and fibroblast growth factors (FGFs)…etc

Proteins and peptides

Although an absolute requirements for protein and peptides by cells in culture has not been established, relatively few media have been established in which the cells grow rapidly in the total absence of proteins or polypeptides. Common examples of protein supplements used are fetuin,alpha globulins fibronectin, albumin and transferrin.

Fatty acids and lipids

As with proteins and peptides, there is no consensus regarding an essential role for lipids in cell culture. However, fatty acids and lipids are important components of several serum free media.

Accessory factors

Amongst these are the trace elements, especially iron, copper, zinc, and selenium. A variety of other compounds, including nucleosides and intermediates of tricarboxylic acid cycle may also be used.

Antibiotics

Antibiotics were originally introduced in to the culture media to reduce the frequency of contamination. However, the use of laminar hoods, coupled with strict aseptic technique, makes antibiotics unnecessary. Indeed, antibiotics have number of significant disadvantages ,

1.They encourage development of antibiotic development resistant organisms

2.They hide the presence of low level ,cryptic contaminations that can become fully operative if the antibiotics were removed, the culture conditions change or resistant strains developed.

3.They may hide mycoplasma infections

4.They have antimetabolic effects that can cross react with mammalian cells

5. They encourage poor aseptic techniques

For all these reasons, it is strongly recommended that routine cultures can be performed in the absence of antibiotics and their use be restricted to primary cultures or large scale labor- intensive experiments with a high cost of consumables. If the conditions demand the use of antibiotics, then they should be removed as soon as possible, or if they are used in long term, parallel culture should be maintained free of antibiotics. A number of antibiotics used in tissue culture are moderately effective in controlling bacterial infections. However, a significant number of bacterial strains are resistant to anti biotic ,either naturally poor by

selection, so the control that they provide is never absolute. Fungal and yeast contaminations are particularly hard to control with antibiotics, they may be held in check abut seldom eliminated when antibiotics are to be used in cell cultures, the key factors governing their choice are

1.Absence of cytotoxicity

2. Broad anti-microbial spectrums

3.Acceptable cost

4.Minimum tendency to induce formation of resistant microorganisms

8.4.3.3 Serum media

Historically the first tissue culture experiments were performed using animal body fluids such as lymph to support cell growth. When Eagle and others, in the late 1950s, produced basal media containing amino acids, carbohydrates, vitamins and minerals, it became apparent that supplementation of medium with body fluid was still needed to provide un identified, but essential, factors needed for efficient cell growth Supplementation of basal medium up to 20%of animal serum

became widely used. Serum contains growth factors, which promotes cell proliferation, and adhesion factors and anti trypsin activity, which promotes cell attachments. Serum is also a source of minerals, lipids, hormones, many of which may bound to proteins. The sera used in most tissue culture laboratory are calf (bovine), fetal bovine, horse and human serum. Calf(CF) and fetal bovine serum(FBS) are the most widely used, the later for more demanding cell lines and for cloning. Human serum is some times used in conjunction with some human cell lines, but it needs to be screened for viruses, such as HIV and hepatitis horse serum is preferred to calf serum by some workers, as it can be obtained from a closed herd and is often consistent from batch to batch Horse serum is also be less likely to metabolize poly amines due to lower levels of poly amine oxidase, Polyamines are mitogenic for more cells.

8.4.3.4 Advantages of serum

1. Serum represents a cocktail of most of the factors required for cell proliferation and maintenance

2. Serum is an all most universal growth supplement which is effective with most cells. Using serum supplemented medium there fore reduces

the need to spend time developing a specific, optimized medium formulation for every cell type under investigation.

3. Serum buffers the cell culture system against a variety of perturbations and toxic effects such as pH change, or presence of heavy metal ions, photolytic action or end toxin.

The use of serum also imposes a number of difficulties which impacts on the safety, reproducibility, and cost of bio pharmaceutical as produced in animal cells .these difficulties can be minimized by careful selection and validation serum sources although almost all new manufacturing processing's using animal cells are designed for serum-free media in order to avoid these difficulties, many existing processes still use FBS supplemented medium. This situation is unlikely to change fundamentally in near future since regulatory constraints generally make it impractical and uneconomic to alter existing processes.

Despite its high cost FBS remains the most frequently used serum for medium supplementation. Several different types of serum have been proposed as cheaper alter native to FBS Calf serum is quite widely use industrially and as available as either new born serum (which has high level of biotin) or as mature calf serum. New born calf gama globulins are as a result of the colostrums immediately after birth. Adult bovine serum is used occasionally but is not usually as effective as FBS or calf serum Horse serum is also used particularly with some human cell lines .the

use of human serum has been proposed for some fastidious human cell lines, but it is not clearly established that clearly that human serum better in general than FBS.

Constituents of Serum

Serum is an effective growth promoting supplement for practically all types of cells because of its complexity and multiplicity of growth promoting, cell protection, and nutritional factors that it contains .These can be divided in to specific poly peptides which stimulates cell growth (growth factors), carrier proteins, cell protective agents, cell attachment factors, and nutrients (some of which may be small molecules which are attached to carrier proteins). Some serum macro molecules can fill more than one of these roles.

Growth factors

Poly peptide growth factors are of particular importance in serum .These 5-30 kD proteins act via specific cell surface receptors as signals which stimulates cell proliferation or differentiation. In some cases ,the presence of certain growth factors may not be stimulatory as such but may still be essential since deprivation of the factor initiates a pre programmed auto destructive sequence of events (apoptosis) which results in death of cells even though they may be fully provided with nutrients and be maintained under optimal culture conditions.

Different cell types have different growth factor requirements and same growth factors may stimulate or inhibit depending up on cell type and the growth factor concentration. Different types of serum may contain different absolute and relative levels of different growth factors. This is one of the main reason why growth testing of serum batches is necessary to ensure satisfactory performance with the specific cell line of interest.

Albumin

Albumin is the majority protein components of serum and exerts several effects which contribute to the growth and maintenance of cell lines in culture it functions as a carrier protein for a range of small molecules, particularly lipids. Trans port of fatty acids is an important function of albumin since these are essential for cells and but are toxic in the unbound form and are also very poorly soluble in water. Steroids and fat soluble vitamins may also bound to albumin. Albumin also has specific binding site for thyroxin and metal ions such as Ni+2 and Cu+2 .There is evidence that albumin also bind some other metal ions and also carry other, undefined components which supports cells in culture.

8.4.3.5 Disadvantages of serum

Physiological variability :the major constituents of serum such as albumin and transferrin are known, but serum also contains a wide

range of minor components that may have considerable effect on cell growth .These components include nutrients (amino acids, nucleosides, sugars etc), peptide growth factors, hormones, minerals, and lipids, the concentration and action of which have not been fully determined.

2. Shelf life and consistency :Serum varies from batch to batch, and at best a batch will last one year, perhaps deteriorating during that time. It must then be replaced with another batch that may be selected as similar but will never be identical, to the first batch.

3. Quality control: Changing serum batches require extensive testing to ensure that replacement is as close as possible to the previous batch. This can involve several tests (for growth plating efficiency, and special functions)and a number of different cell lines.

4. Specificity: If more than one cell type is used each type may require different batch of serum so that several batches must be held on reserve simultaneously. Coculturing different cell types will present an even greater problem.

5. Availability: Periodically, the supply of serum is restricted due to drought in the cattle rearing areas, the spread of disease among the cattle's, or economic or political reasons. This can create problems at any time, restricting the amount of serum available and the number of batches to choose from, but can be particularly acute at times of high

demand To day demand is increasing and it will probably exceed supply unless the majority of commercial users are able to adopt serum-free media. While an average research laboratory may reserve 100-200 liters of serum per year, a commercial laboratory can use that amount or more in a weak.

6. Down stream processing: To any one interested in recovering cell products, the presence of serum creates a major problem to purification and even limit the pharmaceutical acceptance of the product..

7. Contamination : Serum is frequently contaminated with viruses, which may be harmless to cell culture, but represents an un known additional factor out side the operators control. Fortunately improvement in serum sterilization techniques have virtually eliminated the risk of mycoplasma infection from sera from most reputable suppliers, but this cants be guaranteed for viral contamination, in spite of claims that some filters claim removal of viruses. Because of the risk of the spreading of bovine spongiform encephalitis among cattle's, cell cultures and serum supplied to united states and Australia require information about the country of origin and the batch number of the serum. Serum derived from cattle in New Zealand probably has the lowest endogenous viral contamination, as many of the found in European and North American cattle are not found in New Zealand.

8.Cost: Cost is often cited as disadvantage of serum supplementation.C3ertainly serum constitutes the major part of the cost of the bottle of the medium (more than 10 times the cost of chemical constituents) but it is replaced by definite constituents, the cost these may as high as that of the serum. However, as the demand for such items as transferrin, selenium, insulin, etc. rises the cost is likely to come down with increasing market size, and serum free media will become relatively cheaper. The availability of recombinant growth factors, coupled with market demand, particularly in the form of growth factors in the form of pharmaceuticals, may help to reduce their intrinsic cost.

9.Growth inhibitors : As well as its growth promoting activity, serum contains growth inhibiting activity, and although stimulation usually predominates, the net effect of the serum is unpredictable combination of both inhibition and stimulation of growth. While substances such as PDGF may be mitogenic to fibroblasts, other constituents of serum can be cytotstatic. Hydrocortisone, present at around 10-8 M in fetal serum is cytostatic to many cell types such as glia and lung epithelium, at high level densities (though it may be mitogenic at low cell densities), and TGF-beta, released from platelets, is cytostatic to many epithelial cells.

10. Standardisation: Standardization of experimental and production protocols is difficult, both at different times and among different laboratories, due to batch to batch variations.

8.4.3.6 Replacement of serum in medium

Much of the present requirements for cells in culture system comes from Eagles work on the fundamental requirements for growing mammalian cells. Based on this information many attempts have been made to replace serum in part or in full by serum –derived factors or by completely synthetic media. One approach is to reduce the serum requirement by supplementing the culture medium with processed serum products. Controlled processed serum replacement (CPSR)are prepared by processes that yield defined products with much higher batch to batch consistency than serum CPSR products are derived from bovine plasma, and have lower protein and endotoxin levels than serum. Natural serum can also be replaced by supplemented or fortified serum. Serum may be fortified with mitogens, growth factors, hormones, proteins other protein stabilizers, and trace elements. Such fortified serum can be used at a much lower concentration than normal serum.

8.4.3.7 Serum-free media

For the disadvantages discussed in serum media when ever possible it is desirable to culture cells in serum free medium. A properly designed serum-free medium :

1. Is reproducible

2. Is not reliant on the economics of the world cattle market

3. Simplifies down stream purification

4. Has no unknown factors e.g. viruses or growth inhibitors

A number of cell types have been grown success fully in serum-free media, usually in a medium specifically developed for one cell line. The requirements of cell lines differ greatly and success of a serum-free medium formulation with one cell line does not guarantees with other, even closely similar cell lines. A great deal of effort has gone into developing serum free media, but until recently, success has been limited .However, with he identification of essential growth factors and nutrients required by different cells, several very effective serum-free media have been formulated. A variety of tissue sources and species are represented clearly there are unique combinations of growth factors and hormones that promote optimal proliferation of specific cell types. The most consistent requirement appears to be for the polypeptide hormone, insulin and iron trans port protein transferrin. Other supplements includes polypeptides and steroid growth hormones, polypeptide growth factors ,trace elements reducing agents, diamines, vitamins and albumin complexed with unsaturated fatty acids. An important consideration for some application is that animal derived supplements or proteins can pose contamination risks similar to those of serum.

Several commercially produced ,ready to use serum-free media are now available which have been designed for a particular cell types .It

should how ever be remembered that different strains of the same cell type may have different medium requirements now, and that fine tuning of these commercial media may be necessary to optimum results with specific individual strain or construct.

8.4.3.8 Design of serum-free media

A defined serum free media is one in which a group of components are formulated together to optimize performance of single cell type .Each components included of known purity and is present at a known concentration. Several important factors must be considered to achieve this goal. Amongst these the origin of cell lines, i.e. species and tissue, the compatibility of media components and their interaction., and the specific application for which the cell line being cultured, e.g. production of biomass or generation of products. The two approaches generally followed in designing a free media are :

a) reduced serum: In this approach the concentration of serum in the basal medium is progressively reduced while other components, e.g. growth factors and hormones are added to identify the factors capable of restoring the growth to the level obtained in the presence of serum. This process can be very lengthy because at each change, growth assays using the serum –supplemented control, and repeat the verification assays need to be done.

b) Basal medium: A different approach i9s to add components (singly or in combination)to a basal medium in a step wise manner until a medium is progressively built up to give a similar or equal at cell growth to the serum supplemented medium.

Both of these approaches, the following critical factors need to be considered in designing an efficient serum-free medium.

Basal medium: The selection of basal medium is extremely important in terms of energy sources, buffers, and inorganic ions. Generally the starting basal medium formulation is chosen on the basis of the known preference of the required cell line.

Lipids:

These include ethonolamine,phosphoethanolamine,sterols, fatty acids and phosphot lipid. In serum supplemented media, they are usually carried on macro molecules, principally proteins. In serum free media fatty acids are usually provided in a bound form (either to albumin or to the other serum proteins) or in the form of phospholipids enclosed vesicles (liposomes). If serum albumin is used directly as a lipid source, it should be noted that the endogenous lipid content of may be dependent on the methods used for its [purification; the solvent precipitation frequently used may results in substantial stripping of lipid protein. It should also be noted that pasteurized human albumin will

have been stabilized with octanoic acid or other hydro phobic stabilizing agents prior to heating and that it may be important to replace these with more physiologically relevant lipids before use in cell culture. Resent developments have permitted the use of totally synthetic hydrophilic carriers such as cyclodextrins for the trans port.

Buffering :

Buffers maintain a proper environment for the metabolism,growth,and functioning, of cells. major ions (Na+,K+,HCO3- and HPO4-2) are usually regarded as the principle components, in pH control, along along with H+ and OH-, which enters into ionic balance. Other components, including amino acids, if present in high concentrations, can contribute to the buffering power of a medium. Besides bicarbonate, zwitter ionic buffers like Hepes, Bes and Tes may be used in systems in may be used in which the strict control of gas phase in\s not required. However, careful consideration must be given to the concentration of these buffers which can be toxic to the cells. Some of these buffer chelate biologically important cautions. A useful buffer for the use in the presence of low or no bicarbonate is sodium glycerophosphate.

Trace elements :

The major ions, i.e. Na+,K+,Ca+,Mg+2,Cl-,HPO4-and HCO3-, are principally involved in maintaining electrolytes balance and contributing to osmotic equilibrium, of the system. Trace elements are also included in many serum free -media, because of their beneficial effects. Inter relation ships exists between Fe+2,Zn+2,and Cu+2ions which are needed for many cells. Most serum free media also include, Co+2 and SeO3-2. Cells derived from heart and kidney tissue have high requirement for K+ whilst Ca+2, is require for control of mitosis and the Ca+2/Mg+2 ratio is important in controlling cell proliferation and transformation. Selenium is proving to be important for many cell types. Other trace elements include Sn, V, Al, and As. Iron is frequently added as a transferrin but can also be added in other forms such as ferric citrate, ferrous nitrate, or ferrous sulphate. Mechanical stabilizers and adhesion factors : For optimal growth suspension culture require protection from shear due to agitation (air bubbles, stirrer, shaker). Shear damage can be reduced by increasing the viscosity of the medium.Carboxy methyl cellulose and polyvinylpyrrolidone have been used for this purpose. The most widely used shear protectant is Pluronic F-68. This is a non ionic block copolymer with an average molecular weight of 8400Da, consisting of a central block of polypropylene (20% by weight) and blocks of polyoxyethylene at both ends. Pluronic F-68 has been demonstrated to have a significant effect in protecting animals grown in suspension in sparged or stirred bioreactors. The protective effect is thought to be

exerted through the formation of an interfacial structure of adsorbed molecules on the cell surface. It is thought that the hydro phobic portion of molecule interacts with the cell membrane, while poly oxymethylene oxygen may form hydrogen bonds with water molecules to generate a hydrogen sheath, which provides the protection from laminar share stress and cell bubble interactions .

Cell attachment and growth of anchorage dependent cells can be improved by pretreatment of the substrate in a variety of ways. the substrate can be treated with adhesive glycol proteins such as fibronectin ,laminin ,chondroitin.epibolin,or serum spreading factor.

8.4.3.9 Difficulties that may be encountered with serum free media

When cells are grown in serum free- conditions, they no longer benefit from the multiple protective and nutritional effects that serum provides. The robustness of the process in serum free medium depends on attention on the following points;

1) Cells appear more fastidious in the absence of serum: design of dedicated medium for each cell type is usually necessary for optimal results.

2) Cultured conditions become more critical in serum free medium: better control of key processes parameters (PH,oxygenation etc) is there fore necessary.

3) Serum free media has a reduced capacity to inactivate or to absorb the toxic materials (e.g. heavy metals, endotoxin, etc.) Greater attention to the purity of components and depyrogenation is required .Antibiotics may exhibit cytotoxicity in serum free medium

4) Specific shere protective agents may need to be added.

5)A significant adaptation may need to be required before cells are fully weaned to serum-free medium .This makes the design and testing of serum free medium a ling and labor intensive process.

Summary

The medium is by far the most important in culturing cells and tissues. The function of this medium is to provide the physical conditions of pH, osmotic pressure ..etc for their survival and the complicated chemical substances by the tissue which can't be synthesized by the tissue itself. The serum and serum free media includes these substances in required concentrations for cell culture. The serum itself is having potential nutrients in it as it is a cocktail of most of the factors required for cell proliferation. As the availability and Shelf life are the major problems with the with serum now people are trying to adapt serum free media**.**

Questions

1. Write about the different components of serum media.

2. Discuss the advantages and disadvantages of serum free media.

Lesson 8.4.1

Growth of Animal Cell lines in laboratory

Objective:

Animal cells behaves in a different way than plant cells in the culture. This chapter includes the growth, behavior and requirements of animal cells in culture. Constituents of serum and serum free media were also explained.

8.4.1.1. Introduction

8.4.1.1. Rich media are required for culture

8.4.1.2. Most cultures grow only on solid surfaces

8.4.1.5. Summary

Animal cells are more difficult to culture than microorganisms because they require many more nutrients and typically grow only when attached to specially coated surfaces. Despite these difficulties, various types of animal cells, including both undifferentiated and differentiated ones, can be cultured successfully.

8.4.1.1. Rich Media Are Required for Culture of Animal Cells

Nine amino acids, referred to as the *essential amino acids,* cannot be synthesized by adult vertebrate animals and thus must

be obtained from their diet. Animal cells grown in culture also must be supplied with these nine amino acids, namely, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. In addition, most cultured cells require cysteine, glutamine, and tyrosine. In the intact animal, these three amino acids are synthesized by specialized cells; for example, liver cells make tyrosine from phenylalanine, and both liver and kidney cells can make glutamine. Animal cells both within the organism and in culture can synthesize the 8 remaining amino acids; thus these amino acids need not be present in the diet or culture medium. The other essential components of a medium for culturing animal cells are vitamins, which the cells cannot make at all or in adequate amounts; various salts; glucose; and *serum*, the noncellular part of the blood.

Serum, a mixture of hundreds of proteins, contains various factors needed for proliferation of cells in culture. For example, it contains insulin, a hormone required for growth of many cultured vertebrate cells, and transferrin, an iron-transporting protein essential for incorporation of iron by cells in culture. Although many animal cells can grow in a serum-containing medium, such as Eagle's medium, certain cell types require specific protein growth factors that are not present in serum. For instance, precursors of red blood cells require the hormone erythropoietin, and T lymphocytes of the immune system require interleukin 2 (IL-2). These factors bind to receptor proteins that span the plasma membrane, signaling the cells to increase in size and mass and undergo cell division. A few mammalian cell types can be grown in a completely defined, serum-free medium supplemented with trace minerals, specific protein growth factors, and other components.

8.4.1.2 Most Cultured Animal Cells Grow Only on Special Solid Surfaces

Within the tissues of intact animals, most cells tightly contact and interact specifically with other cells via various cellular junctions. The cells also contact the extracellular matrix, a complex network of secreted proteins and carbohydrates that fill the spaces between cells. The matrix, whose constituents are secreted by cells themselves, helps to bind the cells in tissues together; it also provides a lattice through which cells can move, particularly during the early stages of animal differentiation.

The extracellular matrices in various animal tissues consist of several common components: fibrous collagen proteins; hyaluronan (or hyaluronic acid), a large mucopolysaccharide; and covalently linked polysaccharides and proteins in the form of proteoglycans (mostly carbohydrate) and glycoproteins (mostly protein). However, the exact composition of the matrix in different tissues varies, reflecting the specialized function of a tissue. In connective tissue, for example, the major protein of the extracellular matrix is a type of collagen that forms insoluble fibers with a very high tensile strength. Fibroblasts, the principal cell type in connective tissue, secrete this type of collagen as well as the other matrix components. Receptor proteins in the plasma membrane of cells bind various matrix elements, imparting strength and rigidity to tissues. The tendency of animal cells in vivo to interact with one another and with the surrounding extracellular matrix is mimicked in their growth in culture. Unlike bacterial and yeast cells, which can be grown in suspension, most cultured animal cells require a surface to grow on. Many types of cells can adhere to and grow on glass, or on specially treated plastics with negatively charged groups on the surface (e.g., SO_3^{2-}). The cultured cells secrete

collagens and other matrix components; these bind to the culture surface and function as a bridge between it and the cells. Cells cultured from single cells on a glass or a plastic dish form visible colonies in 10 – 14 days. Some tumor cells can be grown in suspension, a considerable experimental advantage because equivalent samples are easier to obtain from suspension cultures than from colonies grown in a dish.

Primary Cell Cultures Are Useful, but Have a Finite Life Span

Normal animal tissues (e.g., skin, kidney, liver) or whole embryos commonly are used to establish *primary cell cultures.* To prepare tissue cells for culture (or to remove adherent cells from a culture dish for biochemical studies), trypsin or another protease is used to destroy the proteins in the junctions that normally interconnect cells. For many years, most cell types were difficult, if not impossible, to culture. But the identification and preparation of various protein growth factors that stimulate the replication of specific cell types, as well as other recent modifications in culture methods, now permit experimenters to grow various types of specialized cells.

Many studies with vertebrate cells, however, still are performed with those few cell types that grow most readily in culture. These are not cells of a defined type; rather, they represent whatever grows when a tissue or an embryo is placed in culture. The cell type that usually predominates in such cultures is called a fibroblast because it secretes the types of proteins associated with fibroblasts in fibrous connective tissue of animals. Cultured fibroblasts have the morphology of tissue fibroblasts, but they retain the ability to differentiate into other cell types; thus they are not as differentiated as tissue fibroblasts.

Some studies are conducted with primary cultures of epithelial cells. In general, external and internal surfaces of tissues and organs are covered by a layer of epithelial cells called an epithelium. These highly differentiated cells are said to be *polarized* because the plasma membrane is organized into at least two discrete regions. For example, the epithelial cells that line the intestine form a simple columnar epithelium. That portion of the plasma membrane facing the lumen of the intestine, the *apical* surface, is specialized for absorption; the rest of the plasma membrane, the *basolateral* surface, mediates transport of nutrients from the cell to the blood and forms junctions with adjacent cells and the underlying extra cellular matrix called the basal lamina. Certain cells cultured from blood, spleen, or bone marrow adhere poorly, if at all, to a culture dish but nonetheless grow well. In the body, such nonadherent cells are held in suspension (in the blood), or they are loosely adherent (in the bone marrow and spleen). Because these cells often come from immature stages in the development of differentiated blood cells, they are very useful for studying normal blood cell differentiation and the abnormal development of leukemias.

When cells are removed from an embryo or an adult animal, most of the adherent ones grow continuously in culture for only a limited time before they spontaneously cease growing. Such a culture eventually dies out after many cell doublings, even if it is provided with fresh supplies of all the known nutrients that cells need to grow, including serum. For instance, when human fetal cells are explanted into cell culture, the majority of cells die within a relatively short time; “fibroblasts,” although also destined to die, proliferate for a while and soon become the predominant cell type. They divide about 50 times before they cease growth. Starting with 10^6 cells, 50 doublings can produce $10^6 \times 2^{50}$, or more than 10^{20}

cells, which is equivalent to the weight of about 10^5 people. Thus, even though its lifetime is limited, a single culture, if carefully maintained, can be studied through many generations. Such a lineage of cells originating from one initial primary culture is called a cell strain.

Transformed Cells Can Grow Indefinitely in Culture

To be able to clone individual cells, modify cell behavior, or select mutants, biologists often want to maintain cell cultures for many more than 100 doublings. This is possible with cells derived from some tumors and with rare cells that arise spontaneously because they have undergone genetic changes that endow them with the ability to grow indefinitely. The genetic changes that allow these cells to grow indefinitely are collectively called *oncogenic* transformation, and the cells are said to be *oncogenically transformed,* or simply *transformed.* A culture of cells with an indefinite life span is considered immortal; such a culture is called a cell line to distinguish it from an impermanent cell *strain.*

The ability of cultured cells to grow indefinitely or their tendency to be transformed varies depending on the animal species from which the cells originate. Normal chicken cells rarely are transformed and die out after only a few doublings; even tumor cells from chickens almost never exhibit immortality. Among human cells, only tumor cells grow indefinitely. The HeLa cell, the first human cell type to be grown in culture, was originally obtained in 1952 from a malignant tumor (carcinoma) of the uterine cervix. This cell line has been invaluable for research on human cells.

In contrast to human and chicken cells, cultures of embryonic adherent cells from rodents routinely give rise to cell lines. When adherent rodent cells are first explanted, they grow

well, but after a number of serial replatings they lose growth potential and the culture goes into crisis. During this period most of the cells die, but often a rapidly dividing variant cell arises spontaneously and takes over the culture. A cell line derived from such a variant will grow forever if it is provided with the necessary nutrients. Cells in spontaneously established rodent cell lines and in cell lines derived from tumors often have abnormal chromosomes. In addition, their chromosome number usually is greater than that of the normal cell from which they arose, and it continually expands and contracts in culture. Such cells are said to be *aneuploid* (i.e., have an inappropriate number of chromosomes) and are obviously mutants.

Although most cell lines are undifferentiated, some can carry out many of the functions characteristic of the normal differentiated cells from which they are derived. One example is certain hepatoma cell lines (e.g., HepG2) that synthesize most of the serum proteins made by normal hepatocytes (the major cell type in the liver) from which they are derived. These highly differentiated hepatoma cells are often studied as models of normal hepatocytes. Cultured *myoblasts* (muscle precursor cells) are another example of transformed cells that continue to perform many functions of a specialized, differentiated cell. When grown in culture, transformed myoblasts can be induced to fuse to form myotubes. These resemble differentiated multinucleated muscle cells and synthesize many of, if not all, the specialized proteins associated with contraction. Certain lines of epithelial cells also have been cultured successfully. One such line, Madin-Darby canine kidney (MDCK) cells, forms a continuous sheet of polarized epithelial cells one cell thick that exhibits many of the properties of the normal canine kidney epithelium from which it was derived.

This type of preparation has proved valuble as a model for studying the functions of epithelial cells.

Fusion of Cultured Animal Cells Can Yield Interspecific Hybrids Useful in Somatic-Cell Genetics Cultured animal cells infrequently undergo cell fusion spontaneously. The fusion rate, however, increases greatly in the presence of certain viruses that have a lipoprotein envelope similar to the plasma membrane of animal cells. A mutant viral glycoprotein in the envelope promotes cell fusion. Cell fusion also is promoted by polyethylene glycol, which causes the plasma membranes of adjacent cells to adhere to each other and to fuse As most fused animal cells undergo cell division, the nuclei eventually fuse, producing viable cells with a single nucleus that contains chromosomes from both "parents." The fusion of two cells that are genetically different yields a hybrid cell called a heterokaryon.

Because some **somatic cells** from animals can be cultured from single cells in a well-defined medium, it is possible to select for genetically distinct cultured animal cells, just as is done with bacterial and yeast cells. Moreover, during mitosis the chromosomes in an animal cell are large and highly visible after staining, making it easy to distinguish individual chromosomes. Genetic studies of cultured animal cells are called *somatic-cell genetics* to distinguish them from *classical genetics,* which deals with whole organisms derived from **germ cells** (sperm and eggs).

Cultured cells from different mammals can be fused to produce interspecific hybrids, which have been widely used in somatic-cell genetics. For instance, hybrids can be prepared from human cells and mutant mouse cells that lack an enzyme required for synthesis of a particular essential metabolite. As the human-mouse hybrid cells grow and divide, they gradually lose human

chromosomes in random order, but retain the mouse chromosomes. In a medium that can support growth of both the human cells and mutant mouse cells, the hybrids eventually lose all human chromosomes. However, in a medium lacking the essential metabolite that the mouse cells cannot produce, the one human chromosome that contains the gene encoding the needed enzyme will be retained, because any hybrid cells that lose it following mitosis will die. All other human chromosomes eventually are lost.

By using different mutant mouse cells and media in which they cannot grow, researchers have prepared various panels of hybrid cell lines. Each cell line in a panel contains either a single human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes. Because each chromosome can be identified visually under a light microscope, such hybrid cells provide a means for assigning, or "mapping," individual genes to specific chromosomes. For example, suppose a hybrid cell line is shown microscopically to contain a particular human chromosome. That hybrid cell line can then be tested biochemically for the presence of various human enzymes, exposed to specific antibodies to detect human surface antigens, or subjected to DNA hybridization and cloning techniques to locate particular human DNA sequences. The genes encoding a human protein or containing a human DNA sequence detected in such tests must be located on the particular human chromosome carried by the cell line being tested. Panels of hybrids between normal mouse and mutant hamster cells also have been established; in these hybrid cells, the majority of mouse chromosomes are lost, allowing mouse genes to be mapped to specific mouse chromosomes. Hybrid Cells Often Are Selected on HAT Medium

One metabolic pathway has been particularly useful in cell-fusion experiments. Most animal cells can synthesize the purine and pyrimidine nucleotides de novo from simpler carbon and nitrogen compounds, rather than from already formed purines and pyrimidines. The folic acid antagonists amethopterin and aminopterin interfere with the donation of methyl and formyl groups by tetrahydrofolic acid in the early stages of de novo synthesis of glycine, purine nucleoside monophosphates, and thymidine monophosphate. These drugs are called *antifolates,* since they block reactions involving tetrahydrofolate, an active form of folic acid. Many cells, however, contain enzymes that can synthesize the necessary nucleotides from purine bases and thymidine if they are provided in the medium; these *salvage pathways* bypass the metabolic blocks imposed by antifolates. A number of mutant cell lines lacking the enzyme needed to catalyze one of the steps in a salvage pathway have been isolated. For example, cell lines lacking thymidine kinase (TK) can be selected because such cells are resistant to the otherwise toxic thymidine analog 5-bromodeoxyuridine. Cells containing TK convert 5-bromodeoxyuridine into 5-bromodeoxyuridine monophosphate. This nucleoside mono- phosphate is then converted into a nucleoside triphosphate by other enzymes and is incorporated by DNA polymerase into DNA, where it exerts its toxic effects. This pathway is blocked in cells with a *TK* mutation that prevents production of functional TK enzyme. Hence, TK^- mutants are resistant to the toxic effects of 5-bromodeoxyuridine. Similarly, cells lacking the HGPRT enzyme have been selected because they are resistant to the otherwise toxic guanine analog 6-thioguanine. As we will see next, $HGPRT^-$ cells and TK^- cells are useful partners in cell fusions with one another or with cells that have salvage-

pathway enzymes but that are differentiated and cannot grow in culture by themselves.

The medium most often used to select hybrid cells is called *HAT medium,* because it contains hypoxanthine (a purine), aminopterin, and thymidine. Normal cells can grow in HAT medium because even though aminopterin blocks de novo synthesis of purines and TMP, the thymidine in the media is transported into the cell and converted to TMP by TK and the hypoxanthine is transported and converted into usable purines by HGPRT. On the other hand, neither TK^- nor $HGPRT^-$ cells can grow in HAT medium because each lacks an enzyme of the salvage pathway. However, hybrids formed by fusion of these two mutants will carry a normal *TK* gene from the $HGPRT^-$ parent and a normal *HGPRT* gene from the TK^-parent. The hybrids thus will produce both functional salvage-pathway enzymes and grow on HAT medium. Likewise, hybrids formed by fusion of mutant cells and normal cells can grow in HAT medium.

Hybridomas Are Used to Produce Monoclonal Antibodies

Each normal B **lymphocyte** in an animal is capable of producing a single type of antibody directed against a specific determinant, or epitope, on an antigen molecule. If an animal is injected with an antigen, B lymphocytes that make antibody recognizing the antigen are stimulated to grow and proliferate. Each antigen-activated B lymphocyte forms a clone of cells in the spleen or lymph nodes, with each cell of the clone producing identical antibody, termed monoclonal antibody. Because most natural antigens contain multiple epitopes, exposure of an animal to an antigen usually stimulates formation of several different B-lymphocyte clones, each producing a different antibody; a mixture

of antibodies that recognize different epitopes on the same antigen is said to be *polyclonal.*

For many types of studies involving antibodies, monoclonal antibody is preferable to polyclonal antibody. However, biochemical purification of monoclonal antibody from serum is not feasible, in part because the concentration of any given antibody is quite low. For this reason, researchers looked to culture techniques in order to obtain usable quantities of monoclonal antibody. Because primary cultures of normal B lymphocytes do not grow indefinitely, such cultures have limited usefulness for production of monoclonal antibody. This limitation can be avoided by fusing normal B lymphocytes with oncogenically transformed lymphocytes called *myeloma cells,* which are immortal.

Fusion of a myeloma cell with a normal antibody-producing cell from a rat or mouse spleen yields a hybrid that proliferates into a clone called a hybridoma. Like myeloma cells, hybridoma cells are immortal. Each hybridoma produces the monoclonal antibody encoded by its B-lymphocyte partner. Many different myeloma cell lines from mice and rats have been established; from these, $HGPRT^-$ lines have been selected based on their resistance to 6-thioguanine as described above. If such mutant myeloma cells are fused with normal B lymphocytes, any fused cells that result can grow in HAT medium, but the parental cells cannot. Each selected hybridoma then is tested for production of the desired antibody; any clone producing that antibody then is grown in large cultures, from which a substantial quantity of pure monoclonal antibody can be obtained.

Such pure antibodies are very valuable research reagents. For example, a monoclonal antibody that interacts with protein X can be used to label, and thus locate, protein X in specific cells of an

organ or in specific cell fractions. Once identified, even very scarce proteins can be isolated by affinity chromatography in columns to which the monoclonal antibody is bound (see Figure 3-43c). Monoclonal antibodies also have become important diagnostic and therapeutic tools in medicine. Monoclonal antibodies that bind to and inactivate toxic proteins (toxins) secreted by bacterial pathogens are used to treat diseases caused by these pathogens. Other monoclonal antibodies are specific for cell-surface proteins expressed by certain types of tumor cells; chemical complexes of such monoclonal antibodies with toxic drugs are being developed for cancer chemotherapy.

8.4.1.5 SUMMARY

- Growth of vertebrate cells in culture requires rich media containing essential amino acids, vitamins, and peptide or protein growth factors, frequently provided by serum. Most cultured vertebrate cells will grow only when attached to a negatively charged substratum that mimics the extra cellular matrix in animal tissues.

- Primary cells, which are derived directly from animal tissue, have limited growth potential in culture and may give rise to a cell strain.

- Transformed cells, which are derived from animal tumors or arise spontaneously from primary rodent cells, grow indefinitely in culture. They usually have an unstable, aneuploid complement of chromosomes, including abnormal

chromosomes. Transformed cells derived from a single parental cell are called *cell lines.*

- Cultured cells can be induced to fuse into heterokaryons (hybrids) by treatment with certain viruses or polyethylene glycol. Heterokaryons between cells of different species tend to lose the chromosomes of one species as they divide.

- Panels of hybrid lines prepared from mutant mouse cells and normal human cells, each containing different human chromosomes, can be used to map the gene encoding a specific human protein to a specific human chromosome.

- Fusion of an $HGPRT^-$ myeloma cell and a single B lymphocyte yields a hybrid cell that can grow on HAT medium and proliferate indefinitely, forming a clone called a *hybridoma.* Since each individual B lymphocyte produces antibodies specific for one antigenic determinant (epitope), a hybridoma produces only the monoclonal antibody synthesized by its original B-lymphocyte parental cell.

8.4.1.6 Keywords

Differentiation, serum,osmolality, myelomacells, matrix, hyaluronicacid, hybridoma, epitope, cell line, myoblasts, aneuploid, proliferation, transferrin, monoclonal, polyclonal.

8.4.1.7 Model Questions

1)Explain the different conditions required for cell culture.

2)Write a note on different components of serum and serum free media.

Lesson 8.4.2

Techniques of Animal Tissue Culture

Objective :

For practicing the animal cell culture one should have clear idea of different techniques of cell culture. The present chapter includes different culture techniques in culturing tissues, organs and embryos etc.

Tissue and Organ Cultures :

Primary Explantation Techniques :

For the development of primary cultures and cell lines, a variety of tissues and disaggregation methods are used to give good yield of separate cells. It is, therefore, obvious that for any exercise involving animal cell and tissue culture, tissue needs to be obtained under aseptic and sterile conditions, although it may or may not be cultured, depending upon whether or not it is immediately subjected to disaggregation to give an yield of cells. However, even for obtaining cell cultures, often the tissue is cultured using **'primary explantation technique'** developed by Harrison (1907), Carrel (1912) and others. This technique is used for cultivation of pieces of fresh tissue derived from the organism, and this was almost the exclusive technique used for animal tissue culture till about 1945. Different forms of primary explantation techniques are still widely used and will continue to be used for a very

long time. These techniques differ only in the type of vessels (flasks, test tubes, etc.) used for growing the tissue, but are uniform in principle. The primary explantation technique is also used for embryo and organ culture, but are variously modified to become specialized techniques. The different explantation techniques are classified into the following: (i) **slide cultures**, (ii) **carrel flask cultures** and (iii) **roller test tube cultures**. These three techniques of tissue culture and the techniques of organ culture (including tissue engineering) will be discussed in this chapter.

TISSUE CULTURE

Slide or coverslip cultures

In this technique, slides or cover slips are prepared by placing a fragment of tissue (**explantation**) onto a coverslip, which is subsequently inverted over the cavity of a depression slide. This is the oldest method of tissue culture and is still quite widely sued. This has a number of advantages and disadvantages listed in Table_____. From this Table, it is obvious that the application of slide culture is limited but it may be very useful for morphological studies through the use of time-lapse cinemicrographic investigations. There are several general methods for preparation of slide culture; three of these methods will be briefly described in this section.

Table : Advantages and disadvantages of slide and coverslip cultures of tissue.

S.No.	ADVANTAGES	DISADVANTAGES

1	It is simple and relatively inexpensive.	Supply of oxygen and nutrients is rapidly exhausted, so that the medium quickly becomes acidic and requires transfer of rapidly growing tissue.
2	Cells in living state are spread out in a manner suitable for microscopy and photography.	Sterility can not be maintained for a long period.
3	Cells grow directly on coverslip and can be fixed and stained to make permanent slides.	Only very small amounts of tissue can be cultured.

Single coverslip with plasma clot.

This technique developed by Harrison (1907) has been most commonly used during the last more than fifty years and includes the following steps (Figure _____):

Figure :

Figure :

(i) prepare medium in two parts, one containing 50% plasma in BSS (Balanced Salt Solution) and the other containing 50% embryo extract in serum; (ii) under sterile and aseptic conditions, using a capillary pipette, place one drop of plasma containing solution in the centre of each of one, or more coverslips (22mm); (iii) transfer a fragment (one or two pieces) of tissue (called explant) to this drop without crushing the tissue; (iv) add the embryo extract containing solution and mix thoroughly before clotting starts and then locate the explant; (v) place two small spots of petroleum jelly (using a glass rod) near the concavity of a depression slide and invert this slide over the coverslip; apply gentle pressue, so that jelly sticks to coverslip; (vi) allow culture medium to clot; (vii) turn over the slide and seal the margins of coverslip with paraffin (viii) label and incubate at 37^{O}C.

Double coverslip with plasma clot

This technique was developed by Maximov and resembles the single coverslip method described above. It includes the following steps: (i) a small drop of BSS is placed on a large coverslip (40mm); (ii) a square or round coverslip (22mm) is placed over BSS in the centre of large coverslip. These two steps are then followed by the steps listed above for single coverslip method. A large depression slide is used and the entire preparation is attached to it by petroleum jelly and wax in such a way

that the small coverslip is not in contact with the slide at any point (Figure _____).

Single coverslip with liquid medium (lying and hanging drop cultures)

Following steps are involved in this method: (i) prepared explants are placed in culture medium in a watch glass; (ii) the explants are drawn into the tip of a capillary pipette, and one explant is deposited in the centre of each coverslip; (iii) the liquid medium can be spread out in a very thin circular film with the explant protruding above the surface; (iv) a depression slide with petroleum jelly is applied immediately and preparation turned over with a quick flip to prevent the fluid from running into the crevice between the slide and the coverslip; (v) the coverslip is sealed and the slide incubated at 37^{O}C, upright or inverted; the tissue grows on the coverglass.

After-care of slide cultures

Single coverslip cultures are very useful for short term studies but they are difficult to handle subsequently except by a process of transfer. Therefore, double coverslip method is recommended, whenever it is desirable to leave the explant in its original location to obtain long term tissue cultures. This would require washing, feeding, patching and transfer of cultures.

(a) Washing and feeding double coverslip cultures

Washing and feeding involves the following steps: (i) remove seal using razor blade and remove large coverslip with small one still attached and flip it over, so that culture is uppermost in orientation; (ii) detach small coverslip from the large coverslip using needle and forceps and transfer it to a Columbia staining dish (watch glass or Petri dish may also be used) containing balanced salt solution (BSS); (iii) remove small coverslip treated as above (one at a time) and place it on a large coverslip with culture up; while removing small coverslip from dish with BSS, the mount of BSS brought with it may be controlled by the rate of removal (too much liquid will interfere in feeding operation and too little will allow air bubbles); (iv) feed the culture by adding a drop of feeding solution (e.g. BSS : serum : 50% embryo extract = 1:1:1) to the small coverslip; (v) attach a clean depression slide, using petroleum jelly, as done earlier.

(b) Patching the plasma clot in slide cultures

If there is evidence of liquefaction, plasma clot should be patched as followed: (i) wash small coverslip with culture as above; (ii) in a watch glass take two drops of a mixture of plasma and BSS and to this add two drops of a mixture of serum and embryo extract; mix this quickly and place a drop on each washed coverslip having a culture; (iii) a clean depression slide with petroleum jelly is then attached, as usual.

(c) Transfer of slide culture

The coverslip culture may need to be transferred using the following steps; (i) remove and wash coverslip with culture, and using a Bard-Parker knife, cut through the outgrowth; (ii) the square tissue may be cut into two or four pieces, each transferred to new coverslip and treated as a new explant.

Flask cultures

The main use of flask cultures is in the establishment of a strain from fresh explants of tissue. A good Carrel flask has excellent optical properties for microscopic examination, even though polystyrene culture flasks can also be used, provided they have a wide neck for handling the explants. The flask technique has the following advantages: (i) tissue can be maintained in the same flask for months or even years; (ii) large number of cultures can be easily prepared and large amount of tissue can be grown with large amount of medium.

Figure :

There are two types of flask techniques: (i) **`thick clot cultures'**, which allow rapid growth suitable for short term cultures and (ii) **`thin**

clot cultures', which can be maintained for a considerable period of time.

Preparation of flask cultures

Following steps are involved in the preparation of flask cultures (Figure _____): (i) place upto six D3.5 **Carrel Flasks** in a rack with their necks flamed and pointing to the right; (ii) place a drop of plasma on the floor of flask and spread this plasma out in a circle; (iii) with the help of spatula, transfer the desired number of explants to the plasma and allow clotting to occur; (iv) after the plasma clots and explants fixed in position, and extra medium; for thick clots 1.2ml of dilute plasma and for thin clots 1.2ml of dilute serum is added instead of plasma; the whole thing is left for clotting; (v) the flasks are gassed with gas phase (5% CO_2 in air).

Renewal of medium

The medium may be periodically replaced in flask cultures as follows: (i) old fluid is drawn off by means of a pipette; (ii) 1.2ml of fluid medium is added as replacement; (iii) the flask is gassed as above.

Transfer of Culture

The culture grown in a flask culture need to be removed and cut into pieces, when there is a need for transfer. These pieces are then used for replantation as usual.

Test tube cultures

Test tubes are cheap and convenient vessels for tissue culture and can be used for preparing a large number of cultures, which can be placed in stationary racks or roller drums. However, this technique has disadvantages like poor optical property for microscopy, difficulty in quantitation due to the curvature and high risk of contamination. Cultures on plasma clots in test tube are prepared just like those in flasks, but tissues may be grown on the wall of test tube without a plasma clot. Test tubes may also be used for developing suspension cultures. The feeding, patching and transfer of culture is done as in other primary explantation techniques.

Figure :

Organ Culture

Organ culture usually implies culturing pieces of an organ (not necessarily whole organ) in vitro, and its objective is to maintain the architecture of the tissue and direct it towards normal development such as occurs in vivo. In order to achieve this aim, it is essential that the tissue should never be disrupted or damaged and this requires careful handling. In view of this, organ culture techniques generally demand more careful manipulation than tissue culture techniques discussed

earlier in this chapter. Media used for growing organ cultures are generally the same as those used for tissue culture. The techniques of organ culture can be divided into (i) those employing a solid medium and (ii) those employing liquid medium.

Culture of embryonic organs

It is easier to culture embryonic organs than to culture organs from adult animals and the techniques used in two cases. Embryo organs can be cultured, using any of the following three techniques.

Organ cultures on plasma clots

The organ cultures can be prepared on plasma clots using the following steps (Figure_____): (i) prepare a plasma clots by mixing 15 drops of plasma with five drops of embryo extract in a watch glass; (ii) place the watch glass on a pad of cotton wool in a Petri dish; cotton wool is kept moist to prevent excessive evaporation from the dish; (iii) place a small, carefully dissected piece of tissue on top of the plasma clot in the watch glass. The technique has now been modified, and a raft of lens paper or rayon net is used on which the tissue is placed. Transfer of the tissue can then be achieved by moving the raft easily. This also facilitates feeding of the culture, so that the net may be removed. Excess fluid is removed and the net with the tissue placed again on fresh pool of medium.

Organ cultures on agar

Media solidified with agar are also used for organ culture and these media consist of 7 parts 1% agar in BSS, 3 parts chick embryo extract and 3 parts horse serum. In some other studies, denied media with or without serum are used with agar. The medium with agar has the advantage that the support for organ culture does not liquefy and no additional mechanical support is required. Embryonic organs generally grow well on this medium, but tumours from adults will not survive.

Organ cultures on fluid media

Organ cultures also make use of liquid media. In this method, often a raft made of lens paper, or cellulose acetate net or perforated metal gauze is used.

Culture of adult organs

The culture of organs or parts from adult animal is more difficult due to their greater requirement of O_2. A variety of adult organs (e.g. liver) have been cultured using special media (e.g. T8; see Paul, 1975) with a special apparatus (Towell's Type II culture chamber). Since serum was found to be toxic, serum free media were used, and the special apparatus permitted the use of 95% O_2.

WHOLE EMBRYO CULTURE

Culture of chick embryo

The effect of metabolic inhibitors on embryonic development was studied by Spratt (1956, 1957), using embryo cultures. In this technique, 40-hours old embryos were used and the embryo development could be followed for another 24-48 hours in vitro before the embryo dies. This involves the following steps: (i) prepare a suitable defined medium or a synthetic medium (consult a manual), and 1ml aliquots of the medium are added to sterile watch glasses, placed on moist absorbent cotton wool pads in Petri dishes (as for organ culture); (ii) incubate hen's eggs at 38^{O}C for 40-42 hours to provide about a dozen embryos; (iii) the shell is wiped with alcohol and broken into a sterile evaporating dish containing 50ml chick saline or BSS; (IV) a circular cut is made (using scissors) into the vitelline membrane around the blastoderm and the latter is transferred to a Petri dish containing BSS; (v) the adherent vitelline membrane is removed with the aid of forceps and the embryo is examined under the microscope to determine the stage of development; (vi) the blastoderm is transferred to the top of the medium in the watch glass prepared in (i) above; (vii) the blastoderm is spread on agar gel (ventral side down) and the excess BSS is removed; (viii) culture is incubated at 37.5^{O}C.

Culture of mammalian embryos or ova

The mammalian embryos (rabbit or mouse), as young as 2-8 celled fertilized ova, have been cultured. It was shown in these cases that considerable development can really take place in vitro, and mouse embryos were shown to develop upto blastocysts. Such cultured embryos or ova can be reimplanted and give rise to healthy animals. The

media used for this purpose varied from 100% serum, through the common types of plasma-embryo extract media to simple Krebs-Ringer solutions supplemented with 1% of thin egg white or bovine albumin. The embryo can be cultured either in the pre-implantation stage or at the post-implantation stage. In the latter case, special care is needed to remove the embryo in an undamaged form.

Figure :

Tissue Engineering

As discussed in the last chapter and earlier in this chapter, a variety of animal tissues can now be cultured. The number of types of these human tissues is more than 200. During the last decade, the tissue culture work in animals demonstrated that virtually any human tissue or organ can be grown in culture (Figure _____]. This has become possible when it was found that the ability of cultured cells to undergo differentiation can be restored. These developments led to the emergence of a new science called `tissue engineering'. Some of the goals of tissue engineering are listed in Table_____ `Skin' was the first organ, which could be cultured in artificial media and could be used successfully for transplantation, after serious skin burns. Eugene Bell, who is the pioneer of artificial skin development, feels that eventually a

tissue engineer will be able to reconstitute body parts for (i) use in grafting, and to be used as (ii) better models for drug delivery and action.

Table Some of the technological goals of tissue engineering.

1	Growth of cells in three-dimensional systems
2	Delivery systems for protein therapeutics
3	Cell cultivation methods for culturing `recalcitrant cells'
4	Transgenic protein expression in transplantable cells
5	Vehicles for delivering transplantable cells
6	Avoiding immunogenicity in transplantation systems
7	Development of markers for tracking transplanted cells
8	Developing in vivo and ex vivo biosensors for monitoring cell behaviour during tissue production

It is believed by tissue engineers like Rebort (Bob) Nerem of USA, that after the success of artificial skin, the next step is the growth of functional artificial organs like pancreas and liver damaged due to a disease. Although the clinical application of these other artificial organs is remote, the potential of growing and using these artificial organs does exist.

A `tissue engineering conference' was held in 1990 at Keystone, Colorado (USA). Where possibility of replacing `Cartilage' in stress bearing joints was found to be a reality. Donald Ingber of USA works on

developing `extra cellular matrices (ECMs)' that will allow tissue growth and differentiation in vitro, as a means to organ replacement. However, tissue engineering would need a knowledge of physics and chemistry along with the knowledge about cell and tissue culture. For example, one will have to know, how the cellular environment affects three dimensional structure of tissue grown in culture. On will also have to know the fine structure of carbohydrate aggregates surrounding the cells. Detailed knowledge on these aspects will allow the induction of stem cells to differentiate in required manner. It is also believed that stem cells exist for all tissue types, and if these can be recovered, any human tissue can be induced to reform.

Artificial skin

There are three biotech companies in USA, which are developing artificial skin to the stage of clinical trials (i) ATS (Advanced Tissue Science), located at La Jolla, California which is now merged with Neomorphics (Lexington, MA), (ii) Biosurface Technology (BTI, Cambridge, MA) and (iii) Organogenesis. These three companies used different technological approaches, which are really the basis of all future tissue engineering.

The real breakthrough in culturing artificial skin was made in 1970s, at Harvard. It was demonstrated that when an irradiated 3T3 fibroblast cell line is grown with skin cells (keratinocytes, which make 90% of the skin epidermis), 3T3 cells stimulated growth of skin cells and allowed their differentiation into epidermis. 3T3 cells are believed to

secrete factors which help in growth and differentiation. This technique was used by BTI, where postage stamp sized skin biopsies are taken and expanded 10,000 times within three to four weeks. This gives only the cultured epidermis, which when applied to burnt area, helps regeneration of dermis underneath. Since in severely burned patients, skin biopsies may not be available from the patients, following two approaches were used to overcome the problem: (i) Skin from cadavers (dead bodies) are taken to cover and protect against infection and desiccation, until their own skin is grown. (ii) Neonatal foreskin (loose skin from the tip of penis of newly born child) is cultured, which creates artificial dermis layer. This leads to allogeneic grafts stimulating rapid healing. New born skin cells are used, since they grow relatively more vigorously. ATS company uses a synthetic polymer, PGA, for growing artificial skin from neonatal foreskin. The use of synthetic polymers allows cells to grow into skin without scars, so that it is believed that this technology will be the basis for future organ development.

A further advancement was made by the company `Organogenesis' which claims to have created a `living skin equivalent (LSE)' comprising of both a lower dermis layer and an upper epithelial layer. Collagen matrix is used as a support for the growth of LSE. This LSE when used for grafting may be replaced by host cells eventually, but is not rejected.

Summary :

Since 18th century several technique were used by different people for culturing animal tissues, organ as cells of animals for different

purposes. For culturing tissues coverslip culture and flask cultures techniques were generally used. Organs are generally cultured of plasma clots, agar clots and some times on third media we can also culture entire embryos on suitable media.

Model Questions :

1. Explain different tissue culture techniques?

2. Write about embryo culture?

8.4.4

APPLICATIONS OF ANIMAL CELL CULTURE

Objective: Techniques of animal cell culture and media employed for animal cell line maintenance and growth and applications of animal cell culture in various fields were discussed in this chapter

8.4.4.1 Introduction

Scientists studying the biochemistry and biophysics of cell growth and division have made extensive use of cultured animal cells, as have those engaged in research on animal viruses. However, the value of cultured

animal cells goes beyond this, for they produce a wide range of biological products of commercial interest including immunoregulators, antibodies, polypeptide growth factors, enzymes and hormones. Already they are used in the manufacture of virus vaccines, tissue plasminogen activator (an enzyme which facilitates the destruction of blood clots), interferon-α (for the treatment of cancer), monoclonal antibodies and tumour-specific antigens (for inclusion in diagnostic kits). Consideration is also being given to the possibility of growing fibroblasts in culture from, for example, burns patients. These cultured cells would be used as a reconstituent skin to facilitate wound healing. Another potential application of cultured cells is their use in evaluating new drugs and toxic chemicals. When used in concert with conventional animal models they could reduce much of the time, cost and effort as well as the total number of animals. Even the cells themselves can have commercial value. For example, cultured fibroblasts from burns patients have been used as a reconstituted skin to facilitate wound healing.

8.4.4.2 IN VITRO TOXICITY TESTING

The use of animals in evaluating chemical safety is costly, time-consuming and increasingly criticized by animal welfare groups. As a consequence, alternative test methods are being developed which make use of cultured cells. For example, genotoxicity can be assessed by using incorporation of radioactive nucleotides as a measure of DNA repair. Not

only most chemicals used on large scale, or administered as pharmaceuticals, be free of cyto- or genotoxicity but so must their metabolites. Rather than administer such compounds to test animals, they can be added to cultures of animal or human hepatocytes (liver cells).

8.4.4.3 PRODUCTION OF VIRAL VACCINES

The oldest commercial application of animal cell cultures is for the production of viral vaccines and it could be argued that globally, with the possible exception of antibiotics, they have provided greater benefit than any other pharmaceutical product. The deployment of a single vaccine, that against smallpox, has been of immense benefit to mankind and hopefully has eliminated the virus for evermore. Because viruses are obligate intracellular parasites the earliest viral vaccines, those against smallpox and rabies, were made in intact animals such as calves, sheep and rabbits. Today the only intact host used in advanced production techniques is the developing chick embryo for the manufacture of influenza and yellow fever vaccines. All other vaccines are prepared by the growth of virus in cell culture. Table-1, lists the major human and veterinary virus vaccines and the cell cultures used in their production.

Viral vaccines are of two types. Those which are prepared from live, avirulent (attenuated) organisms often given lifelong protection but carry with them the attendant risk of contamination with other viruses

derived from the cell culture. Clearly, rigorous microbiological and other quality control procedures are essential. Vaccines prepared from killed (inactivated) viruses are less satisfactory since they are not as immunogenic as live vaccines, presumably because the virus does not multiply in the animal host, and have to be injected repeatedly according to carefully balanced immunization schedules. The only inactivated vaccines for human use are those against rabies and, in some instances, polio.

In theory, the production of viral vaccines is relatively simple. The cell cultures principally used for virus vaccine production are prepared from monkey kidney or chick embryos. More recently there has been an increasing use of human diploid cells. Since these cells grow as monolayers all that is required in vaccine preparation is to infect them with the appropriate virus and then harvest the culture fluid after virus multiplication has occurred. The culture fluid containing the virus is clarified by filtration. In the case of killed-virus vaccines a concentration step is usually employed after the inactivation step. When processing is complete the virus suspension is blended with stabilizers to prevent loss of potency, a problem with live attenuated vaccines, and stored at low temperature. A typical production process is shown in Figure.

Table-1: Viral vaccines currently available for human and veterinary use

Human vaccines		Veterinary vaccines	
Virus	**Cell used for culture**	**Virus**	**Cell used for culture**
Measles	Chick embryo fibroblasts	Canien distemper	Chick embryo fibroblasts or dog kidney cells
Polio (inactivated)	Monkey kidney cells	Canine hepatitis	Dog kidney cells
Polio (live)	Monkey kidney or human diploid cells	Foot and mouth disease	Bovine kidney cells
Rabies	Human diploid cells	Rabies	Duck embryo or chick embryo
Rubella	Rabbit kidney, duck embryo or human diploid cells	Feline panleucopenia	Cat kidney cells
		Marek's disease	Chick embryo cells

In practice, the production of a virus vaccine is far more complex, not just because of the labour involved in large-scale animal culture but because of the rigorous quality control and safety procedure which are required; for example, all tissue culture substrates are rigorously examined to exclude contamination with infectious agents from the source animal or, in the case of human diploid cells, to exclude abnormal cellular characteristics. Monkey tissues are tested exhaustively for

extraneous agents, e.g. herpes virus B, simian virus 40, tubercle bacilli and mycoplasmas. Human diploid cells are subjected to detailed karyological examination to exclude cultures with features resembling transformed cell lines or malignant tissues.

Safety of the final product is another important consideration. With killed-virus vaccines the potential hazards are those due to incomplete virus inactivation – a possible cause of recent outbreaks of food and mouth disease in Europe! The tests used to detect live virus consist of the inoculation of susceptible tissue culture and of susceptible animals. The cultures are examined for cytopathic effects and animals for symptoms of disease and histological evidence of infection at autopsy. With attenuated viral vaccines the potential hazards are those associated with reversion of the virus to a degree of virulence capable of causing disease in vaccines. This possibility is minimized by the use of stable virus seed stocks but virulence checks on the final product are necessary; for example, in the production of attenuated poliomyelitis vaccine the neuro virulence of each batch following intraspinal inoculation of monkeys is compared with that of a control vaccine.

Many of the problems associated with the production of viral vaccines, particularly the safety aspects, may disappear now that recombinant DNA technology can be used to enable virus subunits to be produced in bacteria or yeasts. Another advantage of recombinant DNA

technology is that it may permit the production of vaccines against those viruses which cannot be grown in cell culture to a titre high enough to provide sufficient antigen for effective vaccination.

8.4.4.4 PRODUCTION OF HIGH-VALUE THERAPEUTICS

There are many human proteins which have long been believed or known to have therapeutic potential but which are in short supply; for example, human growth hormone can be obtained from the pituitary gland of cadavers and clotting factors, VIII and IX from the plasma of blood donors. The supply limitations of these substances is obvious and there are other complications, e.g. the potential for acquiring AIDS from contaminated blood products. A more satisfactory alternative would be to grow, in large-scale culture, cells derived from the tissue which normally synthesizes the desired protein. The disadvantage with this method is that, generally speaking, such cultured cells produce only low levels of the therapeutic protein: interferon-β is a good example. Using human embryonic lung fibroblasts growing in 201 roller bottles, each containing 750 ml of medium, only 5×10^6 units (0.02 mg) of interferon-β are produced per litre of medium after induction of interferon synthesis with poly I : C. Until the advent of suitable monoclonal antibodies the purification of this interferon, free of serum proteins from the growth medium, was very difficult.

Table-2 : Proteins overproduced by some mammalian cell lines.

Product	Cell line name	Source
Acetylcholinesterase	BP4 IAS	Murine neuroblastoma
Interferon-α	Namalwa	Human blood
Interferon-β	Flow 7000	Human embryonic foreskin
Interleukin	EL4 CL14	Murine blood
Plasminogen activator	GPK	Guinea pig hepatocyte
	BEB	Human breast
Urokinase	HT 1080	Human fibrosarcoma

One solution to the problem of low yields is to produce the protein in bacteria by using recombinant DNA technology. The problem with this approach is that many mammalian proteins undergo post-translational modifications by processes such as glycosylation which are not mimicked in bacterial systems. Although many of the non-glycosylated derivatives of these proteins retain activity, e.g. interferon-γ, urokinase and interleukin-2, their half-life in vitro may be reduced significantly and long-term therapy in humans may result in undesirable immunological reactions. With some proteins, such as human blood clotting factor IX, the situation is more complex: not only is glycosylation essential for activity but so too is the vitamin K-dependent

γ-carboxylation of glutamic acid residues. Clearly, at least for the foreseeable future, the only prospect of manufacturing such molecules is to do so in mammalian cells. However, this will not solve all the above problems. When human protein C was produced by three different cell lines, the levels of γ-carboxylation and glycosylation varied significantly, as did the antithrombotic activity. The method of cell culture can also affect the glycosylation pattern significantly as well as the in vivo halflife. When IgM was produced in serum-supplemented perfusion culture it had an in vivo half-life of 18 minutes but from serum-free culture it was only 3 minutes.

If mammalian cell culture is the only method for obtaining certain therapeutic proteins, then cells which are overproducers are required. It is well known that in some pathological conditions certain cells in the body become neoplastic and overproduce a particular protein. However, transformed cells that will produce many of the mammalian proteins currently in demand are not available. The solution is to clone the relevant genes in mammalian cells rather than microbial cells.

8.4.4.5 CLONING IN ANIMAL CELLS : BASIC PRINCIPLES

Both bacteriophage and plasmid vectors are available for gene manipulation in bacteria but because of ease of use the latter are favoured. However, plasmids do not occur naturally in animal cells and so only virus vectors are available. As for any vector, the virus genome

must be easily manipulated, it must contain convenient sites for the restriction enzymes used in cloning and the location of those sites with respect to vector control sequences must be known. This latter point is particularly important since the gene sequences required for virus replication are far more complex than those required for plasmid replication. With virus vectors there can be an additional complication, the need to package the recombinant genome in viral coat protein. This can lead to size constraints on the amount of foreign DNA which a vector can carry. For this reason considerable emphasis is being placed on the development of vectors which can replicate like plasmids and thus at no stage need to be encapsidated in virus particles.

Whatever kind of vector is used there will always be a need at some stage to introduce unpackaged DNA into animal cells in culture, a procedure known as transfection. The most commonly used procedure is dependent upon the fact that freshly prepared co-precipitates of DNA and calcium phosphate are phagocytosed by cells. The method is not particularly efficient since only a low proportion of cells take up the exogenous DNA; at best only 1-2% of cells are infected but the efficiency can be as low as 1 cell in 10^6. When virions are produced the efficiency of transfection is not a limiting factor as the recombinant virions can subsequently be used to guarantee efficient infection.

It is not essential to have vectors capable of replication in order to stably introduce DNA into mammalian cells. Fragments of DNA can be phagocytosed by means of the calcium phosphate co-precipitation method and in at least a few of the transfected cells will become stably integrated at random in the genome. Identification of these rare cells carrying exogenous DNA is difficult unless the DNA also carries a gene for a selectable marker. A range of selectable genes available, (Table-3) for ligating to test genes prior to transfection. This technique has been used to obtain expression of hepatitis B surface antigen, herpes simplex type I glycoprotein D and human interferon-γ in Chinese hamster ovary cells. However, even though multiple copies of the transfected gene are present, expression levels are low and this technique is better suited to gene therapy.

Table-3: Some markers whose presence on exogenous DNA facilitates the selection of transfected cells.

Selectable marker	Basis of selection
Thymidine kinase (from herpes simplex virus)	Permits growth of cells lacking thymidine kinase
Bacterial neomycin phospho-transferase	Confers resistance to aminoglycoside antibiotics e.g. G418
Bacterial dihydrofolate reductase	Confers resistance to methotrexate

8.4.4.6 VIRUS VECTORS FOR USE WITH MAMMALIAN CELLS

A number of different virus vectors are available for cloning in mammalian cells. Each vector system has its advantages and disadvantages and, as yet, no one type of vector has emerged as clear favourite. To some extent this is a reflection of the different host ranges of the different viruses but personal preference also plays a part. The best example of this comes from work on the expression of clotting factor IX where three different vector systems have been used successfully. It should be borne in mind that the development of animal virus vectors is progressing at a phenomenal rate.

SV40 vectors, Simian virus 40 (SV40) was the first eukaryote virus to be harnessed as a vector simply because it was the first such virus for which a complete nucleotide sequence was available. The genome of SV40 contains very little nonessential DNA so it is necessary to insert the foreign gene in place of essential viral genes in order not to exceed the size limits imposed by the viral protein coat. Consequently the recombinant genome can be propagated only in the presence of a helper virus. In the original SV40 vectors the gene that is replaced is one which is required late in infection. The procedure for using such a vector is to perform a mixed infection between the vector carrying a temperature sensitive mutation in a gene required early in infection. Following infection at the non-permissive temperature virus growth can occur only

by complementation between the helper virus, which provides late functions, and the recombinant, which provides early functions.

An alternative SV40 vector system is one in which the foreign gene insert replaces a gene requires early in infection. The advantage of this system is that, although these recombinant viruses are unable to replicate in most monkey cell lines, they can do so in a special line of cells known as COS. Since helper virus is not required this means that pure recombinant virus stock can be produced easily. However, expression of foreign genes from the early promoter is much lower than from the later promoter.

A whole series of vectors has been constructed which comprise part of the *E.coli* vector plasmid pBR322 and part of the SV40 genome and which permit the exogenous gene to be placed under the control of an SV40 promoter. These vectors are grown and manipulated using *E.coli* as the host and are then transferred back into monkey cells. A similar series of vectors based on a related virus, polyoma, is available for use with cultured mouse cells.

Adenovirus vectors, The major disadvantage with SV40- and polyoma-based vectors is the small size of the genome. Since packaging constraints are strict, only small gene inserts can be made. Adenoviruses are much larger than SV 40 and can accommodate much more extraneous DNA. However, the larger size means that the

adenovirus genome is intrinsically more difficult to manipulate since it contains multiple sites for restriction endonucleases. Using a variety of techniques, vectors are being constructed in which many of these sites have been eliminated. In one set of vectors exogenous DNA is placed under the control of a transcriptional unit which is functional early in the life-cycle of the virus. Although the exogenous gene replaces a viral gene function, no helper virus is required if the recombinants are grown in a special strain of human embryonic kidney cells.

Vaccinia vectors, based on vaccinia virus are used in a slightly different way. Exogenous genes are first cloned adjacent to a vaccinia promoter present in the middle of a vaccinia-derived thymidine kinase gene which is carried on an *E.coli* plasmid (Figure-2). After manipulations are complete in *E.coli* the recombinant plasmid and wild-type vaccinia virus are contransfected into a monkey cell line. Recombination occurs between the plasmid-borne thymidine kinase gene fragment and the viral thymidine kinase gene. Recombinant virions fail to synthesize thymidine kinase and can be selected by virtue of their resistance to 5-bromodeoxyuridine.

BPV vectors, Vectors based on bovine papillomavirus (BPV) are unique in that not only are they propagated as multicopy plasmids but they also cause neoplastic transformation of the host cell, i.e. the cell becomes cancerous. The earliest vectors comprise a large subgenomic

transforming fragment of the BPV genome linked to an *E.coli* plasmid. Selection of cells carrying the vector is easy: being neoplastic they outgrow the normal cells, which only from a monolayer in culture. BPV-derived vectors show great promise because permanent cell lines are obtained which carry the foreign DNA insert at high copy number (up to 200 per cell). An additional advantage is that there is no size limitation on the DNA insert.

Baculovirus vectors, Baculoviruses infect insects, which have large double-stranded circular DNA genomes within a rod-shaped protein coat. A property of those baculoviruses which have been exploited as vectors if that during normal infection they produce nuclear inclusion bodies. These consist of virus particles embedded in a protein matrix of a virus-encoded protein called polyhedrin. Large amounts of virus and polyhedrin are produced. Transcription of the polyhedrin gene is driven by an extremely active promoter, which can be used to drive expression of foreign genes. This is particularly attractive because the polyhedrin gene product is not essential for viral replication. Construction of expression vectors has consisted of inserting a foreign coding sequence just downstream of the polyhedrin promoter. This cannot be achieved directly because the large size (about 130 kb) of the viral DNA precludes simple in vitro manipulation. Thus the strategy for inserting the foreign DNA into the virus is similar to that described above for vaccinia virus.

Baculovirus vectors are available for use either with cultured insect cells or silkworms. The silkworm host has advantages as a production system because silkworms can be cultured easily and at low cost as in the long-established silk industry.

Retrovirus vectors, Retroviruses have single-stranded RNA genomes and, at first sight, would appear to be unpromising candidates as vectors but the peculiarities of the retrovirus life-cycle mean that these viruses provide perhaps the most promising vector system of all. During the process of reverse transcription, a double-stranded proviral DNA molecule is generated which has all the regulatory functions for viral transcription and translation. Thus, any of the normal proviral genes can be replaced with foreign DNA provided the recombinant is propagated in the presence of a helper virus. Retroviruses have a number of features which make them particularly useful as gene transfer vectors.

Table-4: Properties of retroviruses which are of value in gene cloning

1	The normal replication process involves the stable insertion of a DNA copy of their genome into the host genome
2	They can infect and transmit their genetic information into a high proportion of recipient cells
3	Retroviruses have a broad host range which can be easily modified, making it possible to infect cells from a variety of species and cell types
4	The integrated provirus exists as a stable, predictable structure

	within the host genome
5	Infection of mammalian cells with retroviruses does not result in cell death. Rather, infected cells continue to grow and divide while producing large numbers of virus particles
6	A large amount of foreign gene sequence can be packaged within a virus particle
7	Viral gene expression is driven by a strong promoter

8.4.4.6 PRODUCTION OF THERAPEUTICS AND VACCINES IN RECOMBINANT MAMMALIAN CELLS

A whole range of proteins of potential value to the pharmaceutical industry has now been produced in cultured, recombinant mammalian cells. Some of them are listed in Table and several are approved for clinical use. All of the proteins are secreted as glycoproteins but for at least three of them (tPA, interferon-β and erythropoietin) the pattern of glycosylation differs from that seen in the natural molecule. However, since the glycosylation pattern differs for the same protein made in different cell types it is questionable as to the definition of the normal glycoform.

Vaccinia vectors have been extensively used to develop novel vaccines. Some proteins expressed by recombinant vaccinia viruses are listed in Table. The vaccinia virus recombinant expressing the influenza haemagglutinin gene was able to induce resistance to influenza virus

infection in hamsters. More interesting is the work done with the rabies antigen recombinant. This was administered to the wild population of foxes in north-eastern France by providing `bait' consisting of chicken heads spiked with the virus. Subsequently a substantial proportion of the wild foxes were shown to have acquired immunity to rabies. The same virus has been used to eliminate rabies in wild animal populations in other European countries and is being trialled in the USA.

Whether recombinant vaccinia viruses will ever be used for human vaccination remains to be seen. Interestingly in this context, an unplanned trial of recombinant vaccinia in man has been reported. A researcher accidentally inoculated a small cut on his finger with a drop of recombinant vaccinia which expresses the vesicular stomatitis virus (VSV) nucleoprotein. VSV causes a highly contagious vesicular disease of cattle, pigs and horses and flu-like symptoms in man. It was evident that the researcher had become infected with the recombinant but he did not experience malaise. The mildness of the infection was notable because a non-vaccine strain of the virus was involved. This could be related to the fact that the researcher had been vaccinated some 30 years previously, or to attenuation of the virus by the insertional inactivation of the viral tk gene. Importantly, serum from the researcher showed a high antibody titre to the VSV protein. This event showed that despite previous vaccination, a new immune response was elicited to non-vaccinia protein in man.

Table-5: Some mammalian proteins overproduced in cells in culture as a result of in vitro gene manipulation.

Protein	Size	Use	Comments
Tissue plasminogen activator	527 amino acids	Thrombosis	Enzyme overproduced in E.coli or yeast not fully functional
Interleukin-2	133 amino acids	Cancer therapy	
Tumour necrosis factor	157 amino acids	Cancer therapy	
Factor VIII	2332 amino acids	Haemophilia	Normally obtained from plasma of blood donors but now there is concern over potential contamination with AIDS virus
Factor IX	415 amino acids	Christmas disease	Must be made in mammalian cells as glycosylation and conversion of first 12 glutamate residues to pyroglutamate essential for activity
Erythropoietin	166 amino acids	Anaemia	Without glycosylation protein is cleared very quickly from

			plasma
Interferon-β Granulocyte – macrophage colony – stimulating factor	164 amino acids	Cancer therapy Stimulates production of white blood cells after cancer chemotherapy	Many other clinical indications

Table-6: Viral genes expressed in vaccinia recombinants.

Hepatitis B virus surface antigen
Herpes simplex virus glycoprotein D
Rabies virus glycoprotein G
Vesicular stomatitis virus (VSV) nucleoprotein
Epstein – Barr virus glycoprotein
Influenza virus haemagglutinin
Malaria sporozoite antigen
Human respiratory syncytial virus glycoprotein G
Sindbis virus structural proteins
HTLV-III (AIDS virus) envelope gene

8.4.4.8 Summary

The developments in the animal cell culture technology has created a big scope for the production of commercially and medically important compounds which play key role in daily human life. The production of vaccines and therapeutics has remarkably helped the human in reducing the risks for various diseases. The advent of r-DNA technology in this animal cell culture further created wider scope in development of various products, which are not possible earlier by the application of conventional techniques.

8.4.4.9 Questions

1. Write in detail the applications of animal cell culture for the development of vaccines
2. Discuss the various vectors used for gene manipulation in animal cell lines

8.4.4.10 Reference Books

Lesson 8.5.3

PRODUCTION OF ALCOHOL

Objective:

Brewing is the production of malt beverages. Brewing also differs from most other industrial fermentations because flavor, aroma, clarity, color, foam production, foam stability, percentage of alcohol and satiety all are factors associated with the finished product. Various malt beverages like beer are produced by microbial fermentation.

Industrial solvents like ethanol production is a very important fermentation industry. Alcohol production was the first fermentation known to mankind. Distilleries began to appear in Europe in the middle of the seventeenth century. At first alcohol was used only for human consumption. Later the demand for alcohol as a universal solvent and chemical raw material developed. The distillery industry grew very rapidly.

Structure of the lesson

8.5.3.1 Brewing

A. Medium
B. Microorganisms
C. Production
D. Carbonation
E. Byproducts

8.5.3.2 Alcohol Production

A. Microorganisms
B. Raw Materials
   (i) Sugar
   (ii) Starch
   (iii) Cellulose
C. Medium
D. Fermentation conditions
   (i) Carbon source
   (ii) Nitrogen source
   (iii) PH
   (iv) Temperature
   (v) Time
E. Production types
   (i) Batch fermentation
   (ii) Continuous fermentation
F. Recovery

Summary

Model Questions

Reference books

Figures list

1. Figure 1. Ethanol production (Fig.10.3, 148, Waites)

8.5.3.1 Brewing

8.5.3.2 Alcohol production

8.5.3.1 Brewing:

Brewing is the production of malt beverages. This is one of the oldest fermentations, dating back thousands of years and probably originating in the Nile valley. In early times, the type of local agriculture most likely influenced the particular fermented beverage produced. The cereal grains are used for beer production, fruits for wine production.

The various characteristics of a finished malt or other alcoholic beverage are difficult if not impossible to duplicate by chemical synthesis or by mixing chemically synthesized products with natural materials, even though the ethanol itself can be easily synthesized chemically. There is a high demand by the public for this type of fermentation product. Thus these considerations place alcoholic – beverage fermentations in a unique situation in regard to fermentation industries, in that they are among the most stable of the various fermentation processes.

A. Medium

The medium must contain the carbon, nitrogen, vitamin, and growth factors sources. Apart from these common needs it must contribute to the aroma, flavor, foam, color, clarity, stability of the finished product. This will be further processed to produce wort.

B. Microorganisms

Brewing utilizes strains of Sacchoromyces carlsbergensis, and S.cerevisiae. Yeast strains are specially selected for their fermenting ability and for their ability to flocculate at the proper time near the end of the fermentation. Brewing is different from much other industrial fermentation in that the cells for inoculating are often those recovered from a previous fermentation. Before being employed as inoculium, the yeast cells from a previous fermentation are washed with phosphoric acid, tartaric acid or ammonium persulfate by setting. This is a procedure that reduces the pH value to approximately 2.5, and removes bacterial contamination, if present.

C. Production

The aerated medium is cooled to approximately 10 to 11^{O}C and then placed in a closed fermentation tank containing cooling coils. The closed tank is preferred so that evolved carbon dioxide can be collected for later carbonation of the product. Approximately three Quarters to one pound of yeast are added for each 31 gallons of wort, and within 24 hours after inoculation, foam begins to appear on the surface of the medium, first along the wall of the tank and then gradually across the surface. The carbon dioxide evolution then increases so that the yeast cells become suspended in the medium. At this point, the wort is often transferred to a second fermentation tank so that dead and weak yeast cells, precipitation proteins and insoluble hop resins can be left behind as a deposit on the bottom of the original settling or starting tank or trapped in the foam. This procedure also provides some additional aeration to the medium.

By approximately 40 to 60 hrs after inoculation, the surface foam layer becomes very thick and can measure up to almost 12 inches in depth. It is during this time that the most rapid yeast – cell multiplication occurs, and considerable heat is evolved. This heat evolution causes a temperature rise to approximately 12 to 13^{O}C, the peak temperature for this fermentation.

By approximately the fifth day of fermentation, there is no longer enough carbon dioxide evolution to support the heavy foam and the foam begins to collapse. From seven to nine days, the last phase of the fermentation, the yeast become inactive and flocculate, the yeast break. The fermentation is usually completed by approximately five to seven days. The yeasts settle to the bottom, and the medium is further cooled to hasten settling. At this time, some of the surface scum may be removed to improve flavor.

The completed fermentation is transferred to storage flasks and held at approximately 0 to 3^{O}C for a period of time. During this "Cold storage maturation", coagulated nitrogenous substances, resins, insoluble phosphates and yeast cells sediment from the beer. In addition esters are formed and the beer matures so that it looses its harshness. Antioxidants are added during cold storage maturation to prevent later oxidative changes in beer, which affect Havor. Sulfur dioxide and ascorbic acid are commonly used to accomplish this.

D. Carbonation

Carbonation of the beer is accomplished either by injection of cleaned CO_2 recovered from the evolved fermentation gas, or by the Knowsen process in which actively fermenting yeast is added to provide the so called natural carbonation. Addition of carbon dioxide, which is the most common practice, provides a final dissolved CO_2 content of approximately 0.5% in the beer. The CO_2 displaces dissolved oxygen, which is detrimental to the stability of the beer, and helps in the production and retention of foam and in the preservation of the beer.

The Krausening process is accomplished by the addition to beer undergoing cold storage maturation of approximately 15% of active fermentation broth and cells from an early stage in the fermentation. These yeast cells are allowed to slowly ferment and remove residual sugar, a process that requires approximately 3 to 4 weeks of cold storage maturation time. The excess CO_2 evolved during this period is maintained so that the final level will be similar to that for CO_2 injection. After the sugar is gone, the beer undergoes several weeks' further cold storage maturation so as to complete the clarification of beer.

E. By products

The spent grains separated without drying from the wort find commercial use as animal feed. However, they also are dried to about 10% moisture to provide an animal feed product known as dried brewer's grain, which contains protein, fat, carbohydrate, crude fiber, and B complex vitamins.

8.5.3.2 Alcohol Production

1. Figure 1. Ethanol production (Fig.10.3, 148, Waites)

Ethanol is used as a solvent and a precursor of many synthetic chemicals. Fermentation processes have produced this ethanol. Today, fermentation processes have virtually disappeared as a result of the development of chemical production. This chemical production development is because of

(i) High prices of the fermentable substrates

(ii) Increasing economic competition from the petrochemical industry.

Ethanol is an attractive fuel because it may be used alone or mixed with other liquid fuels like gasohol. The gasohol is a blend of 10 – 22% ethanol with gasoline. In the 1970s Basil and a few other countries under took the full-scale production of ethanol from indigenous renewable biomass resources to offset the growing costs of oil imports. Brazil is now responsible for over 46% of annual world production, some 14.5 billion litres of ethanol.

A. Microorganisms

The microorganisms responsible for alcoholic fermentation of molasses are yeast species of Saccharomyces, Torulopsis, Kloekera, Candida and certain species of Mucor and bacteria. The most important among them are the yeast species belonging genus Saccharomyces. The two important species are S. cerevisiae and S. Carls bergensis. The S. cerevisiae is called top fermenter yeasts, as it is active at the top surface of the fermenting liquid. They remain

either in an evenly distributed layer, or as a ring along the walls of the fermentation tank. S. carls bergensis is called bottom fermenters. Bottom fermenters develop at the bottom of the fermentation tanks. These are whirled up in the liquid owing to CO_2 evolution.

The yeast strains used for alcoholic fermentation should possess the following features:

(i) It should produce a large quantity of alcohol.

(ii) It should be a fast growing strain.

(iii) It should have a high tolerance to alcohol.

Usually the strains of yeast, S. cerevisiae, are used for commercial production of a ethyl alcohol. The choice of microorganism also depends on the type of raw material to be used in fermentation. When starch or molasses are used as raw materials S. cerevisiae is selected. When whey and waste sulphite liquor are employed as raw material candida strains are preferred.

B. Raw materials for ethanol fermentations

45 Kg of fermentable sugar yield from 18 to 23 Kg of ethanol. For starchy materials the yield is 40 to 50% on the dry weight of carbohydrate. For cellulosed raw materials, the yields of ethanol are substantially less because α-cellulose is quite resistant to enzymatic attack.

Types of raw materials can be usefully divided into (i) sugar containing materials, which can be fermented directly, (ii) a starchy materials, which can be easily hydrolyzed by enzymes or acids to fermentable sugars and (iii) cellulosed materials, which are difficult to hydrolyze.

(i) Sugar

Ethanol may be produced from any sugar containing fruits, fruit juices or extracts, such as grape juice, apple juice, honey, date syrup or sugar containing effluents of canneries. Such sources are usually too costly in comparison with sugar beets, sugar cane and sweet sorghum. The production of crystalline sucrose yields a by-product, molasses, which until recently has been the cheapest source of fermentable sugar. Molasses containing about 50 – 55% fermentable sugar can be fermented without difficulty.

Fig. Fermentation of pentoses to ethanol (Fig.10.4, 151, waites)

This fermentation is the basis of the production of rum.

(ii) Starch

Starch, which has been gelatinized by heating, can be readily hydrolyzed to fermentable sugars by enzymes. Such starches occur in cereal grains, root crops or tubers. All of these materials have been used for the production of distilled alcoholic beverages; the use of wheat or corn for production of whiskey, and the use of potatoes for production of voolka are well known. In the United States corn is the most abundant source of starch.

(iii) Cellulose

The utilization of cellulosed materials for the production of ethanol is ultimately the most promising, but it also presents the most difficult technological and economic problems. One of the most promising developments in using cellulose is the 2-step

hydrolysis of cellulosed materials with (a) dilute acid to hydrolyze the hemicellulose to pentoses, followed by (b) acid or enzymatic hydrolysis of the separated cellulose.

C. Medium

The optimum sugar concentration ranges between 10 to 18% in the fermentation medium. Also, a suitable source of nitrogen such as ammonium sulfate is added in optimum concentration. When beet – molasses is used, usually a small quantity of black – strap molasses is also added to meet the biotin deficiency in the beet molasses. The pH of the medium is adjusted to 4.8 to 5. The pH can be adjusted by the use of H_2 SO_4 or lactic acid. Alternatively, it can be adjusted by growing lactic acid bacteria prior to fermentation. The high acidic pH in the medium hinders the growth of microorganisms.

D. Fermentation Conditions

(i) Carbon sources

Sugar concentration in the range of 10 to 18% is found satisfactory. Sugar concentration of 12% is often used. For example, cane molasses contain sucrose as a carbon source.

The sucrose content of the raw material is about 48 to 55% wort is prepared to contain about 10% sugars. High concentration affects yeast adversely while low concentration makes the process uneconomical.

(ii) Nitrogen source

Many organic as well as inorganic nitrogenous compounds may be used in this fermentation. Ammonium sulfate is generally used with success to supply nitrogen for growing yeast cells. The level of nitrogen in the medium is limited. Excess nitrogen is not acceptable, since it promotes the growth of yeast, inhibiting the fermentation.

(iii) pH

pH of the fermentation medium is adjusted to 4.8 to 5. The higher pH increases chances of contamination, where as very low pH values inhibit the yield of ethyl alcohol.

(iv) Temperature

Usually, the temperature range of 70^{O} to 80^{O}F is preferred. As there is heat evolution during fermentation, the temperature in the fermentation tank rises gradually and is controlled by means of

cooling coils. Temperature can also be controlled by cold-water spray around the fermentation tank. High temperatures favours the growth of bacteria but causes loss of ethyl alcohol due to evaporation.

(v) Time

Fermentation starts within a few hours after the addition of yeast. The process becomes very rapid after 24 hours to complete the fermentation. Duration for ethyl alcohol fermentation is usually 30 to 72 hours when the specific gravity of the fermented liquid becomes constant.

E. Production types

There are two types of fermentations for alcohol production using microorganisms.

(i) Batch fermentation

(ii) Continuous fermentation

(i) Batch fermentation

If the fermentation is in a tank which filled with media and inoculated with microorganisms. This tank has no continuous supply for substituting the depleted requirements during fermentation in the fermentor. The total fermentation time varies between 48 and 72 hr, and final alcohol concentrations of 6-8% can be obtained. The proteinaceons residue which is a valuable feed product is economically important to the operation of the process.

Batch fermentations are carried out without the need for establishing pure culture conditions that is without the need for complete sterilization. This fermentation inhibits the growth of the other microbes by depleting the available nutrients, by a lowering of the pH and most importantly by the formation of ethanol.

(ii) Continuous fermentation

Most of the experimental work has been carried out with homogenous fermentations with a single stirred fermentor. Fresh medium is continuously pumped into the fermentor and an equal

volume of the fermentor liquid is continuously pumped out for recovery of ethanol and yeast. The rate at which medium is added is usually expressed as dilution rate. The dilution rate is the ratio of withdrawn liquid to the volume of the total liquid in the fermentor.

(F) Recovery

The fermented liquid is allowed to settle for a few hours and then distilled in analyser and rectifier columns to obtain ethyl alcohol. The highest concentration can be achieved by fractional distillation is the constant boiling mixture, which contains 95.6% ethyl alcohol and 4.4% water.

Summary

- Wort is the diluted molasses form, which is the mostly used medial source in Brewing industry.
- The carbonation of the brewing product is needed as this increases the stability and help in the production and retention of foam, and in preservation of beer.
- During ethanol production all the medium components must be maintained at the exact levels otherwise if more

concentration are used it effects the fermentation process and if low levels are used it effects the yield.

- The batch and continuous fermentation systems operation need to have experience in operating the system, as they need the sterilization conditions and continuous monitoring of fermentation.

Model Questions

1. What is brewing ?
2. How can you produce the important industrial solvent ethanol using microbial fermentation ?

Reference Books

1. Industrial microbiology – A.H. Patel
2. Industrial microbiology – L.E. Casida
3. Industrial microbiology – Prescott and Dunn's.
4. Fermentation technology_Stanbury

Author

R.Padmini
Dept. of Microbiology
SGHR & MCMR College
GUNTUR

Lesson 8.5.1

PRODUCTION OF ANTIBIOTICS

Objective:

In this lesson we will deal about the production of antibiotics, applications of antibiotics, physiology of antibiotic production and genetics of antibiotic production .

Structure of the lesson:

8.5.1.1. The microbial groups involved in production of antibiotics.

8.5.1.2. Applications of antibiotics.

8.5.1.3. Steps involved in production of antibiotics

(Physiology of antibiotic production)

(a) Secondary metabolism and its control

(b) Catabolite repression

(c) Nitrogen and phosphate repression

(d) Feedback regulation

(e) Effects of precursors

(f) Conditions for fermentation

8.5.1.4. Genetics of antibiotic production

(a) Stain improvement

(b) Culture preservation and propagation aseptically

(c) Scale up

(d) Fermentation

Reference Books

8.5.1. PRODUCTION OF ANTIBIOTICS:

I. Introduction

Secondary metabolism products which inhibit the growth of other microbial species, even at low levels, are called antibiotics.

In the area of industrial microbiology screening of efficient strain, various molecular biological techniques and other microbial research continues for the effective production of antibiotics. Among large number of known antibiotics, only 100 are produced commercially via fermentation.

Production of antibiotics can be semi synthetic or in few cases they are produced synthetically eg: in case of chloramphenicol, phosphonomycin and pyrolnitrin.

8.5.1.1. The Microbial groups involved in production of antibiotics

Bacteria, actinomycetes and fungi are found to produce antibiotics. In fungi, aspergillaceae and moniliales produce

important antibiotics. Only penicillins, cephalosporirn C, griciofulvin and fusidic acid are of clinical importance. In bacteria, many taxonomic groups produce antibiotics. Varied number of antibiotics is produced by the actinomycetes, of genus S*treptomyces.* Genus *Bacillus* can produce peptide antibiotics which are very important.

The significance of production of secondary metabolites by organism is not certainly clear.

1. Secondary metabolites are formed by only few organisms.
2. They are essential for growth and reproduction.
3. Their production can be depending on environmental conditions.
4. Besides five phases of metabolism [i.e. intermediary, regulatory, transport, differentiation and morphogenesis) secondary metabolism is considered a “playing field” for the evolution of further biochemical developments, without damaging the primary metabolism.

Antibiotic	Organism
1. β-lactum Atibiotics	
(a) Penicillins	Penicillium sp. Aspergillus sp.
(b) Cephalorpolins	Cephalorposium acremonium
(c) Clavulanic acid	S.clavuligerus
(d) Nocardins	Norandia uniformis
(e) Thienamycins	S. cattleya
2. Amini acid & peptide	
(a) Amphomycin	Streptomyces canus
(b) Bacitrain	Bacillus licheniformis
(c) Granicidin A	B.brevis
(d) Viomycin	S.floridae
(e) Nisin	Streptococcus cremories
3. Carbohydrate Antibiotics	
(a) Nojirimycin	Streptomyces reseochromogenes
(b) Vancomycin	S.orientalis
(c) Lincrnycin	S.lincolensis
(d) Meomycin	S.banbergiensis
3i. Aminoglycoride Antibiotics	
(a) Streptomycin	S.griseus
(b) Neomycin	S.fradias
(c) Kanrycin	S.kanamyceticus
(d) Gentamycin	Micromonorposa purpurea
4. Macrolytic Lactone Antibiotics	
(a) Carbomycin A	S.haletedii
(b) Erythromycin	S.erythreus
4ii. Polyene macrolides	

(a) Amphotericin B	S.molosus
(b) Hamycin	S.primprina

4iii. Macrotetrolides

(a) Tetranactin	S.flaveolus

4iv. Typical macrolides

(a) Averunectin	S.avermitilis

5. Tetracyclins & Anthrocyclins

7-chlorotetracyclin (Aureomycin)	S.aureofociens
Daunormycin (daumosubicin)	S.peucetius
Adria mycin (doxorubicin)	

6. Nucleaside Antibiotics

Puromycin	S.aboniger

7. Aromatic antibiotics

Chlosamphenicol	S.venezuelae, S.omiyaeneus etc. Streptamyces sp.
Griseofulvin	Penicillium grieeofulvum P.patulum, other P.sp.
Novoliocin	S.spheroids, other sp.

8. Other cermercially produced

Mitomycin	S.caespitosus
Lasaloside	S.lasaliensis

8.5.1.2. Applications of Antibiotics

1. In the treatment of tumors:

   Many classes of antibiotics, which belongs to peptide, glycoside, anthrocyclin, benzoquinone groups, are used in the treatment of certain kinds of tumors.

   Eg: Chromomycin A_3, Mitomycin C

2. Antibiotics used in plant pathology:

   Initially streptomycin is used against diseases caused by pseudomonas species and *Xanthomonas oryzae*.

   - Blasticidin from *S. griseochromogenes* is used as a rice fungicide on *Pyricularia oryzae* (Rice burn)

   - Cycloheximide acts as leaf fungicide on warm blooded animals.

   - Validomycin – fungicide against *Rhizoctonia solani* which causes leaf drop and stalk disease in rice .

3. Antibiotics are also used in veterinary medicine and as animal growth promoters. Animal feed with antibiotic additive

has shown acceleration weight gain due to the effect on microbial flora in the GIT. Most of the therapeutically important antibioitics like penicillins, tetracyclins, bacitracin, erythomycin were also used in the feed. But due to the development of resistance these are stopped. Some antibiotics like. Enduramycin, Siomycin, Tylosin are used in animal feed which are only for nutritional purse.

4. Food industries also use antibiotics for preservation of food. Which is been controlled by regulatory agencies.

   Eg: Pimaricin used as fungicide, applied to the surface of foods.

   -Tylosin is effective against bacillus spores

   -Nisin against clostridium.

   - Chlortetracyclins are used in fish, meat & poultry industries.

5. Some antibiotics like gentamicin are used in animal cell cultures to control contamination.

6. Antibiotics as selective inhibitors are also used in molecucular biology studies to understand cell functions, replication, transcription, translation, etc.

7. Many antibiotics are used therapeutically to cure human diseases.

-Penicillin is commonly given antibiotic against bacterial infections like “classical” pneumonia caused by streptococcus pneumoniae.

-Streptomycin an amino glycoside antibiotic is active against tuberculosis.

8.5.1.3 Steps Involved in Production of Antibiotics

Most antibiotics are still produced by fermentation or by the chemical modification of fermentation products. Therefore research is devoted mainly on improving the fermentation processes to have a significant beneficial impact.

Antibiotic production depends on the enzyme activity. Hence regulation of activity depends on understanding the physiology of the organism to maximize the production of antibiotics.

(a) Physiology of antibiotic production includes the following steps:

(a) Secondary Metabolism and its control

(b) Catabolic Repression

(c) Nitrogen and phosphate repression

(d) Feed back regulation

(e) Effects of precursors

(f) Conditions or fermentation

(a) Secondary metabolism and its control

Primary metabolites like ethanol and lysine for example are produced in the entire growth phase of a culture. Hence optimizing the conditions of growth is required and continuous processes are feasible in such cases. In contrast secondary metabolite production is complex and poorly understood. Hence designing continuous fermentation is difficult, as cultures do not produce

antibiotics unless they are entering or are already in a stationary phase.

Molecular genetic studies showed some regulatory mechanisms that operate antibiotic production in prokaryotes. In Bacillus the end growth phase requires differential transcription of various types of promoters by several different sigma subunits of RNA polymerase.

(b) Catabolite Repression

In the presence of good carbon source like glucose, E. coli cells stop synthesizing the enzymes for the degradation of the preferable carbon sources, such as lactose. Similarly soil organisms, producing antibiotics, need to produce antibiotics and kill off their competitors only in a nutritionally poor environment and catabolite repression is useful for this arrangement.

In order to overcome catabolite repression, carbon source must be added in a careful manner in small increments so that no large build-up of their compounds occurs. Mutants, which no longer effected by the presence of carbon source, can be constructed.

(c) Nitrogen and phosphate repression

Presence of excess nitrogen or phosphate in the fermentation medium decreases antibiotic production. This leads an ecological advantage similar to catabolite repression. Phosphates are found to inhibit the transcription of some of the genes of antibiotic synthesis. Hence the concentrations of nitrogen and phosphate have to be regulated.

(d) Feedback regulation

It is suspected that the antibiotics themselves, as end products exert negative – feedback regulation on their synthesis. If feed back inhibition plays a role in regulating the production of an antibiotic, it must be possible to increase its production by promoting excretion of the antibiotic and thus lowering its intracellular concentration.

(e) Effects of precursors

Primary metabolites acts as precursors for secondary metabolite production for example α-aminoadipic acid is an intermediate in lysine biosynthesis. Therefore high levels of lysine, inhibits the first enzyme of the pathway, which leads to shortage of

all intermediates, which in turn inhibit production of penicillin. In contrast excess lysine stimulates the production of cephamycin C in Streptomyces.

CYCLE.

(f) Conditions for fermentation

Most fermentation procedures run in two stages

(1) First stage: It starts from spores because antibiotic production is often unstable and can be lost if the stock is kept growing constantly through out culture. Hence organisms are grown under submerged conditions with sufficient aeration and nutrients so that they can attain maximal density in a short time.

(2) Second stage: In which culture reaches the stationary phase, stops growing and starts to produce antibiotics. Hence the concentrations of key nutrients (carbon, phosphate and nitrogen) are to be controlled carefully by continuous feed processes.

(3) **8.5.1.4 Genetics of Antibiotic Production**

The detailed knowledge of synthetic pathways, together with their relevant enzymes and genetic locations had a significant impact on the development of improved strains and optimization of productive fermentations.

(a) Strain improvement

Strain improvement is necessary as high concentrations of the antibiotics increase the volumetric productivity, increases the extraction efficiency, decreases the proportion of unwanted products, makes purification easier and thus reduce cost of the product.

The strain improvement programs involve forced creation of mutations in the DNA material of the microorganism either by UV or chemical mutagens like nitro guanidine.

Traditional method of strain improvement depends entirely on random mutagenesis and screening of high procedures, unwanted mutations may be introduced into the organism, which may result weakened strain. These problems are overcome in classical genetics where desirable mutant alleles of various genes that affect antitibiotic production are identified and recombined them into a single organism. This method is also replaced with back crossing of overproducing strains with parent to improve the vigour of the mutant strain. Though true sexual cycle is not found in penicillin a eukaryotic fungus, because of the identification of Para sexual cycle – resulting in the production of heterokaryons was and to improve the strain. Even strains of *Streptomyces* can also be improved by this proves.

Targeted mutagenesis is one of the classic approach in which mutations are introduced indiscriminately into all the genes. Then transductants that received the donor copy of a gene located next to the antibiotic synthesis gene complex are selected. This is a possible way to recover only those pieces of mutagenized DNA that contained the particular gene used for selection.

Grouping of genes involved in the production of a single antibiotic in closely linked clusters, several complete gene clusters have successfully been cloned into a single plasmid. This type of approach gave insights into the nature of antibiotic and helped to study polypeptide antibiotics like erythromycin A etc.

(b) Culture preservation and propagation aseptically

Attention has to be given to the correct preservation and consistant propagation of high producing strains. Repeated slant-to-slant transfer of high yielding strains can produce sub-populations with lower productivity and appearance of wild type morphology. Hence, stock cultures are maintained in liquid N_2 for a long time. There frozen cultures are further used to prepare working stock cultures.

New master cell lines are prepared though single cell or spore re-isolation and there are evaluated in shake flask and pilot fermentations to confirm superiority and stability before the culture is used in large scale.

Maintaining of aseptic conditions throughout the build-up of culture volumes is essential this is critical at the seed stage where

the cultures are growing fast and scheduled tank transfer occurs before full status of asepsis is known. Impurity profiling by gradient HPLC (high performance liquid chromatography) and MS (mass spectroscopy) is now standard practice in the evaluation of new strains, new media components and any engineering changes.

(c) Scale up

The conditions of shake flask are selected as close as possible to the stirred – tank large – scale fermentations. This may also have many compromises also like for example if the volume in shake – flask decrease by evaporation while in stirred tank volume may increase by sugar feed. Antifoaming agents are required in stirred tank where as in shake flask it is not needed.

Shake flask	Stirred – tank
1. Volume is less	Volume is more
2. Batch only	Batch & feed possible
3. Limited controls	Continuous controls : pH, Temp, dissolved oxygen, pressure
4. Slow metabolizing carbohydrates like lactose starch	Readily metabolized carbohydrates – glucose
5. Ambient pressures	Two atmospheric pressure possible

6. In processes sampling is difficult	In process sampling is easy & necessary
7. Solid growth of side walls	Very uniform growth
8. No control of dissolved oxygen	Control of dissolved oxygen

In scale-up interdisciplinary skills of the bio-engineers, microbiologists and biochemists can prove to be rewarding.

(d) Fermentation

Media for cell mass build-up are designed to provide fact growth with minimal changes in pH. The concentration of individual medium components need not be greater than 3.5%. Readily available carbon source like glucose or sucrose and soluble form of nitrogen like corn steep liquor or yeast extract are used. Calcium carbonate or phosphates can be added as buffers. Ammonium sulphate can be used to provide additional nitrogen.

Components of seed media

Glucose/starch/sucrose	3 – 5%
Corn steep liquor	3 – 5%
Calcium carbonates	0.5 – 1.0%

Phosphate	0.1 – 0.5%
Ammonium sulphate	0.1 – 1.0%
Urea	0.1 – 0.5%
Oil	0.1 – 0.5%

Production Medias have been developed and modified according to the need. There, media should be cost and performance effective. The most suitable media are there that are inexpensive raw materials and can maximize production.

Components of production media: - Shake flack

Glucose	0.2 – 1.0%
Starch	0.5 – 5.0%
Lactose	5.0 – 8.0%
Corn steep liquor	5.0 – 8.0%
Pharma media	1.0 – 5.0%
Soy flour	1.0 – 5.0%
Oil	0.5 – 5.0%

Ammonium Sulphate	0.5 – 1.0%
Calcium carbonate	0.5 – 1.0%
Phosphate	0.1 – 1.0%

In continuously producing large fermentation systems Ammonium sulphate, corn syrup and oil are fed continuously.

Final – stage fermentations are fed – batch, which can be controlled in a number of ways. Physically by temperature, aeration, agitation, pH. Bio chemically by addition of nutrients, precursors, inducers In some highly productive fermentation there is no clear separation of the primary (tropophasic) and secondary (idiophasic) stages, which mainly depends upon the state of the fermentation technology. In batch fermentation, clear primary and secondary stages can be seen, whereas in continuous system these differences are not always apparent.

Raw materials used in the initial batch have to provide immediate utilizable soluble nutrients as well as longer lasting and less soluble sources. Supplemented raw materials are soluble and

rapidly utilized. Suitable carbon sources sucrose, glucose or enzyme hydrolyzed corn syrups can be used. Use of soluble, readily utilizable carbohydrates like glucose can prevent catabolite repression, as the concentrations of the sugar will always be very low.

Addition of oil has the benefit of controlling excessive framing and air hold-up. Oil as triacylglycerol can be lard oil, soy oil, palm oil, peanut oil or rapeseed oil. Other antifoams such as silicone based products or polypropylene glycol can also be used. Antifoam addition should be available on an as – need basis and not simply adding into the start medium due to toxic nature of some antifoams. As protenacious nature of raw materials cause foaming automated feed – back control of antifoaming agent has to be provided. Foam control and minimum air hold-up is important for maximum volumetric output. The final out put expected is 80 – 85%.

The pH of the broth can be controlled to within 0.1 pH units by the addition of acid like sulfuric acid or base ammonia or caustic. The pH can also be controlled by using the cultures own

metabolism of sugars. Excess feeding of sugar will produce acetic acid, which lowers pH.

Dissolved O_2 levels are also critical for maintaining the maximum rate of antibiotic production and culture viability. This is the reason why a fine balance has to be established between aeration and the agitation necessary to distribute the O_2 into the liquid phase, and the back pressure in the tank to increase O_2 solubility, the volume expansion of the fermentation broth, and the compounding of several of there effects on the dissolved CO_2 levels. The dissolved oxygen levels has to be maintained higher than 20% saturation at 1.5-2 atmospheres pressure throughout the fermentation, at air flow rates high enough to sweep out as much CO_2 as possible.

By the use of multiple probes for pH and dissolved oxygen; pressure and temperature fermentation process can be monitored. Aseptic nature of the fermentation can also be assessed by microbial examination of samples. All the probes have to be checked for this correct performance.

Reference Books

1. Industrial microbiology – A.H. Patel

2. Industrial microbiology – L.E. Casida

3. Industrial microbiology – Prescott and Dunn's.

4. Fermentation technology_ Stan bury

Author

R.Padmini

Dept. of Microbilogy

SGHR & MCMR College

GUNTUR

Lesson 8.5.2

Production of Vaccines

Objective

8.5.2.1 Nature of Vaccine:

Living organisms (with attenuated virulence) as Vaccines

Genetically engineered viruses as vaccines

Dead organisms as vaccines

Bacterial toxins and toxoids as vaccines

Immunizing sera as vaccines

8.5.2.2 Vaccines of defined chemical nature

8.5.2.3 Preparations of active immunization products

Bacterial vaccine

Killed bacterial vaccine

Cholera vaccine, Pertusis vaccine

Plague vaccine

Typhoid vaccine

Living bacterial vaccine

BCG Toxoid or toxin preparation

Diphtheria vaccine

Formal toxoid (F.T.)

Alum precipitated toxoid (A.P.T)

Purified Toxoid Aluminium Phosphate (P.T.A.P)

Purified toxoid Aluminium hydroxide (P.T.A.H)

Toxoid Antitoxin Floccules (T.A.F)

Staphylococcus toxoid

Tetanus toxoid

Viral vaccine

Influenza vaccine

Poliomyelitis vaccine

Rabies vaccine

Smallpox vaccine

Yellow fever vaccine

Typhus vaccine

8.5.2.4 Subunit vaccines

Herpes simplex virus

INTRODUCTION :

Vaccination protects a recipient from pathogenic agents by establishing an immunological resistance to infection. An injected or oral vaccine induces the host to generate antibodies against the disease-causing organism; therefore, during future exposures, the infectious agent is inactivated (neutralized or killed), its proliferation is prevented, and the disease state is not established.

Just over 200 years ago, in 1796, Dr. Edward Jenner experimentally tested the folklore-based notion that human infection with a milk cattle disease called cowpox would protect infected individuals against the human disease smallpox. Smallpox is an extremely virulent disease with a high death rate; if

one survives, permanent disfigurement, mental derangement and blindness often follow. Jenner inoculated James Phipps, an 8-year-old with exudates from a cowpox pustule. In two separate trials after the initial vaccination, the body was fully protected against human smallpox. This country doctor had discovered the principle of vaccination.

Such as tuberculosis, smallpox, cholera, typhus, bubonic plague and poliomyelitis. With the advent of vaccination, antibiotics, and effective public health measures, these epidemic diseases been brought under control.

Modern vaccines typically consist of either a killed (inactivated) or a live, nonvirulent (attenuated) form of an infectious agent. Traditionally, the infectious agent is grown in culture, purified and either inactivated or attenuated without, of course, losing the ability to evoke an immune response that is effective against the virulent form of the infectious organism. Notwithstanding the considerable success that has been achieved in creating effective vaccines against diseases such as German measles, diphtheria, whooping cough, tetanus, smallpox and

poliomyelitis, there are a number of limitations to the current mode of vaccine production:

- Not all infectious agents can be grown in culture, and so no vaccines have been developed for a number of diseases.
- Production of animal and human viruses requires animal cell culture, which is expensive.
- Both the yield and rate of production of animal and human viruses in culture are often quite low, thereby making vaccine production costly.
- Extensive safety precautions are necessary to ensure that laboratory and production personnel are not exposed to a pathogenic agent.
- Batches of vaccine may not be killed or may be insufficiently attenuated during the production process, thereby introducing virulent organisms into the vaccine and inadvertently spreading the disease.
- Attenuated strains may revert a possibility that requires continual testing to ensure that the reacquisition of virulence has not occurred.

- Not all diseases (e.g. AIDS) are preventable through the use of traditional vaccines.

- Most current vaccines have a limited shelf life and often require refrigeration to maintain potency.

This requirement creates storage problems in countries with large, unelectrified rural areas.

Within the last decade, recombinant DNA technology has provided a means of creating a new generation of vaccines that overcome the drawbacks of traditional vaccines. The availability of gene cloning has enabled researchers to contemplate various novel strategies for vaccine development.

- Virulence genes could be deleted from an infectious agent that retains the ability to stimulate an immunological response. In this case, the genetically engineering agent could be used as a live vaccine without concern about reversion to virulence, because it is impossible for a whole gene to be required spontaneously during growth in pure culture.

- Live non-pathogenic carrier systems that carry discrete antigenic determinants of an unrelated pathogenic agent can be created. In this form, the carrier system facilitates the induction of a strong immunological response directed against the pathogenic agent.

- For infectious agents that cannot be maintained in culture, the genes for the proteins that have critical antigenic determinants can be isolated, cloned, and expressed in an alternative host system such as Escherichia coli or a mammalian cell line. These cloned gene proteins can be formulated into a “subunit” vaccine.

- There are some infectious agents that do not damage host cells directly; instead, the disease condition results when the host immune system attacks it own (infected) cells. For these diseases, it may be possible to create a targeted cell-specific killing system. Although not a true vaccine, this type of system attacks only infected cells, thereby removing the source of the adverse immunological response. In these cases, the gene for a fusion protein is constructed. First, one

part of this fusion protein binds to an infected cell. Then, the other part kills the infected cell.

Because of less stringent regulatory requirements, the first vaccines that were produced by recombinant DNA techniques were for animal diseases such as foot-and-mouth disease, rabies, scours, and a diarrheal disease of piglets. In addition, many more animal vaccines are currently being developed. For human diseases, a large number of recombinant vaccines were to be made available by the year 2000.

8.5.2.1 Nature of vaccines

In a broader sense, the term vaccine is used for any biological material injected in the body to stimulate active immunity. Immunization of the body through vaccination is generally done for the prevention of a disease. The vaccine is administered in advance so as to give the body time to set active immunity, before invasion by the pathogen occurs. The objective of vaccination is to stimulate the production of antibodies without the person actually having to develop the disease. The vaccines may be of the following types:

Living organisms (with attenuated virulence) as Vaccines

Some vaccines involve use of organisms, whose virulence is greatly attenuated or diminished. These organisms are unable to produced disease but induce immunity when injected in the host. There are at least five different ways of producing attenuated strains of pathogens: (i) Many successive passages of pathogen in some animals other than the usual host of the pathogen or in cultured cells, (ii) Selection of mutants of low virulence, (iii) Treatment of pathogens with chemicals, (iv) Cultivation of pathogens under unfavorable conditions such as high temperature and (v) Deletion or insertion of genes leading to attenuation of pathogens as tried for vaccinia virus.

The concept of recombinant `vaccinia' virus as alive vaccine gained momentum in the 1990s due to its earlier effective use as a smallpox vaccine. Its host range makes it suitable for livestock also. Following attributes of Vaccinia made it attractive for vaccine development: (i) stimulation of humoral and cell mediated immunity, (ii) low cost, (iii) heat stability, (iv) simple method of administration, (v) visible proof of successful vaccination and (vi) long duration of effectiveness after a single dose.

Genetically engineered viruses as vaccines

Although in the past, attenuated strains of vaccinia virus were obtained by serial virus passage, genetic engineering of vaccinia has been used for this purpose in the 1980s and 1990s. Deletion of several genes decreases its virulence. Similarly insertion of some lymphokine genes also leads to decreased virulence. While reducing virulence or pathogenicity, immune response of the recombinant protein (coded by gene inserted in the virus for developing specific vaccine) must be increased.

More than one gene can also be stably integrated into attenuated vaccinia virus for developing recombinant virus to be used as a vaccine against multiple pathogens. A glycoprotein gene or rabies virus has been integrated and the resulting rabies virus vaccine has been field tested in Europe and U.S.A. giving promising results. Other vaccines against HIV are being developed using the gene for HIV-1 envelope glycoprotein. In future, the widespread use of Vaccinia as a live vaccine will depend on improving safety.

Dead organisms as vaccines

In some cases, the dead bodies of the pathogen, when infected into a host, stimulate the production of antibodies without

causing any infection. This method has been used in immunizing against typhoid fever, whooping cough, blackleg in cattle and a few other diseases.

Bacterial toxins and toxoids as vaccines

Exotoxins of most pathogenic bacteria are highly antigenic; they stimulate the production of antibodies in animal or human body. These exotoxins are highly unstable and they lose toxicity during storage and are converted gradually into toxoids. Toxoids retain the antigenic power but they are not toxic. Therefore, they can be used in producing immunity against corresponding toxins. The transformation of toxins into toxoids is accelerated by heat, formaldehyde and other chemicals. Alum precipitated toxoids are highly antigenic. Toxoids have been used to develop immunity against several diseases including diphtheria, tetanus, gingrene, etc.

Immunizing sera as vaccines

An immunizing serum is the actual blood serum of a person or animal and contains an immunizing material or antibody. It is generally obtained by injecting a toxoid into an animal (usually a horse), permitting establishment of active immunity in the animal,

and then withdrawing some of its blood. The corpuscles are removed from serum, which is then standardized as to the strength of antibodies and stored in sterile tubes or bottles.

Immunizing sera are used mostly for cure rather than prevention. They may be given for prevention after exposure has taken place, but their immunizing effects are too short-lived to give any benefit, if administered much in advance of the disease. As a curative agent, the action of immunizing serum is very rapid. Immunizing serum has been used in the cure of diphtheria and tetanus.

8.5.2.2 Vaccines of defined chemical nature

Since the chemical nature of many antigens is now known, it has been possible to produce these antigens either by biological or by chemical means. Perhaps the first defined molecular species to be used as a vaccine were the polysaccharide vaccines for Neisseria meningitides (meningitis) and Streptococcus pneumoniae (pneumonia). The pure polysaccharide preparations from the bacterial capsule could be used as an antigen in adults or children of over two years of age. Addition of some other chemicals or biological principles often increases the potency of the

polysaccharide antigen. For example, Haemophilus influenzae (influenza) B polysaccharide is very poor in inducing antibodies in the susceptible host on its own. However, its antigenic property is greatly enhanced if it is mixed up with diphtheria toxoid. Proteins and nucleic acids have also been identified as immunogenic molecules. An outer membrane protein-polysaccharide complex from meningococci bacteria in association with aluminium phosphate or hydroxide elicits immunogenic responses and hence the mixture can be used as a vaccine. Antigens produced using cloned genes, synthetic peptides and proteins along with certain adjuvants are also often used as vaccines. It has also been proposed that the nucleoprotein of influenza virus can be used as a universal vaccine for influenza.

The advent of recombinant DNA and monoclonal technologies coupled with the recent advances in immunology has opened a new era into the production of vaccines. It seems that isolated immunogens can now be produced in unlimited quantities and vaccine development can proceed without hindrance. Several vaccines based on the new technologies have been licensed for commercial production. Some of these include the following: (i) Yeast cells could be used for the production of vaccine for hepatitis

B virus based on cloning the DNA sequence for the antigen; (ii) Yeast cells could be used for the production of vaccine for hepatitis B virus based on cloning the DNA sequence for the antigen; (iii) Escherichia coli vaccines could be produced for pigs and carried extra plasmids encoding the antigen for K88 strain E.coli; (iv) Monoclonal antibody against K99 strain of E.coli could be produced for protection of calves.

8.5.2.3 Preparations of active immunization products

Preparations containing antigens or antigenic products or mixtures of antigens that induce active immunity are called vaccines. Vaccines are of two types: toxoids and suspension of microorganisms. Many bacterial pathogens produce disease conditions because of the chemical substance they produce called toxin. The toxins synthesized outside the cell are called as exotoxin and those inside the body are called as endotoxin. The exotoxins are highly toxic and thus they are never administered as such but as toxoids. Toxoids are preparations in which toxicity of the toxin is destroyed or reduced to a safe-level without altering the antigenic property of toxin.

When immunization against whole organisms is required, the vaccines are administered as suspensions of microorganisms. In this type of vaccines, either live or killed suspensions of microorganisms are used. Immunization with live antigens is generally more effective than dead. Many times it is possible to isolate a strain of a pathogens (mutants) that has lost its virulence but not antigenic property, such type is called attenuated strains.

The various types of vaccines used for active immunization are as follows:

1. **Bacterial vaccine containing suspension of living bacteria or killed bacteria.**
2. **Viral vaccines and**
3. **Rickettssial vaccines.**

Bacterial vaccine

This type of preparation is a living or killed suspension of bacteria. Bacterial vaccines are simple or mixed and univalent or polyvalent. Simple vaccines are prepared from one species only. Mixed vaccines are mixtures of two or more species. Univalent vaccines are those that contain single strain of one species and

polyvalent vaccines are those that contain more than one strain of one species.

Killed bacterial vaccine

Cholera vaccine

Pure culture of suitable strains of V.cholera is grown on solid media at 37^{o}C for some days. The strains used are carefully selected for retention of antigenic property. After the incubation period, the organisms are harvested in saline solution, the suspension is centrifuged, the supernatant fluid is discarded and cells are resuspended in isotonic saline. The cells are killed by minimal heat treatment i.e., 1 hr at 56^{o}C or by treatment with chemical bactericide. The approximate number of dead organisms in the suspension is determined by a total count method and the suspension is then diluted with bacteriostatic saline to contain standard number of organisms. The final product is a polyvalent suspension of different strains. Official books such as Indian Pharmacopoeia (I.P) or British Pharmacopoeia (B.P) sets the standards for toxicity, sterility and requirements for storage and labeling.

Pertusis vaccine

Suitable strains of Haemophilus pertusis are grown on solid or liquid medium for 24 to 72 hours. The organisms are harvested in saline, the suspension is centrifuged, the supernatant fluid is discarded and the cells are resuspended in isotonic saline containing the bactericide thiomersalate. The suspension is stored in cold for several months to reduce its toxicity and then a final dilution is made with thiomersalate solution. The product can be univalent or polyvalent killed vaccine. The B.P. or I.P. sets the standards for toxicity, sterility and requirements for storage and labeling.

Plague vaccine

The preparation is a simple, killed vaccine of Pasteurella pestis. The organisms are grown on suitable medium and harvested when capsule production appears to be at maximum. The capsulated organisms are killed by harvesting them in formaldehyde – saline and kept at room temperature for 24 hrs before being diluted with phenol-saline to contain the standard number of microorganisms. The B.P. or I.P. sets the standards for toxicity, sterility and requirements for storage and labeling.

Typhoid vaccine

These are mixed, polyvalent, killed vaccines of Salmonella typhi and Salmonella paratyphi A and B. The harvested bacteria are killed either by heat treatment or by the action of a bactericide and the suspensions diluted to the standard and mixed.

Living bacterial vaccine

BCG

A pure culture of an authentic strain of attenuated Mycobacterium tuberculosis of Calmette and Guerin is grown on the suitable medium for not more than 14 days. The growth is harvested and suspended, at a suitable concentration, in a liquid medium, designed to preserve the antigenicity and viability of vaccine. It is issued in freeze-dried form and liquid form. B.P. or I.P. sets the standards for skin-sensitizing potency, toxicity, virulence and sterility and states requirements for storage and labeling.

Toxoid or toxin preparation

Diphtheria vaccine

A pure culture of Colnebacterium diphtheriae is grown in suitable fluid culture medium for several days and a filtrate is obtained from this, which contains the specific exotoxin. The toxin is converted to the non-toxic, yet antigenically active, toxoid form and the vaccine issued as following preparations.

Formal toxoid (F.T.)

A quantity of filtrate containing bacterial exotoxin is rendered non-toxic by adding a suitable concentration of formaldehyde solution and incubating the mixture at 37^{o}C for 4 weeks or until the toxicity is completely removed.

Alum precipitated toxoid (A.P.T)

The formal toxoid is precipitated with sufficient potash alum; the precipitate is collected, washed alternately with saline solution and phosphate buffer and resuspended in thiomersalate-saline.

Purified Toxoid Aluminium Phosphate (P.T.A.P)

Purified formal toxoid is absorbed onto a suspension of hydrated aluminium phosphate in saline. Thiomersalate is added as a bacteriostatic.

Purified toxoid aluminium hydroxide (P.T.A.H)

Purified formal toxoid is adsorbed onto a suspension of aluminium hydroxide in saline and thiomersalate is added.

Toxoid antitoxin floccules (T.A.F)

The toxicity of the filtrate containing exotoxin is reduced to a low value or completely destroyed, by the addition of formalin solution. A quantity of diphtheria antitoxin, which is about 80% of the toxoid, is added to the inactivated toxin. The mixture flocculates, and the reaction is allowed to proceed for few weeks before the floccules are separated, washed with water and resuspended in bacteriostatic-saline.

Staphylococcus toxoid

The filtrate from a culture of suitable toxigenic strain of S. aureus is prepared and the α-toxin is treated with formaldehyde solution and rest of the preparation method is same as diphtheria F.T.

Tetanus toxoid

The filtrate from the culture of Clostridium tetani is used for the preparation of F.T, A.P.T, P.T.T.P and P.T.A.H tetanus toxoid. The method of preparation is similar to diphtheria toxoid.

For the diphtheria toxoid, staphylococcus toxoid and tetanus toxoid preparation I.P or B.P sets the standards for potency, sterility, toxicity and requirement for storage and labeling.

Viral vaccine

Two types of viral vaccines are available: inactivated (killed) vaccines and attenuated (living) vaccines. Virus, being intracellular parasites, can grow only within other living cells. Thus, viruses are cultivated in free-living animals, fertile eggs or tissue cultures.

Influenza vaccine

The strains of influenza virus used in the preparation of the vaccine are those recommended by World Health Organization (W.H.O). Each strain is grown separately in the cells lining the allantoic cavity of 10-13 day old fertile chicken-embryo. After incubation at optimum temperature for 2-3 days, the allantoic fluids are collected, cooled and inactivated by the addition of

suitable concentration of formaldehyde solution. Inactivation is allowed to proceed for 2-3 days at cold temperature. Virus particles are collected by centrifugation and suspended in saline solution containing bactericide. Polyvalent vaccines are prepared by mixing different strains of the virus. B.P gives test for identification, toxicity, viral inactivation, haemagglutinating activity, sterility and requirements for storage and labeling.

Poliomyelitis vaccine

Strains of poliomyelitis virus types 1, 2 and 3 are grown separately in the cultures of healthy kidney tissue obtained from suitable species of monkey. After a suitable incubation period, the tissue cells lyse and liberate virus. The virus suspension is harvested and passed through bacteria proof filter. After harvesting, but before filtration, the virus suspension is tested for presence of M. tuberculosis, hepatitis B virus and L.C.M virus. The TCID of each suspension is determined and if satisfactory, suspension is inactivated by the addition of suitable concentration of formaldehyde solution. Inactivation is effected at 37^{o}C for usually not less than 12 days. A second filtration is made on the 6th day of inactivation to remove aggregates, which may protect the

virus. Inoculating them into tissue cultures performs safety test for inactivation. Free formaldehyde is removed from blended vaccine by the addition of sodium metabisulphite.

Rabies vaccine

Suitable animals are injected intra-cerebrally with fixed rabies viruses. The subsequently paralyzed animals are killed if they show typical symptoms of rabies lasting for 24 hours. The animal brains are removed and are suspended in the saline solution. Viruses are killed or attenuated by the treatment with phenol or other chemical substances. The product is diluted to contain a specified amount of brain material.

Smallpox vaccine

It is prepared by growing vaccine viruses on chorio-allantoic membrane of fertile chicken egg. After a suitable incubation time, the membranes are separated, chilled, grounded to uniform suspension with 50% glycerol and with or without addition of bactericide. Preparation is issued either as freeze-dried or liquid preparation.

Yellow fever vaccine

Fertile chicken eggs are incubated for 7 to 8 days and the embryos are injected with a strain of yellow fever viruses that are virulent for mice, but not for a man. After incubation for 3-4 days, the embryos are grounded and extracted with purified water. The resultant suspension is centrifuged and supernatant fluid is freeze-dried. The containers are filled with nitrogen before sealing.

Typhus vaccine

Fertile chicken eggs are incubated for 7 days and the yolk sacs are injected with virulent Rickettssia prowazeki. Following 1-2 weeks of incubation, the yolk sacs of the dead embryos are subjected to treatment to release rickettsia. The disintegrated material is suspended in saline solution and formaldehyde solution is added in a concentration suitable to inactivate the suspension. Fat is removed from the yolk sac suspension by purification with solvent ether or trichlorotrifluroethane. The aqueous middle layers of the resultant mixture constitute the vaccine.

8.5.2.4 Subunit vaccines

Vaccines generally consist of either killed or attenuated forms of the whole pathogenic agent. The antibodies elicited by these vaccines initiate and immune response to inactivate (neutrailize) pathogenic organisms by binding to proteins on the outer surface of the agent. So do vaccines need to contain the whole organism, or will specific portions of pathogenic organisms are sufficient. For disease-causing viruses, it has been shown that purified outer surface viral proteins, either capsid or envelope proteins, are alone sufficient for eliciting neutralizing antibodies I the host organism. Vaccines that use components of a pathogenic organism rather than the whole organism are called “subunit” vaccines; recombinant DNA technology is very well suited for developing new subunit vaccines.

Herpes simplex virus

Herpes simplex virus (HSV) has been implicated as a cancer-causing (oncogenic) agent – in addition to its more common roles in causing sexually transmitted disease, severe eye infections and encephalitis – so that prevention of HSV infection by vaccination with either be killed or attenuated virus may put the recipient at

risk for cancer. Thus, protection against HSV would be best achieved by a subunit vaccine, which would not be oncogenic.

The primary requirement for creating any subunit vaccine is identification of the component(s) of the infectious agent that elicits antibodies that react against the intact form of the infectious agent. The HSV type 1 (HSV-1) envelope glycoprotein D (gD) is such a component, because, after injection into mice, it elicits antibodies that neutralize intact HSV. The HSV-1 gD gene was isolated and then cloned into a mammalian expression vector and expressed in Chinese hamster ovary (CHO) cells, which, unlike the Escherichia coli system, allows foreign proteins to be properly glycosylated. The complete sequence of the gD gene encodes a protein that normally is bound to the mammalian host cell membrane. However, a membrane-bound protein is more difficult to purify than a soluble protein, and so the gD gene was modified by removing the nucleotides encoding the C-terminal transmembrane binding domain. The modified gene was then transformed into CHO cells, where the product was glycosylated and secreted into the external medium since it could not be incorporated into the cell membrane. In laboratory trials testing its neutralizing ability, the modified form of gD was effective against both HSV-1 and HSV-2.

Foot-and-Mouth Disease

Foot-and-mouth disease virus (FMDV) has a devastating impact on cattle and swine and is extremely virulent, but it has been possible to keep the negative effects of this virus to a minimum by using formalin-killed FMDV preparations as a vaccine. Approximately 1 billion doses of this killed-virus vaccine are used worldwide each year.

Research on FMDV found that the major antigenic determinant inducing neutralizing antibody is the capsid viral protein 1 (VP1). Although purified VP1 is a much less potent antigen than intact viral particles, it can still induce neutralizing antibodies by itself and therefore can protect animals from infection by FMDV. Thus, the gene for VP1 became a target for cloning.

Tuberculosis

Tuberculosis, one of the most important infectious diseases worldwide, is caused by the bacterium Mycobacterium tuberculosis. This bacterium can form lesions in any tissue or organ, which leads to cell death. The lungs are most commonly affected. In addition, patients suffer from fever and loss of body weight, and without treatment tuberculosis is often fatal. It is

estimated that approximately 2 billion people are currently infected with this organism and that nearly 3 million deaths a year result from these infections.

In an initial attempt to determine whether a safer and more effective subunit vaccine against tuberculosis might be developed, one group of researchers decided to first examine the immunoprotection that was provided by purified M. tuberculosis extracellular proteins. Following growth of the bacterium in liquid culture, six of the most abundant of the approximately 100 secreted proteins were purified. Each of these proteins was used separately and then in combination with the others to immunize guinea pigs. The immunized animals were then challenged with an aerosol containing approximately 200 cells of live M. tuberculosis-a large dose for these animals. The animals were observed for 9 to 10 weeks before their lungs and spleen were examined for the presence of disease-causing organisms. In these experiments, some of the purified protein combinations provided approximately the same level of protection against weight loss, death and infection of lungs and spleen as did the live BCG vaccine. A safe and efficacious subunit vaccine for the prevention of tuberculosis in human can be developed once it is determined whether the

protective M. tuberculosis proteins produced by recombinant DNA technology are as effective as secreted proteins.

Peptide vaccines

Short peptides that mimic epitopes (antigenic determinants) will be immunogenic and could be used as vaccines (peptide vaccines).

Chemically synthesized domains of FMDV VP1 were tested as potential peptide vaccines. Peptides corresponding to amino acids 141 to 160, 151 to 160 and 200 to 213 which are located near the C-terminal end of VP1, and amino acids 9 to 24, 17 to 32, and 25 to 41, which are located near the N-terminal end of VP1, were each bound to a separate inert carrier protein (key-hole limpet hemocycanin) – very small peptides are usually rapidly degraded unless they are bound to the surface of a larger carrier molecule – and injected into guinea pigs. A single inoculation with peptide 141 to 160 elicited sufficient antibody to protect the animal against subsequent challenges with FMDV. By contrast, inoculation with complete VP1 or peptides 9 to 24, 17 to 32 or 25 to 41 yielded a lower level of neutralizing antibodies.

In an additional experiment, a longer peptide consisting of amino acids 141 to 158 joined to amino acids 200 to 213 by two proline residues elicited high levels of neutralizing antibodies in guinea pigs, even when it was injected without any carrier protein. This “two-peptide” molecule was more effective than either of the single peptides alone and prevented FMDV proliferation in cattle as well as in guinea pigs.

There are certain limitations to using short peptides vaccines:

- To be effective, an epitope must consist of a short strech of contagious amino acids, which does not always occur naturally.
- The peptide must be able to assume the same conformation that the epitope has in the intact viral particle.
- A single epitope may not be sufficiently immunogenic.

In the future, synthetic peptide vaccines could become highly specific, relatively inexpensive, safe and effective alternatives to traditional vaccines.

Genetic manipulation may be used to construct modified organisms (bacteria or viruses) that are used as live recombinant

vaccines. These vaccines are either non-pathogenic organisms that have been engineered to carry and express antigenic determinants from a target pathogenic agent or engineered strains of pathogenic organisms in which the virulent genes have been modified or deleted. In these instances, as a part of a bacterium or a virus, the important antigenic determinants are presented to the immune system with a conformation that is very similar to the form of the antigen in the disease-causing organism. By contract, purified antigen alone often lacks the native conformation and elicits a weak immunological response.

8.5.2.5 Cholera

Cholera is a fast-acting intestinal disease characterized by fever, dehydration, abdominal pain and diarrhoea. It is transmitted by drinking water contaminated with faecal matter. In developing countries, the threat of cholera is a real and significant health concern whenever water purification and sewage disposal systems are inadequate.

The bacterium Vibrio cholerae, the causative agent of cholera, colonizes the small intestine and secretes large amounts of a hexameric enterotoxin, which is the actual pathogenic agent.

This protein consists of one subunit, the A subunit, that has ADP-ribosylation activity and stimulates adenylate cyclase, and five identical B subunits that bind specificially to an intestinal mucosal cell receptor. The A subunit has two functional domains: the A_1 peptide, which contains the toxic activity and the A_2 peptide, which joins the A subunit to the B subunits. The cholera vaccine that I currently used consists of phenol-killed V. cholerate. This vaccine generates only moderate protection, which normally lasts from about 3 to 6 months. However, other possible types of cholera vaccines have been examined.

A strain of V. cholerae in which part of the coding sequence for the A_1 peptide was deleted was created. This strain cannot produce enterotoxin; therefore; its is non-pathogenic and is a good candidate for a live vaccine.

In this experiment, a tetracycline resistance gene was incorporated into the A_1 peptide DNA sequence on the V.cholerae chromosome. This insertion inactivated the A_1 peptide activity and also made the strain resistant to tetracycline. Although the A_1 peptide sequence was disrupted, this strain is not acceptable as a vaccine because the inserted tetracycline resistance gene can excise

spontaneously, thereby restoring enterotoxin activity. Consequently, it was necessary to engineer a strain carrying a defective A_1 peptide sequence that could not revert.

1. A plasmid containing the cloned DNA segment for the A_1 peptide was digested with the restriction enzymes Clal and Xbal, each of which cut only within the A_1 peptide-coding sequence of the insert.

2. To recircularize the plasmid, an Xbal linker was added to the Clal site and then cut with Xbal.

3. T4 DNA ligase was used to join the plasmid at the Xbal sites, thereby deleting a 550-base-pair (bp) segment from the middle of the A_1 peptide – coding region. This deletion removed 183 of the 94 amino acids of the A_1 peptide.

4. Then, by conjugation, the plasmid containing the deleted A_1 peptide-coding sequence was transferred into the V.cholerae strain carrying the tetracycline resistance gene within its A_1 peptide DNA sequence.

5. Recombination can occur between the remaining A_1 coding sequence on the plasmid and the Test gene-disrupted A_1

peptide gene on the chromosome. Such a double-crossover event replaces the chromosomal A_1 peptide-coding sequence with the homologous segment on the plasmid carrying the deletion.

6. After growth for a number of generations, the extra chromosomal plasmid, which is unstable in V. cholerae, was spontaneously lost.

7. Cells with an integrated defective A_1 peptide were selected on the basis of their tetracycline sensitivity. The desired cells no longer had the tetracycline resistance gene but carried the deleted A_1 peptide sequence.

A stable strain with a deleted A_1 peptide sequence was selected in this way. This strain did not produce active enterotoxin but nevertheless retained all the other biochemical features of the pathogenic form of V. cholerae. This strain is currently being evaluated in clinical trials to test its effectiveness as a cholera vaccine. The results so far have been equivocal. While this vaccine conferred nearly 90% protection against diarrhoeal disease in volunteers, it induced side effects in some of those who were tested.

It is possible that this strain will require modification at an another chromosomal locus before it can be used as a vaccine.

8.5.2.6 Salmonella species

It is assumed that a "doubly deleted" strain would have a limited ability to proliferate after its injection as a vaccine, thereby curtailing its pathogenicity while allowing it to stimulate an immunological response.

Strains of the Salmonella genes cause enteric fever, infant death, typhoid fever and food poisoning. Therefore, an effective vaccine against these organisms is needed. Deletions in the aro genes, which encode enzymes involved in the biosynthesis of aromatic compounds, and in the pur genes, which encode enzymes involved in purine metabolism, have been used to attenuate various Salmonella strains. These doubly deleted strains, which can be grown on a complete and enriched medium, generally establish only low-level infections; overall their virulence is reduced by 10^6-fold or more. These attenuated Salmonella strains have been shown to be effective oral vaccines in mice, sheep, cattle, chicken and more recently, in humans.

8.5.2.7 Leishmania species

Attenuated strains of Leishmania are sometimes effective as vaccines; however, they often revert to virulence. Also, the attenuated parasite can persist for long periods in an infected but apparently asymptomatic individual. Such individuals can act as reservoirs for the parasite, which can be transferred to other people by an intermediate host. To overcome these problems, an attenuated strain of Leishmania that is unable to revert to virulence can be created by targeted deletion of an essential metabolic gene such as the one encoding dihydrofolate reductase-thymidylate synthase. In one of these attenuated strains. Leishmania major E10-5A3, the two-dihydrofolate reductase-thymidylate synthase genes that are present in wild-type strains are replaced with the genes encoding resistance to the antibiotics G-418 and hygromycin. For growth in culture, it is necessary to add thymidine to the medium used to propagate the attenuated, but not the wild-type strain. In addition, unlike the wild type, the attenuated strain is unable to replicate in macrophages in tissue culture unless thymidine is added to the growth medium. Importantly, the attenuated strain survives for only a few days when inoculated into mice; in that time, it does not cause any

disease. This period is sufficient to induce substantial immunity against leishmania lesions in BALA/c mice after administration of the wild-type parasite. Since the attenuated parasite did not establish a persistent infection or cause disease, even in the most susceptible strains of mice tested, it is considered to be a strong candidate vaccine. Following additional experiments with animals, it should be possible to test whether this attenuated parasite is effective as a vaccine in humans.

REFERENCE BOOKS

1. Industrial microbiology – A.H. Patel
2. Industrial microbiology – L.E. Casida
3. Industrial microbiology – Prescott and Dunn's.
4. Fermentation technology - Stan bury

Author

R.Padmini
Dept. of Microbilogy
SGHR & MCMR College
GUNTUR