UNIT 6

CELL CULTURE AND GENETIC MANIPULATION

CHAPTER 1

MICROBIAL CELL CULTURE AND ITS APPLICATIONS

6.1.1. Introduction

In your previous class you have studied that microorganisms like bacteria and fungi are cultured to obtain a number of products, which are beneficial for human beings. Some of the commonest examples are antibiotics, ethanol and enzymes. With the advent of biotechnology and genetic engineering techniques, microorganisms are also being used for the production of recombinant molecules such as insulin, hepatitis B vaccine, growth hormones and interferons. Microorganisms are also grown or cultured for production of biomass per se such as the single cell protein (SCP). Besides these, scientists culture microbes in their laboratories for research proposes. It is therefore very important to understand how microbes are cultured in the laboratory or in the industry for production of various important molecules.

A microbial culture works as a factory in which the metabolism of a microorganism is exploited to convert raw material into products. There is a maximal capacity for a single microbial cell to convert raw material into products in a given period of time. Hence, to produce a given amount of product within a given time, it is necessary to calculate the number of cells as well as doubling time of the particular bacterium used in the process. Like any other chemical reaction, which requires an appropriate temperature, pressure, pH and solvent, microbes also grow in an appropriate environment of pH, temperature, nutrients (provided by the growth medium) and the substrate (raw material), which is converted by the bacterium into the desired product.

This chapter deals with basic principles of culturing microbes in the laboratory, large-scale industrial productions, and their applications. An important point that should be kept in mind is that, the requirements for culturing microbes on a large or industrial scale are different from culturing them in a laboratory.

6.1.2. Microbial culture techniques

Nutrients for microbial culture:

All growing microorganisms require water, sources of energy, carbon, nitrogen, oxygen and mineral elements. For culturing microbes on a small scale such as in a laboratory, it is relatively simpler to devise nutritive medium using pure chemicals such that full composition of the medium is known. Such media are called **synthetic media**. Alternatively, we may use media commonly available in the market such as nutrient broth, typticase soya broth (TSB) or brain heart infusion (BHI) broth. These media contain highly complex components such as peptone,

beef extract, yeast extract or casein digest. Such media are called **semi-synthetic media**. These media are well suited for culturing most microbes in the laboratory. However, when one wants to culture microbes on a large scale for production of useful metabolites, one uses sources of nutrients, which are economical and available readily. In some cases, these nutrient sources or raw materials may need a pretreatment before use. Other considerations while selecting the growth medium are:

- Should yield maximum product or biomass per gram of the substrate used.
- Should be of consistent quality and available throughout the year.
- Cause minimum problems during preparation and sterilization.
- Give minimum problems during production process particularly aeration, agitation, extraction and purification of the product.

Carbon sources: Cereal grains, starch, cane molasses, glucose, sucrose and lactose are commonly used as carbon sources.

Nitrogen sources: Ammonium salts, urea, corn steep liquor or slaughterhouse waste are used as nitrogen sources.

The carbon and nitrogen sources mentioned above are cheap and easily available.

Trace elements: Trace elements like Fe, Cu, Zn, Mn, Mo are also needed in small quantities.

Growth factors: Some microorganisms cannot synthesize specific nutrients like some amino acids or some vitamins. Such growth factors are incorporated into the medium in adequate amounts as a pure compound or as a component of complex mixture.

Antifoaming agents: In most microbiological processes, foaming is a problem. Foaming may be due to the components of the culture medium or some molecules produced by the microbes. The most common cause of foaming is the presence of proteins in the culture medium. Excess foaming denatures proteins and provides hindrance to free diffusion of oxygen in the medium. Commonly used antifoams are fatty acids, such as olive oil or sunflower oil. Silicones are also used as antifoams.

Energy sources: The carbon sources such as carbohydrates, lipids and proteins in the culture medium are the major sources, which provide energy for the growth of microbes.

Water: Water is the major component of the culture media. When required for culturing microbes in the laboratory, single distilled or double distilled water would suffice. When assessing its suitability for large-scale microbial culture as in industry, we should consider the pH and dissolved salts. Water is also required for ancillary services such as heating, cooling and rinsing. Clean water of consistent composition is therefore a prerequisite for large-scale cultivation of microbes.

Culture Procedures

Sterilization procedures: When microbes are cultured in laboratory in 100 -1000 ml flasks, sterilization of the nutrient medium can be easily done in an autoclave at a pressure of 15 pounds psi (per square inch) for 15-20 minutes. However, when microbes are cultured on a large scale such as for fermentation processes in the industry, we need to sterilize thousands of liters of culture medium or the substrate. There is also the problem of sterilizing the huge culture vessel i.e. the fermentor. Steam is used almost universally for sterilization of the fermentation media. Medium may be sterilized *in situ* in the fermentor itself. But if the medium is sterilized in a separate vessel, the fermentor must be sterilized before sterile medium is added to it. The fermentor is sterilized by passing steam through its jacket or coils. The steam is also sparged into the vessel through all entries allowing it to exit slowly from the air outlet. Steam pressure is held at 15 lbs psi in the vessel for 20 minutes.

The air used in the fermentation process should also be sterilized. This is done by filter sterilization.

Aeration and mixing: When microbes are cultivated in the laboratory, aeration and mixing can be easily achieved by putting the flasks on shakers (shake culture). This may be further augmented by the use of baffle flasks **(Fig. 1)**. In large-scale bioreactors however, transfer of oxygen to microorganisms is particularly difficult because the microorganisms must be well mixed and the oxygen dispersed to achieve relatively uniform concentration. Many fermentor designs have mechanical stirrers to mix the medium, baffles to increase turbulence, which ensure adequate mixing. Forced aeration also provides mixing and the needed oxygen.

Equipment for microbial culture

In the laboratory, microbes can be cultured in test tubes or ordinary Erlenmeyer flasks. Such cultures are usually carried out in 100 -1000 ml volumes. Growth of microbes in the laboratory can be augmented by simple improvements in the design of the flasks or by the use of shakers.

Baffle flask: One of the simplest ways is to produce a V- shaped notch or indentation in the sides of the flask. Such flasks are called baffle flasks **(Fig. 1)**. This improves the growth of the microbes by improving the efficiency of oxygen transfer due to increased turbulence of the agitated culture medium.

Fig. 1. A Baffle flask.

Shakers: Continuous agitation of the culture medium also greatly improves the efficiency of the oxygen transfer and this improves the growth of the microbes. In the laboratory, this is done by the use of shakers **(Fig. 2)**. Shakers may be end-to-end type or rotatory type. These may be designed for use at the ambient temperature or in a controlled temperature environment (incubator shaker).

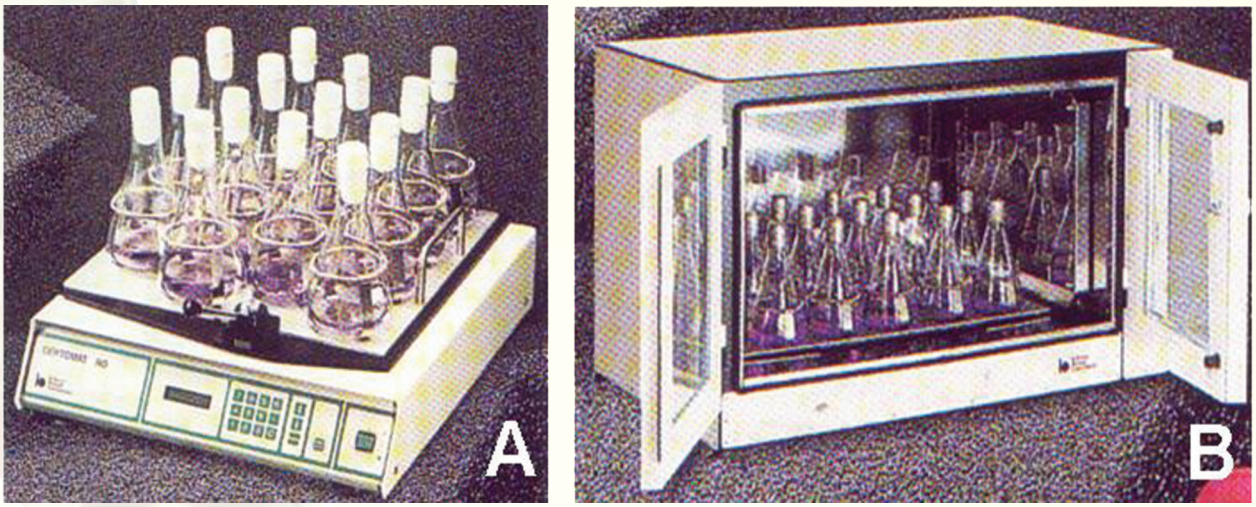

Fig. 2. (A) Shaker; (B) Incubator Shaker.

Fermentors (bioreactors): These are vessels, which are used for large-scale growth of microorganisms under a controlled environment. You have read about the basic design of a fermentor in your previous class in Unit I. It is a closed vessel with adequate arrangement for aeration, agitation, temperature and pH control, and drain or overflow vent to remove the cultured microbes along with their products. The vessel is capable of being operated aseptically for a number of days. These are also equipped with sampling ports, which allow withdrawal of the fermentation broth at regular intervals while the fermentation is in progress.

There are four main types of fermentor or bioreactor designs. The most common of these is a stirred tank bioreactor in which the culture medium is stirred with an impeller. In the bubble column reactor, the air is forced through a bottom sparger that creates enough agitation to ensure proper aeration. Basic features of a fermentor are shown in **Fig. 3.**

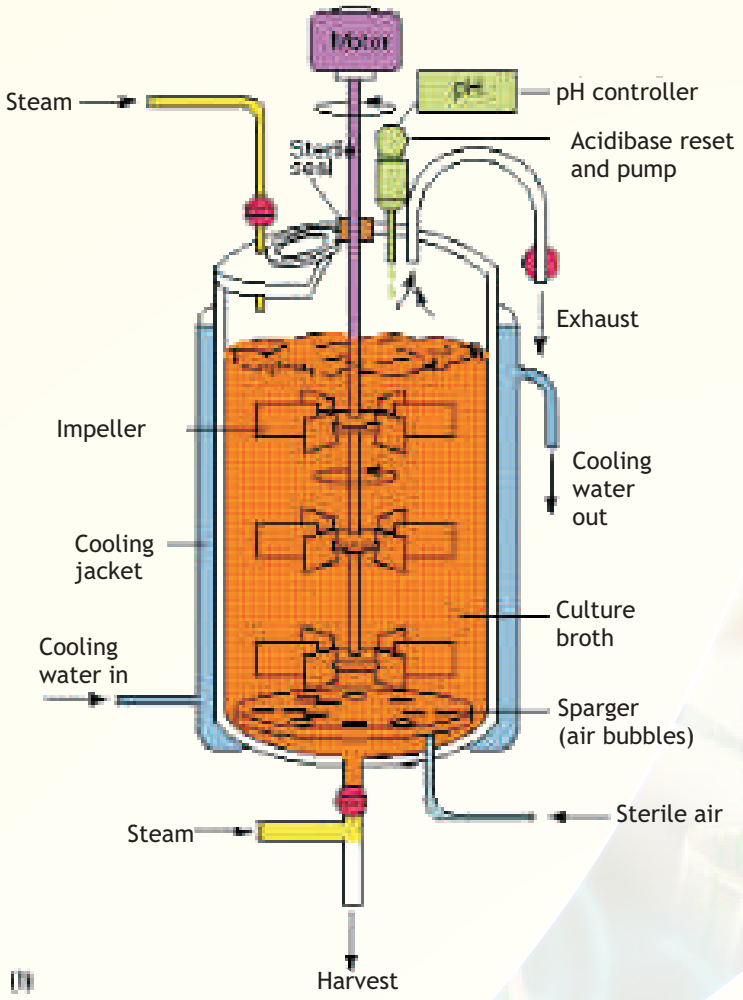

Fig. 3. Basic features of a fermentor (Bioreactor).

It should be noted that a high concentration of the microbial cells, as is achieved in a fermentor, could rapidly deplete the soluble oxygen in the medium, creating anaerobic conditions that may not be favorable to the growth of microorganisms and/or production of the desired products. So, forced aeration is done. The rapid growth of the microorganisms in the fermentor quickly alters the pH of the medium. So pH of the growing culture is continuously monitored and acid or alkali is added as required.

However, you should not harbor any notion that fermentors are used only for large-scale growth of microbes on an industrial scale **(Fig. 4)**. Small scale fermentors of capacity 10 -100 liters are also used in research laboratories These are used by the scientists in research, to optimize various parameters for the growth of microbes. The laboratory scale fermentors are also used by scientists, to produce enough quantities of metabolites from microbes for research purposes **(Fig. 4)**.

Fig. 4. (A) Laboratory-scale fermentor; (B) Industrial-scale fermentor.

Types of microbial culture

Batch culture: Batch culture is a closed culture system, which contains an initial limited amount of nutrients. After the medium is inoculated with the bacterial inoculum, the organism will grow and show usual growth phases viz. lag, log (exponential), stationary and decline. You have read about these in your class XI. Growth results in the consumption of nutrients and excretion of microbial products. At stationary phase, the growth is zero. This means, that in such a culture, growing cells are exposed to continually changing environment due to gradual consumption of nutrients and the accumulation of metabolites. The cell density [X], the concentration of substrate [S] and cell-specific substrate turnover rate [QS] during such a system are shown in **Fig.5**. Culturing microbes in the laboratory, in an ordinary flask, is nothing but an example of batch culture.

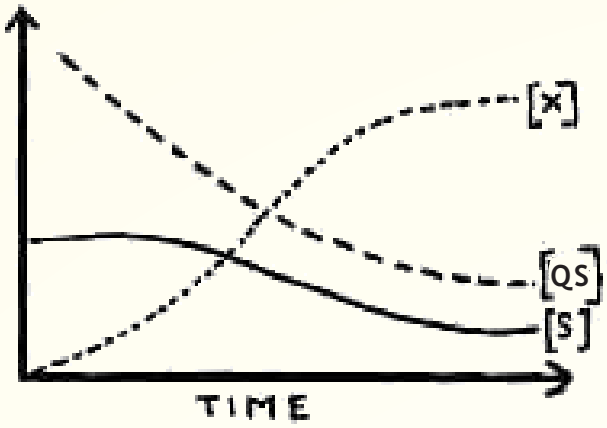

Fig. 5. Characteristic features of a batch culture.

Fed-batch culture: If a batch culture described above is continuously or sequentially fed with fresh medium without removing the growing culture, it is called fed-batch culture. In this system, substrate concentration remains constant and the cell density keeps increasing over time **(Fig. 6)**. This also means that over the period of time, the volume in the culture vessel goes on increasing.

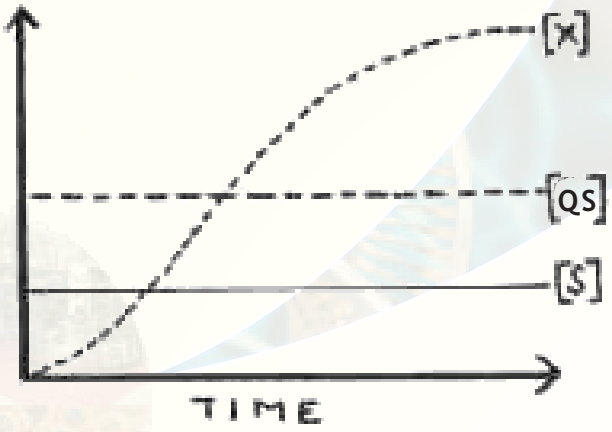

Fig. 6. Characteristic features of a fed-batch culture.

Continuous culture: This is a very interesting way of getting a continuous supply of microbial growth and/or products. The growth medium is designed in such a way that one of the nutrients is in limited quantity. Thus, during the exponential growth, as this nutrient is exhausted the growth will stop. However, just before the nutrient is fully exhausted, fresh medium containing the limited nutrient is added. This is repeated every time the limited nutrient is about to exhaust. This system is also fitted with an overflow device. This means that the added volume displaces out an

equal volume of culture from culture vessel. That is, formation of new biomass by the culture is balanced by the loss of culture from the vessel. In continuous culture, cells can be grown at a particular growth rate for an extended period of time. Most of the time, the chemical environment inside a continuous culture is constant. In a **chemostat**, constant chemical environment is maintained whereas in a **turbidostat** constant cell concentration is maintained. If medium is fed to such a culture at a suitable rate, a **steady state** is achieved eventually. At steady state, the cell growth and substrate consumption takes place at a fixed rate **(Fig. 7)**. Growth rate of cells remains constant during steady state operation. This means that at steady state, the concentration of cells, metabolites and other nutrients inside the reactor remain constant. That is, formation of new biomass by the culture is balanced by the loss of the culture from the vessel. Continuous culture is most suitable for production of biomass or metabolites. Continuous culture is widely used for production of both microbial biomass as well as their metabolites.

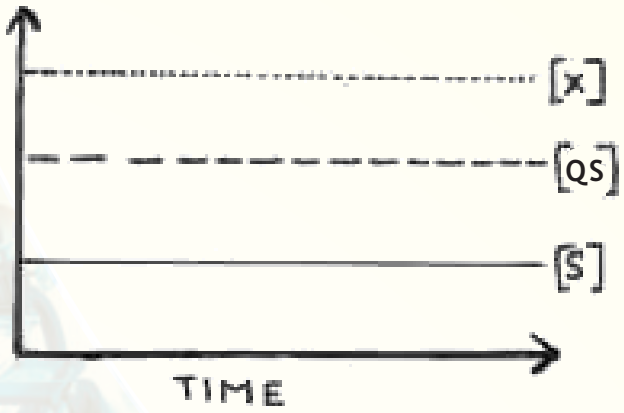

Fig. 7. Characteristic features of a continuous culture at steady state.

6.1.3. Measurement and kinetics of microbial growth

A clear understanding of microbial growth is necessary for proper utilization of biological processes for production of metabolites. In this section, the methods used for the measurement and quantitative evaluation of microbial growth will be discussed. There are four general patterns of microbial growth exemplified by bacteria, yeast, mold and viruses. All these microorganisms grow in different ways: bacteria grows by binary fission, yeast divide by budding, fungi divide by chain elongation and branching whereas viruses normally do not follow a regular growth pattern as they grow intracellularly in host cells.

Measurement of microbial growth

Microbial growth is defined as an orderly increase in all chemical components in the presence of suitable medium and environment. During the period of balanced growth, doubling of biomass is accompanied by doubling of other measurable properties of the microbe such as protein, DNA, RNA or intracellular water. In general, measurement of cell mass or cell number is used for

quantitative measurement of cell growth. The parameter that characterises microbial growth is the doubling time. It is the time required for the cell mass or number to double its original value during the balanced growth (i.e., log phase) of the organism.

Measurement of cell mass or number is one of the easiest ways to measure microbial growth.

(1) It is carried out by measuring the dry weight of the cell material in a fixed volume of the culture by removing the cells from the medium, and drying them till constant weight is obtained. For *E. coli*, the bacterium that is extensively used in laboratories, the dry cell weight of 1 billion cells is approximately 150 mg.

(2) Cell growth is also measured by measuring the absorbance of cell suspensions in a spectrophotometer. This principle is based on the fact that small molecules scatter light proportionate to their concentration. When light passes through a suspension of bacteria, there is a reduction in light transmitted as a consequence of scattering. Thus, with different cell concentrations, the absorbance at a particular wavelength will be proportional to the cell concentration. If you have a standard graph, plotted with absorbance versus cell concentration, the cell concentration of the unknown microbial sample can be calculated by measuring the absorbance at the same wavelength.

Apart from the methods described above, other methods are also used for measuring cell growth. Some of these are: measurement of wet weight of cells, turbidity measurements, ATP measurement, viable plate count **(colony forming units or cfu)** and use of Coulter counter. Coulter counter is an electronic instrument and is used for direct counting of microbial cells in suspension. In Coulter counter, the cells are made to pass in a single file through electrical field and the electrical impedance thus generated is recorded.

Growth kinetics and specific growth rate

In order to understand microbial growth kinetics, we shall take bacterial **binary fission** as the example where each cell division produces two identical daughter cells. The time taken for the cell to divide is called **generation time**. The generation time is also known as **doubling time (t_d)**, because the population of cells doubles over this time. For example, one cell of *E.coli* put in a nutritive medium will divide every 20 minutes. After one hour of growth (i.e. after three generations), one cell will have become eight (1 to 2, 2 to 4, 4 to 8). Because cell number doubles with each division, the increase in cell number over time is **exponential** or **logarithmic**.

A typical bacterial growth curve is depicted in **Fig. 8,** about which you have already learnt in Unit I (Chapter 1) of the textbook for class XI. In the lag phase, the microbial population remains almost constant. It is however a period of intense metabolic activity as the bacteria adapt to the culture conditions. Once the cells have adapted to the culture, cell division occurs with increasing frequency until the maximum growth is reached. This is called the log phase. At this

point **exponential growth** occurs and cell biomass or cell number increases at a constant rate.

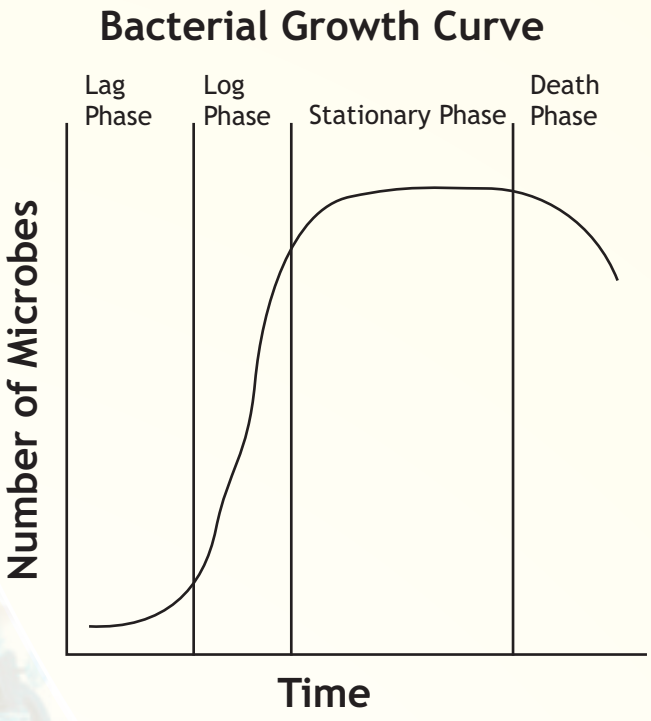

Fig. 8. A typical bacterial growth curve.

Mathematically, exponential growth can be described by two methods:

One related by biomass (X) and the other by cell numbers (N).

Let us first consider the exponential growth as related to biomass.

Balanced growth is like a chemical reaction where substrate is getting converted into product i.e. cell biomass in this case. Thus, growth behaves like an auto-catalytic reaction and the rate of growth will be proportional to the cell biomass present at that time. This can be described as follows:

rate of change of biomass $dX / dt = \mu X$ Eq.------- 1

where

X : concentration of biomass (g/L)

t : time (h)

μ : specific growth rate ($hour^{-1}$)

The equation 1 can be rearranged to estimate the specific growth rate (μ) as:

The specific growth rate $\mu = 1 / X \cdot dX / dt$ Eq.------- 2

Specific growth rate is an index of rate of growth of the cells in particular environment. In other words, specific growth rate is characteristic of the microorganism and is a function of the growth environment including temperature, pH, medium composition and levels of dissolved oxygen.

During the period of true exponential growth, equation 1 can be integrated to provide the following equation:

$X_t = X_0 e^{\mu t}$ Eq. ------- 3

where

X_t: biomass conc. after time t

X_0: biomass conc. at the start of the exponential growth

e : base of the natural logarithm

Taking natural logarithm:

$\ln X_t = \ln X_0 + \mu t$ Eq. -------- 4

This equation is of the form of straight-line y = mx + c.

Thus, for an exponentially growing culture, a plot of natural log of biomass concentration against time (i.e. a semilog plot) will give a straight line with the slope equal to μ.

or $\mu = (\ln X_t - \ln X_0) / t$

Please note that if $\log_{10}$ values were plotted instead of natural log the slope would be equal to μ/2.303.

$\mu = 2.303 (\text{Log } X_t - \text{Log} X_0) / t$ Eq. -------- 5

If we consider a situation where at time zero, the cell biomass is X_0, then after exponential growth equivalent to one doubling time (t_d), the microbial biomass will become $2 X_0$.

i.e. $X_t = 2X_0$ when $t = t_d$

Substituting these parameters in Equation 3 gives:

$2X_0 = X_0 e^{\mu t d}$ Eq. ---------- 6

Taking natural logarithm gives:

$$\ln 2X_0 = \ln X_0 + \mu t_d$$

or $$\mu t_d = \ln 2$$

Thus,

$$t_d = 0.693 / \mu \qquad \text{Eq. ---------- 7}$$

Where t_d is the doubling time of the culture.

By calculating μ from the graph or from Equation 5, and substituting it in Equation 7, we can calculate the doubling time of the culture. From the Equation 7, we can also say that doubling time and specific growth rates are inversely related. Higher the doubling time, lower will be the specific growth rate and vice versa. Thus, the microbial culture having high specific growth rate will have low doubling time.

Specific growth rate of microbial cells, in general, is calculated during the balanced growth of cells particularly during the exponential phase of growth. It signifies growth capacity of the culture in the particular environment. Specific growth rates of microbial cells change during different phases of batch growth, having a maximum value during the exponential phase. It is also affected by growth temperature, medium composition and other environmental parameters that affect the microbial growth.

Let us now study the second approach, which examines growth in relation to cell number, where number of the cells at the start of the exponential growth is N_0.

Let us take an example where $N_0 = 1$.

As the cell divides, we shall have:

No. of cell division	0	1	2	3	n
No. of cells	1	2	4	8	2^n
Mathematically	N_o	$N_o \times 2$ $N_o 2^1$	$N_o \times 2 \times 2$ $N_o 2^2$	$N_o \times 2 \times 2 \times 2$ $N_o 2^3$	- $N_o 2^n$

Thus, after a period of exponential growth time (t), the number of cells (Nt) will be given by:

$$N_t = N_0 2^n \qquad \text{Eq.-------- 8}$$

Where,

n = the number of the divisions

N_0 = the initial cell number

Taking logarithm

$$\text{Log } N_t = \text{Log } N_0 + n \text{ Log } 2$$

Thus, the number of divisions (n) that have taken place is given by:

$$n = (\text{Log } N_t - \text{Log } N_0) / \text{Log } 2$$

$$= (\text{Log } N_t - \text{Log } N_0) / 0.301$$

$$= 3.3 (\text{Log } N_t - \text{Log } N_0) \qquad \text{Eq.-------- --- 9}$$

The mean generation time or doubling time (t_d) i.e. the time taken to undergo single generation that doubles the population is:

t_d = Total growth time / number of divisions

$$= t / n \qquad \text{Eq.---------- 10}$$

The specific growth rate and doubling times of the organism decide the medium requirements and fermentation batch time for the production of biochemical molecules, and thus, are important parameters for large-scale production process.

Example: Calculate the generation time (doubling time or t_d) of a bacterial population in which the number of bacteria increases from 10^4/ml to 10^7/ml during four hours of exponential growth.

Answer: First calculate the number of divisions the population must have undergone to increase from 10^4 to 10^7 in 4 hours.

Using the equation 9: $n = 3.3 (\text{Log } 10^7 - \text{Log } 10^4)$

$= 3.3 (7 - 4)$

$= 10$

Using the equation 10: $t_d = 240$ minutes / 10

= 24 minutes

6.1.4. Scale-up of microbial processes

In your class XI text book (Chapter I: Introduction to Biotechnology), you were introduced to the early attempts on large-scale production of acetone from the bacterium *Clostridium acetobutylicum*. This chapter also brings out a number of products, which are produced from microbes for which a large-scale cultivation of microbes is necessary. You were also introduced to

the quantitative approach of producing just 1 liter of curd at home. Imagine the issues involved when one has to produce thousands of liters of curd in dairy industry.

Direct production of microbes on a large or commercial scale has the risk of not only large investments, but also producing products, which may not be of appropriate quality so that there are problems in their commercialisation. To avoid these risks, the manufacturers try to validate laboratory process on an intermediate scale before attempting commercial production. This step is carried out in a **pilot plant**, which is a mini version of the commercial plant.

Before setting up a commercial scale plant the results, which are derived from pilot plant, are theoretically extrapolated to the commercial scale in terms of equipment size (fermentor / bioreactor), utilities like steam, water, electricity, fittings etc, labour (technical and non-technical) and market surveys. All these data are compiled into a techno-economic report to assess the feasibility of the project. Such a report is also important for seeking financial assistance for the project from banks or other financial institutions.

The following example will give you an idea about the importance of scale-up calculations for the bioprocess industry in terms of reactor size:

Recombinant insulin is produced at 100 mg/L by *E. coli* at a cell concentration of 1 g/L. Calculate the volume of reactor (size of the fermentor) needed to produce 1 Kilogram of insulin in the following conditions:

(a) When the cell concentration is 1 g/L and insulin production is 100 mg /L.

(b) When the cell concentration is 50 g/L and insulin production is 100 mg /L.

(c) When the cell concentration is 50 g/L and insulin production is 500 mg /L.

Answer:

(a) Insulin production is 100 mg/L; so fermentor volume needed for 1 Kg of insulin is 1 Kg / 100mg = 1000, 000mg/100,g = 10,000mg = 10,000L.

So we need 10,000-litre fermentor to produce 1 Kilogram of insulin in one batch.

(b) In this case the cell concentration is increased to 50 g/L; so insulin production per liter will be 50 X 100 = 5000 mg = 5 g / L;

Thus, to produce 1 Kilogram of insulin we need 1 Kilogram / 5 g = 1000 g / 5g = 200 g. So, if the cell concentration is increased 50 times, we need 200-litre reactor to produce 1 Kilogram of insulin.

(c) In this case cell concentration is high (50 g/L) and the insulin concentration is also very high (500 mg/L). Thus insulin yield per liter of culture is 500 X 50 = 25, 000 mg / L which is 25 gram/L. Thus, to produce 1 Kilogram of insulin we need 1 Kilogram / 25

gram1000g/25g = 40g thus, we need a 40 liter reactor to produce 1 kilogram of insulin.

Please notice that the reactor volume decreases when we have high cell density or high concentration of insulin production as in case of (c). Such calculations are useful in deciding the reactor size required to produce a desired amount of the metabolite. Usually 30% extra space is recommended in the fermenter vessel for air, froth etc.

6.1.5. Isolation of microbial products

Once the fermentation is complete, it is necessary to recover the desired metabolite. Minimally, this will involve separation of the cells from the fermentation broth. But it may also include, purification of the metabolite with or without cell disruption; cell disruption will be necessary if the metabolite is intracellular. Such operations are referred to as **downstream processing**. The steps involved in isolation of the desired microbial product are: (1) separation of cells from the fermented broth, (2) cell disruption if the product is intracellular or concentration of the broth if the product is extracellular (3) initial purification of the metabolite, (4) metabolite-specific purification in which the metabolite of interest is purified to a high degree, and (5) polishing of the metabolite (bringing it to 98 -100% purity) where it is further concentrated and formulated for use.

Fig. 9 and 10 depict the steps involved in isolation of microbial products or metabolites of extracellular and intracellular origin respectively. The important steps are: separation of microbial cells (biomass / pellet) from the fermentation broth, concentration, metabolite-specific purification and final purification. Isolation of cells from the fermented broth is, in general, carried out by either centrifugation or ultra filtration. Some cells rapidly settle out of suspension once aeration and agitation of the fermented broth ceases. The settling of cells may also be assisted by the addition of certain flocculating agents. Where cell settling does not occur, cell removal can be effected by centrifugation. An alternative to centrifugation is ultra filtration. The term ultra filtration describes processes in which particles significantly greater in size than the solvent are retained when the solution is forced through a membrane of very fine pore size, usually less than 0.5 mm. Microbial cells can be concentrated using ultra filtration so that the fermented broth is separated from cells.

The clarified fermentation liquor will contain microbial metabolites and extra cellular enzymes. Several methods are available for recovery of metabolites such as precipitation, solvent extraction and ion exchange chromatography.

Different downstream operations are available for concentration as well as purification of the metabolite. But it is always advisable to use lesser number of steps to achieve desired purity of the metabolite or product. This is because, more the number of steps involved, more will be the cost of the production and lower would be the yield.

Most of the antibiotics are secreted into the medium, so their isolation mainly involves steps

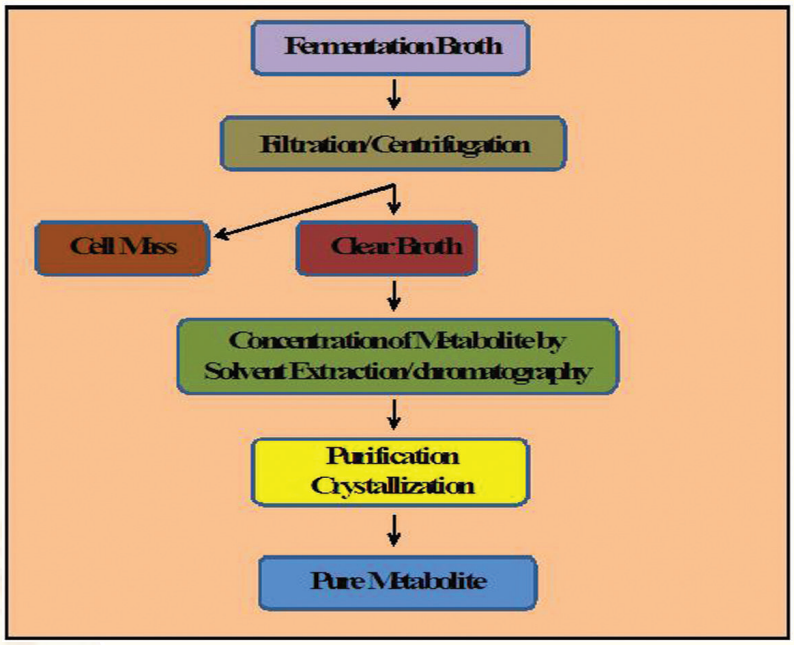

Fig. 9. Isolation of an extracellular microbial product (cell mass is discarded). Example: Streptomycin from *Streptomyces gresius*.

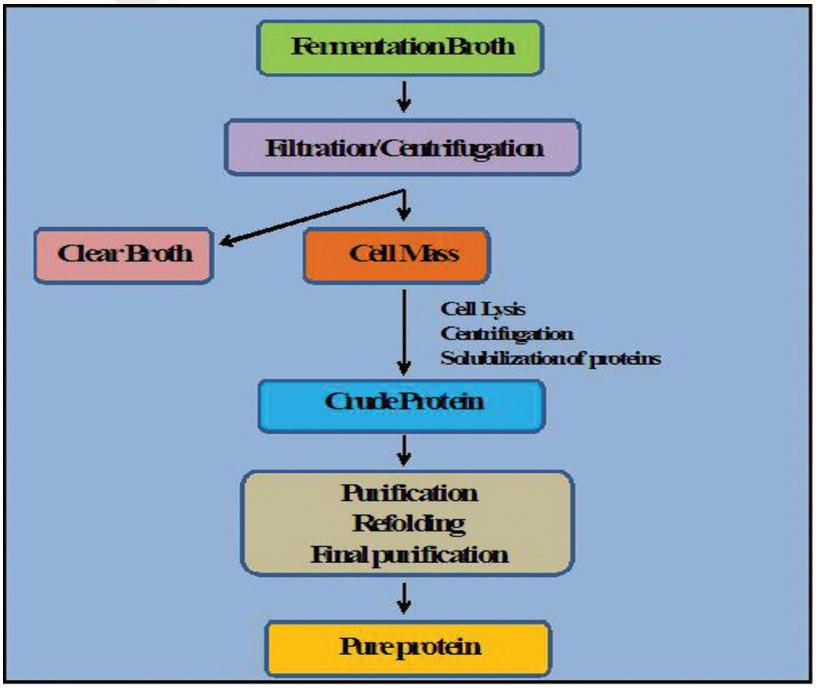

Fig. 10. Isolation of an intracellular microbial product (clear broth is discarded). Example: Recombinant insulin (Humulin®) from *E. coli*.

depicted in **Fig. 9**.

Most of the recombinant proteins expressed in *E. coli* accumulate intracellularly as protein aggregates. So their isolation and purification involve steps as described in **Fig. 10**. Once the pure metabolite is obtained, a stabilized formulation is made using several ingredients known as excipients.

6.1.6. Strain isolation, improvement and preservation

By this time, you must have realized that one of our major purposes to culture microbes is to produce useful products. But, do you think that all microbes produce useful or novel products? The answer is no. So, we have to search for those microbes, which produce substances of our interest. Where do we get these microorganisms?

As you know, microbes are wide spread in natural habitats especially in soil and in aquatic environments. They are also found in extreme environments namely arctic waters and hot springs. These natural habitats are our source of microbes. Once we have isolated microbes of desired interest, we can further improve their desired traits using a variety of methods.

Strain isolation:

The sample containing the microbes (e.g. soil) is put in a nutritive medium and allowed to grow in shake cultures. The growth conditions (e.g. temperature) or nutrients in the medium are provided such that these favour the growth of microbes of our interest. This is called **enrichment technique**. The enriched culture can further be sub-cultured by taking a small inoculum and putting it into fresh medium. In this way, the growth of the desired organisms improves successively. Further screening is done using a method where the organism will show its desired properties. For example, if we are looking for a microorganism, which produces an antibiotic, we may detect it by growing the culture on an agar plate in the presence of that bacterium against which antimicrobial activity is desired. Immunological methods are also available in which the microbes producing products are detected using **specific antibodies**. Molecular biology has made available a variety of **probes**, which enable the detection of organisms capable of producing specific products. Recently some of these methods have been adapted to robotic automation resulting in enormous throughput screening of microbes for newer / novel molecules.

Strain improvement:

Strain isolation procedure described above only identifies a strain, which has the capability or potential to produce a desired molecule. It does not ensure that it produces molecule in sufficient quantities to be economically viable. Techniques of classical genetics and genetic engineering are used to improve the desirable characteristics of the strain.

Mutation Selection: This is one of the oldest methods of strain improvement. The strain is exposed to chemical (e.g. nitrosoguanidine or NTG) or physical (e.g. UV rays) mutagens and the mutants having improved characteristics are selected. It is often necessary to carry out multiple successive mutations before we get the desired results. One of the classical examples of strain improvement using this methodology is the production of antibiotic penicillin. Several successive mutations were necessary to develop a strain of *Penicillium chrysogenum* capable of producing nearly 100 times the concentration of penicillin produced by the original strain *(Penicillium notatum)*, thus making production of penicillin commercially feasible.

Genetic Engineering Techniques: Until the recent breakthroughs in the techniques of genetic engineering, a bacterium could produce only substances coded for in its genome. Genetic engineering techniques about which you have learnt in the unit V (Chapter 1) allow totally new properties or capabilities to be added to the microorganisms giving rise to **recombinant strains.** Using these techniques, microorganisms may be manipulated to, synthesize or secrete enhanced quantities of biomolecules, facilitate production of novel compounds or allow utilization of cheaper substrates. Using these techniques, the microorganisms may also be utilized to produce plant, animal or human proteins. Some of the valuable human proteins which are being produced in microorganisms using this technology include recombinant human insulin (Humulin), hepatitis B surface antigen, human growth hormone and interferons. These proteins can now be produced in large quantities. Consequently the cost of the therapies which make use of these proteins viz. insulin (diabetes), hepatitis B surface antigen (vaccination against hepatitis B virus), human growth hormone (growth retardation) and interferons (immunotherapy) has been reduced considerably.

The tools, which are used for genetic engineering viz. the restriction enzymes, cloning and expression vectors and introduction of recombinant DNA into host cells, have been discussed fairly in detail in the unit V (Chapter 1). However there are many practical problems, which must be taken care of before a foreign **(heterologous)** gene may be expressed in a microorganism to make it commercially viable. For example, when a foreign gene is introduced into a host bacterium, it may not be expressed there. This problem is overcome by placing foreign gene under regulatory controls recognized by the host microorganism. To maximize production of foreign protein, the **expression vector** used is such that it replicates to **high copy number** and is stable. The foreign gene should ideally be linked to a strong promoter that has high affinity for RNA polymerase. The foreign gene may also be put under the control of a regulatory switch such that production of recombinant protein does not occur until required.

When a eukaryotic gene (e.g., plant, animal, human) is expressed in prokaryotic (bacterial) host, there are additional problems to be tackled. The non-coding region of eukaryotic gene must be excised. This requires use of reverse transcription of mRNA into cDNA. Additionally, the recombinant protein may not be secreted into the medium or its incorrect folding and accumulation intracellularly may generate **inclusion bodies**. All these problems make

downstream processing difficult and costly. Thus, an alternative would be to use a eukaryotic expression host. For this purpose, *Saccharomyces cerevisiae* has been quite popular because it is safe and scientists have long experience of using this yeast in industrial fermentations. Detailed information on biochemistry, physiology and genetics of this yeast is also known. Moreover, this yeast can be manipulated genetically rather easily. However, product yields are relatively low at 1-5% of the total protein. Other yeasts like *Pichia pastoris* has a number of advantages: it has strong inducible promoters; it is capable of making post-translational modifications similar to those performed by human cells; downstream processing is simpler as *Pichia* does not secrete its own proteins into the fermentation medium.

Metagenomics

In the last few years, another approach has been developed to identify and select microbial genes synthesizing novel molecules. This approach directly utilizes the large number of microbial genomes present in an environmental niche, for example in soil, in water such as ocean or in human gut. These genomes are contributed by both the culturable and the non-culturable variety of microbes and together constitute what has been termed as **metagenome**. The collective DNA is extracted from a sample of soil, water or any other environmental niche. It is subjected to restriction digestion using restriction endonucleses and the fragments are cloned as described in unit V (Chapter 1) of this book. The clones are then screened for presence of a variety of molecules. The clones expressing novel molecules or molecules with improved characteristics are used for large-scale production by fermentation techniques described in this chapter.

The metagenomic approach not only give the scientists an opportunity to cast a wider net on microbial resource present in the environment to fish out genes of their interest, it also gives them the opportunity to analyze the genomes of the microbes without culturing these in the laboratory. Thus, it is really a very useful approach to study those microbes, which are difficult to culture in the laboratory or have never been cultured in the laboratory as yet, and analyze these to see if they carry any genes, which may be exploited for human use. A typical procedure depicting metagenomic approach is shown in **Fig. 11**.

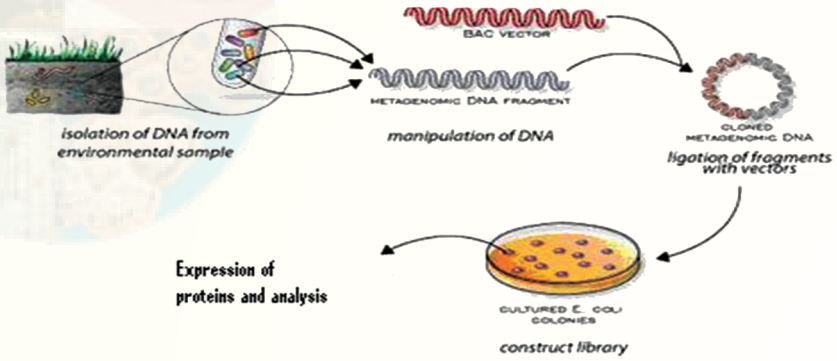

Fig. 11. A typical procedure depicting metagenomics approach .

Strain preservation:

Once a strain producing a novel or desired product has been obtained, it must be appropriately preserved for future use. If not done properly, the strain may be lost through loss of viability or even show decline in the production of the product for which it was isolated.

Storage on agar: Cultures are grown on agar slants or stabs & stored at 5 to -20 °C. These must be sub-cultured at approximately 6-month interval. The time of sub-culture may be extended to 1 year if cultures are covered with sterile mineral oil.

Storage in liquid nitrogen: The culture is grown and a cryoprotective agent like glycerol (10-30%) is added. These are dispensed in sealed ampoules & frozen in liquid nitrogen. (-176 to -196 °C).

Lyophilisation: Lyophilization or freeze-drying involves freezing of a culture followed by drying under vacuum. This results in sublimation of cell water. Lyophilised culture may remain viable for 5-10 years or more.

Culture Collections Centers

Cultures may be deposited to culture collection centers. These centers safely maintain cultures for years. The cultures are also made available to prospective investigators. With the advent of the modern biotechnology and the associated commercial and financial implications, the culture collection centers are governed by stringent rules & regulations to protect the intellectual property rights of the depositors. Some of the well-known culture collection centers of international repute are ATCC (American Type Culture Collection, USA), NCIB (National Collection of Industrial Bacteria, UK) and DSM (Deutsche Sammlung von Mikroorganismen and Zelkulturen, Germany). The National culture collection of India is called **MTCC (Microbial Type Culture a Collection and Gene Bank)** and is located at Institute of Microbial Technology, Chandigarh. Recently another National culture collection centre named **NBAIM (National Bureau of Agriculturally Important Microorganisms)** has been established in India at Mau in Uttar Pradesh (U.P.)

6.1.7. Applications of microbial culture technology

Microbial cultures have immense potential for production of very useful compounds. Once the microbial culture is established, depending on its metabolic activity it can be used for the production of numerous compounds. In general, microbial cultures can be exploited primarily in six different ways for the production of metabolites. They are listed below:

1. Production of whole microbial cells (for food, vaccines).
2. Production of primary metabolites (acids, alcohol).
3. Production of secondary metabolites (antibiotics).
4. Biotransformation reactions (enzymatic, steroid).
5. Exploitation of metabolism (microbial leaching, biodegradable waste treatment).
6. Synthesis of recombinant proteins (therapeutic proteins).

The most ancient use of microbial cultures is for the production of fermented foods such as curd and cheese where the whole bacteria are used as starter cultures. A complete list of such foods has been given in Unit I (Chapter 1) of the textbook for the class XI. The whole microorganisms are also used for preparations such as bacterial vaccines, e.g. vaccines for typhoid and tuberculosis. Single cell protein (SCP) is another example where the whole microorganisms are used a source of protein. Production of alcohol and acids are examples of primary metabolic products, whereas antibiotics are examples of secondary metabolites produced by different microorganisms. Microbial metabolism has also been exploited for the microbial production of vitamins. Extraction of metals from ores and treatment of liquid waste are also examples where microbial metabolism is used to convert unsuitable substrates to useful products.

Table 1. Some microbial species used for producing commercial products

Microorganisms	Products
Saccharomyces cerevisiae	Ethanol
Aspergillus niger	Citric Acid
Penicillium chrysogenum	Penicillin
Streptomyces griseus	Streptomycin
Corynebacterium glutamicum	L-Lysine
Propionibacterium shermanii	Vitamin B12
Aspergillus oryzae	Amylases
Leuconostoc mesenteroides	Dextran
Escherichia coli (via recombinant technology)	Insulin, growth hormones and interferons
Saccharomyces cerevisiae (via recombinant technology)	Hepatitis B surface antigen
Alcaligenes eutrophus	Poly 3-hydroxybutyrate (PHB)

One of the most important uses of microorganisms in recent years has been as hosts for production of recombinant proteins using genetic engineering techniques. Expression of human insulin in *Escherichia coli* and hepatitis B surface antigen in Yeast for making hepatitis B vaccine are the most notable examples of applications of microbes for human use. Some specific examples of products derived from microbial cultures have been given in **Table 1**.

6.1.8. Biosafety issues in Microbial Technology

Till date, all the products of the biotechnology industry have been found to be safe. Nevertheless, for all biotechnology processes, safety is of paramount importance. The main areas of consideration for safety aspects specific to biotechnology are:

- Potential of genetically modified organisms (GMO) or recombinant strains to infect humans, animals and plants to cause diseases.
- Toxicity and allergy associated with the use of microbially produced biomolecules especially the recombinant molecules.
- Other medically relevant implications like increasing the environmental pool of antibiotic resistant microorganisms or transfer of antibiotic resistant genes.
- Problems associated with the disposal of spent microbial biomass.
- Safety aspects associated with contamination, infection or mutation of process strains.

When the microbes are to be used in industrial processes, it is preferred to use only those microorganisms, which are included in the **'GRAS' (generally regarded as safe)** category. Also, when microbes are to be used as hosts for production of recombinant molecules, this should be carried out using GRAS organisms.

The main fear associated with the genetically engineered microorganisms (GMO) or recombinant strains is that they could escape from the laboratory into the environment with unpredictable and perhaps catastrophic consequences. It was believed that such released microorganisms could upset the balance of nature or that foreign DNA in the new microorganism could alter the metabolic activity of the host microbe in unpredictable and undesirable ways. Guidelines have been established to ensure safe working practices and levels of containment based on potential hazards. Many of the GRAS organisms found in nature have been genetically modified so that these may be used as host for production of recombinant biomolecules. At present, all new releases of genetically modified organisms are evaluated by expert committees on a case by case basis for various safety aspects before approval for their use is granted.

Besides these, whenever we are required to culture microbes whether for research or for industrial applications, we must follow **good microbiological practices (GMP)**. These include:

i. Persons must wash their hands with germicidal soap after handling viable microorganisms.

i. Eating, drinking, smoking etc. are not permitted in the working area.

ii. Food is to be stored outside the work area in cabinets / refrigerators designated for this purpose only.

iii. Mouth pipetting is strictly prohibited.

iv. All procedures are carried out in a way, so as to minimize splashes and generation of aerosols.

v. After work with viable microorganisms, work surfaces are decontaminated thoroughly.

vi. It is recommended that laboratory coats / aprons should be worn while working.

vii. All cultures, stocks or other waste are decontaminated and autoclaved before disposal.

Review Questions

1. While culturing microbes in the laboratory in a flask, what measures do you suggest to enhance their growth?
2. Differentiate between:
   a. Batch and Fed batch culture.
   b. Chemostat and turbidostat.
3. How is a continuous culture better than batch or fed batch cultures?
4. Explain what is meant by steady state in relation to the growth of microbial cultures?
5. How is the large quantity of air, required in industrial fermentors, sterilized?
6. What problems must be tackled while expressing a eukaryotic gene in a prokaryotic host?
7. How would you ensure that production of a recombinant molecule does not occur until required?
8. What problems make the downstream processing of recombinant proteins difficult and costly?
9. How is *Pichia pastoris* better expression host compared to *Saccharomyces cerevisiae*?

10. How metagenomic approach helps to identify newer/novel genes?
11. Suggest two methods of preserving microbial strains.
12. What is lyophilisation?
13. Explain what is meant by GMP, GRAS?
14. What are the functions of the microbial culture collections?
15. Enlist five good laboratory practices, which need to be followed while working with microbes.
16. Why lyophilised cultures of microbes remain viable for several years?

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CHAPTER

2

PLANT CELL CULTURE AND APPLICATIONS

6.2.1. Introduction

The plant cell culture is based on a unique property of the cell, i.e., totipotency which may be defined as the ability of a plant cell to regenerate into whole plant on a defined artificial nutrient medium under the suitable physical conditions in the laboratory. In fact, it was Schwann who first drew attention to the fact that a single cell has the capacity to both grow and divide in a self regulatory fashion and that a single cell is also totipotent. This was stated in the famous 'cell theory' which was proposed in the beginning of 19th century by Schleiden and Schwann. **Gottlieb Haberlandt,** who is regarded as **'Father of Plant Tissue Culture',** first attempted in 1902 to cultivate the mechanically isolated plant leaf cells on a simple nutrient medium. Though unsuccessful in achieving the growth and differentiation of the cultured cells, he has made several predictions in plant tissue culture like the concept of growth hormones, the use of embryo sac fluids, the cultivation of artificial embryos from somatic cells, etc. and indeed all of his predictions were found to be true as demonstrated by later researchers. In the first phase during the period 1902 - 1930s, attempts were made by several scientists to culture the isolated plant organs such as roots and shoot apices (organ culture). In the second phase during 1940s - 1970s, the extensive studies were undertaken to develop the suitable nutrient media to culture plant tissues, embryos, anthers, pollen, cells and protoplasts, and the regeneration of complete plants (in vitro morphogenesis) from cultured tissues and cells. Since 1980s, a new era has started involving the introduction of foreign genes into crop plants using cell and tissue culture systems to develop genetically modified (GM) or transgenic crops with improved characteristics, which may be responsible for the 'second green revolution.

6.2.2. Cell and Tissue Culture Techniques

Basic technique

The whole plants can be regenerated virtually from any plant part (referred to as explant) or **cells**. The basic technique of plant tissue culture **(Fig. 1)** involves the following steps:

1. Selection of suitable explants like shoot tip, leaf, cotyledon and hypocotyls.
2. Surface sterilization of the explants by disinfectants (e.g. sodium hypochlorite) and then washing the explants with sterile distilled water.
3. Inoculation (transfer) of the explants onto the suitable nutrient medium (shoot regeneration medium, which is sterilized by autoclaving or filter-sterilized to avoid

microbial contamination) in culture vessels under sterile conditions (i.e., in laminar flow cabinet).

4. Growing the cultures in the growth chamber or plant tissue culture room **(Fig. 2A)**, having the appropriate physical conditions [i.e., artificial light (16 h photoperiod), temperature (~26°C) and relative humidity (50-60%)].
5. Regeneration of shoots from cultured plant tissues and their elongation.
6. Rooting of regenerated shoots on rooting medium.
7. Transfer of plants to the transgenic green-house **(Fig. 2B)** or field conditions following the acclimatization (tissue hardening) of the regenerated plants.

Nutrient media

The *in vitro* culture of plant parts or cells require a variety of nutrients and suitable physical conditions, unlike the intact plants which can synthesize their own food and many other essential compounds needed for their growth and development using light, CO_2, water and minerals. The composition of plant tissue culture medium can vary, depending upon the type of plant tissues or cells that are used for culture.

The typical plant tissue culture nutrient medium consists of inorganic salts (both micro- and macro-elements), a carbon source (usually sucrose), vitamins (e.g. nicotinic acid, thiamine, pyridoxine and myo-inositol), amino acids (e.g., arginine) and growth regulators (e.g. auxins, cytokinins and gibbrellins). Other compounds like casein hydrolysate, coconut milk, malt extract, yeast extract, tomato juice, etc. may be added

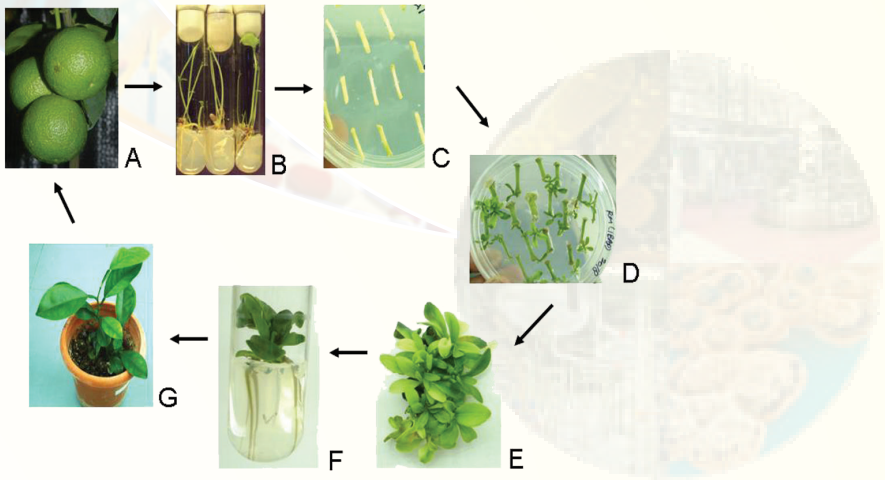


Fig. 1. Various steps involved in the regeneration of whole plants using tissue culture techniques. (A) Citrus plant with fruits, (B) Seedlings raised by germinating Citrus seeds on nutrient medium, (C) Transfer of epicotyl explants onto shoot regeneration medium, (D) Induction of shoots from explants, (E) Elongation of shoots, (F) Rooting of *in vitro* shoot, (G) Regenerated plant in a pot.

Fig. 2. Plant tissue culture room (A) and transgenic green-house (B).

for specific purposes. Plant hormones play a pivotal role in growth and differentiation of cultured cells and tissues. An optimum pH (usually 5.7) is also very important.

There are two kinds of media - **liquid and solid media**, and in the later case a gelling agent such as **agar** (a polysaccharide obtained from a red algae, *Gelidium amansii)* is added for solidification of the medium. There are several types of media like MS, LS, B5 and Nitsch's medium, and the choice of media is dictated by the plant species, variety or plant part. However, the most extensively used nutrient medium is MS medium, which was developed by Murashige and Skoog in 1962. The success of plant regeneration from cultured cells and tissues are largely governed by the composition of the culture medium. The sterilization of nutrient media by autoclaving or membrane filter-sterilization is utmost important to avoid microbial contamination.

Types of cultures

Organ culture: It deals with the culture of the isolated organs (like roots) under laboratory conditions *(in vitro)*, and different names are given depending upon the organ utilized for the culture. For instance, the culture of roots, endosperm, ovary, ovule and anther are called as i) Root culture, ii) Endosperm culture, iii) Ovary culture, iv) Ovule culture and v) Anther culture respectively.

Explant culture: The culture of plant parts **(explants)** is known as explant culture. The explants can be any part of the plant like the piece of stem, leaf, cotyledon, hypocotyls, etc. The explant cultures are generally used to induce callus or plant regeneration.

Callus culture: Callus refers to an unorganised mass of cells, which are generally parenchymatous in nature **(Fig. 3A)**. A variety of plant parts can be induced to show callusing response, but the response varies with the composition of culture media. Generally, auxins are added to culture medium for callus induction but the nature and quantity of auxin added depends on the nature and source of explant and its genotype besides other factors. Callus cultures can be maintained for prolonged period of time by repeated sub-culture. Callus cultures are used for

- plant regeneration.
- preparation of the single cell suspensions and protoplasts.
- genetic transformation studies.

Cell suspension cultures: Single cells can be isolated from either callus or any other part of the plant (e.g. leaf) and cultured in liquid medium. Both mechanical and enzymatic methods can be used for isolation of plant cells. Mechanical method involves grinding of the tissue to a fine suspension in a buffered medium followed by filtration/centrifugation to get rid of cell debris. The enzymatic method is based on the usage of enzymes (pectinase/macerozyme), which dissolve the middle lamella between the cells, i.e., the inter-cellular cement, to release single cells. Once the cells have been isolated, they may be cultured by **batch cultures** or **continuous cultures**, which have been discussed in detail (see the previous chapter). The cell suspension cultures can be used for:

- induction of somatic embryos/shoots.
- *in vitro* mutagenesis and mutant selection.
- genetic transformation.
- production of secondary metabolites.

Mass cell culture: It involves the large-scale culture of cells in specially designed 'plant bioreactors', which essentially do not have a stirrer (as plant cells are shear sensitive). In place of stirrer, gas is gently bubbled, which provides stirring as well as meet the demand of a higher oxygen supply. Mass cell cultures are ideal for producing pharmaceutically important secondary metabolites.

Protoplast culture: Protoplasts are plant cells without cell wall **(Fig. 3B)** and can be isolated from a variety of plant tissues (usually leaves, callus pieces, single cells or pollen grains) by enzymatic method using cell wall digesting enzymes (cellulases, hemicellulases and pectinases). Protoplasts are usually cultured by suspension culture in petri plates. As the protoplasts lack cell wall, they can be utilized for many purposes such as:

- Various biochemical and metabolic studies.
- Fusion of two somatic cells to produce somatic hybrids.
- Fusion of enucleated and nucleated protoplasts to produce cytoplasmic hybrids (Cybrids).
- Genetic transformation.

Protoplast fusion: The protoplasts from two different plant genotypes can be fused in the presence of fusogenic agents like polyethylene glycol (PEG - most widely used and most successful method for protoplast fusion) or by electro-fusion. The hybrid cells **(heterokaryons)** can be selected by various methods such as the use of different antibiotic markers or fluorescent dyes for two different protoplasts. Then, regenerated somatic hybrids from hybrid cells are characterized by morphology and molecular analysis (e.g. RAPD). This technique offers a unique method to raise new genotypes (i.e. somatic hybrids between distantly related plants or cybrids) with useful agronomic traits.

Plant regeneration pathways

The plants can be regenerated by either **organogenesis** and **somatic embryogenesis**.

Organogenesis means formation of organs like shoots from the cultured explants **(Fig. 3C)**. Miller and Skoog experimentally proved that formation of shoot or root first on the cultured tissue depends on the relative concentration of auxin and cytokinin. If auxins are high in the medium, then it promotes rooting while if cytokinins are high, shoot formation is promoted.

In **somatic embryogenesis**, the totipotent cells may undergo embryogenic pathway to form somatic embryos **(Fig. 3D-G)**, which can be grown to regenerate into complete plants. Generally, somatic embryos resemble the zygotic embryos (seed embryos) except in their place of origin and larger size. For the first time, Steward in 1958 and Reinert in 1959 independently reported the somatic embryogenesis from carrot cultures.

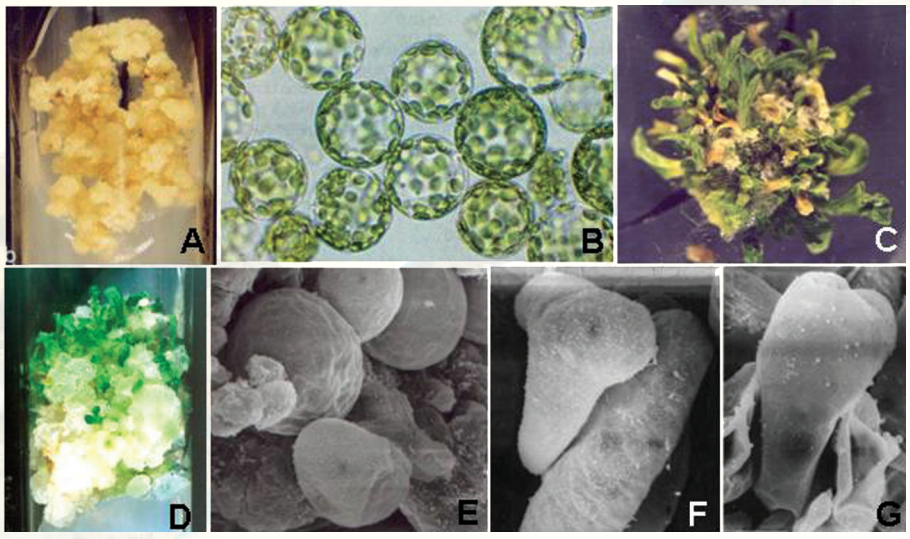


Fig. 3. Callus culture of indica rice (A), protoplasts isolated from leaf mesophyll cells of tobacco (B), multiple shoot regeneration from leaf explants of brinjal (C), regeneration of somatic embryos from leaf explants of brinjal (D) and scanning electron microscopy photographs of the various stages of somatic embryogenesis - globular (E), heart-shape (F) and torpedo (G) stage embryos from the brinjal culture (D).

6.2.3. Applications of Cell and Tissue Culture

The plant cell culture offers many potential applications in agriculture and health-care. Some of the important applications of plant cell and tissue culture are given below.

Micropropagation

Vegetative propagation of plants is of considerable importance in agriculture, horticulture and forestry as it provides the multiplication of uniform material for crop planting **(Clones)**. Traditionally, it is done by using cuttings, budding, grafting, corms, tubers and other vegetative propagules. The main problem with this method is that it is labour-intensive, low productivity and seasonal. Thus, tissue culture method of plant propagation, known as '**micropropagation**' can be used to overcome the problems mentioned above. This technique utilizes the culture of apical shoots, axillary buds and meristems on suitable nutrient medium, and has been adopted for commercialisation of important plants such as banana, apple, pears, strawberry, cardamom, many ornamentals (e.g. Orchids) and other plants.

There are four defined steps in micropropagation method. These are:

- **Initiation of culture -** from an explant like shoot tip on a suitable nutrient medium.
- **Shoot multiplication -** multiple shoots formation **(Fig. 4A)** from the cultured explant.
- **Rooting of shoots -** rooting of *in vitro* developed shoots on rooting medium.
- **Transplantation -** the hardening of tissue culture raised plants **(Fig. 4B)** as they are tender and subsequent transplantation to the green-houses or field.

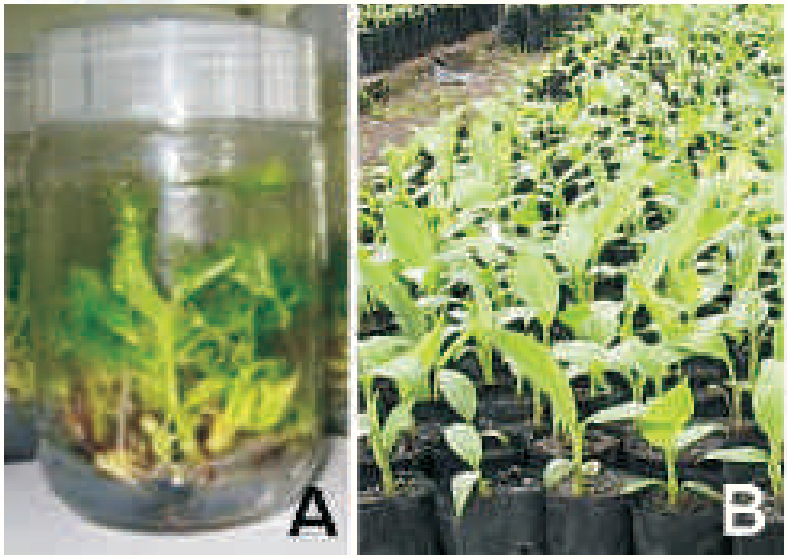


Fig. 4. Micropropagation of banana using tissue culture techniques. Multiple shoot regeneration (A) and acclimatized regenerated banana plants in green-house (B).

In recent years, much effort has been devoted to develop automated, robotised and more efficient transplant production methods. In India, the Department of Biotechnology (DBT) initiated a major network programme in 1989, and so far propagation protocols have been developed for more than 60 elite plant species.

Virus-free plants

Most of the crops plants, particularly vegetatively propagated plants are systemically infected with viruses. If the stock of a cultivar (vegetative propagules) are infected with a virus, the entire clonal population raised with such stock will also be infected and reduce the yield and quality significantly. Therefore, the production of virus-free plants is important to increase yield and quality. Interestingly, the distribution of viruses in plants is uneven, and the apical or axillary meristems are generally free from viral particles. This has enabled scientists to produce virus-free plants by culturing small meristems (usually less than 1 mm long) collected from virus-infected plants. Meristem culture technique is now successfully used to produce virus-free plants (but not virus-resistant plants) in a number of important clonal plants like potato, sugarcane, banana and apple. The scheme used for the production of virus-free plants by meristem culture is essentially same as micropropagation technique, except that the starting material (explant) is meristem which is dissected out from shoot tips of infected plants.

Artificial seeds

The artificial seeds (also called as synthetic seeds or somatic seeds) can be utilized for the rapid and mass propagation of elite plant species as well as hybrid varieties. Artificial seeds are produced either by encapsulating the somatic embryos in a protective coating, i.e., calcium alginate beads or by desiccating the somatic embryos with or without coating. A typical artificial seed is shown in **Fig. 5**.

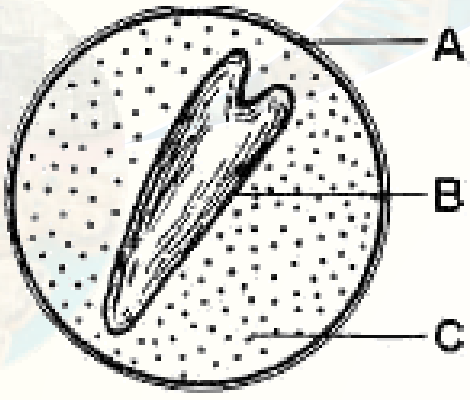


Fig. 5. The diagrammatic representation of an artificial seed. The artificial seed coat (A), somatic embryo at torpedo stage (B) and artificial endosperm (C).

Embryo rescue

It is very difficult to produce hybrids in case of inter-specific and inter-generic crosses (crosses between distantly related plants) because of abnormal development of the endosperm, which causes premature death of the hybrid embryo and leads to sterile seeds. The embryo from such sterile hybrid seeds can be excised at an appropriate time and cultured on a suitable nutrient medium to produce novel hybrids which is otherwise not possible. This is known as embryo rescue. Several useful hybrids were produced in a variety of crops using this technique.

Haploids and triploids

The technique of haploid production through anther and pollen culture as well ovary culture is of immense use in plant breeding to improve crop plants. It enables raising plants expressing traits that are otherwise recessive. The genetically homozygous diploid plants, which serve as parents in cross breeding can also be produced by diploidisation of haploid plants using colchicine chemical.

The endosperm is a triploid tissue and an excellent material to produce triploid plants by culturing endosperm tissue. Triploid plants usually show seed sterility or seedlessness, which is desirable in crops like citrus, apple and pear.

Somatic hybrids and cybrids

For many years, sexual hybridisation has been successfully used for crop improvement, however this method is limited in most cases, especially inter-specific and inter-generic crosses. The **somatic cell hybridisation** (also known as **parasexual hybridisation**) offers an excellent alternative for obtaining distant hybrids with useful agronomic traits (known as **somatic hybrids** or **parasexual hybrids**), which would never be formed in nature through sexual fertilization. Essentially, the protoplasts are isolated from the two different plants and are allowed to fuse with each other and the required fusion products (hybrid cells) are selected as discussed earlier in this chapter (see protoplast isolation and fusion).

The first inter-specific somatic hybrid between *Nicotiana glauca* and *N. langsdorffii* was produced by Carlson and his associates in 1972. Later, Melchers and his team in 1978 developed the first inter-genetic somatic hybrids between *Solanum tuberosum* (potato) and *Lycopersicon esculentum* (tomato) and the hybrids are known as '**Pomatoes** or **Topatoes**'. Several inter-specific somatic hybrids have been produced in number of crops like brinjal **(Fig. 6)**.

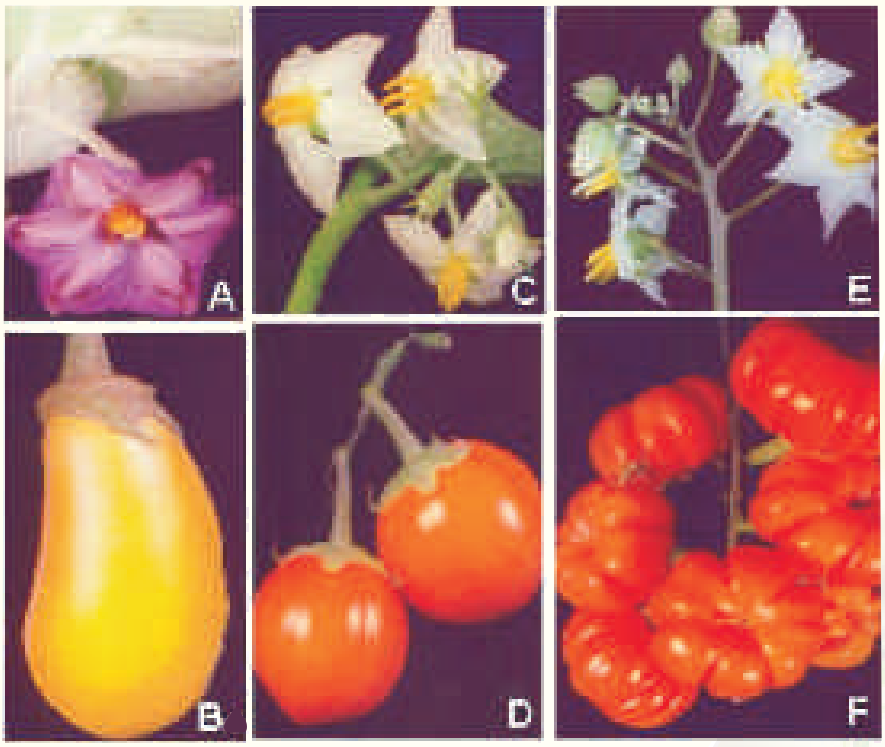


Fig. 6. Development of somatic hybrids in brinjal for transferring disease resistance from wild species to the cultivated variety. Flowers and fruits of the cultivated brinjal **(A & B)**, somatic hybrids **(C & D)** and wild-type brinjal **(E & F)**.

It is also possible to produce cytoplasmic hybrids **(cybrids)** through protoplast fusion in which the genomes of one of the partners is lost. Alternatively, the isolated and purified organelles - chloroplasts or mitochondria from one species can be fused with the recipient protoplasts from a different plant species (known as **organelle transfer** or **organelle uptake**) to transfer useful cytoplasmic traits like herbicide tolerance and cytoplasmic male sterility.

Production of secondary metabolites

Plants produce throusands of sophisticated chemical molecules. These include the chemicals required for a plant's basic metabolic processes such as sugars, lipids, amino acids and nucleic acids **(primary metabolites)** and also some other additional products such as alkaloids, resins, tannins, latex, etc. **(secondary metabolites)**.

The function of secondary metabolites in plants is not clearly understood, although they have been implicated in defence mechanism of the plant against pests and pathogens as well as

feeding by animals. However, many of the secondary products, especially various alkaloids are of immense use in medicine **(Table 1)**. Such compounds are produced in plants only in small amounts, and therefore, they are quite expensive.

Table 1. Few examples of industrially important plant secondary metabolites produced through cell and tissue cultures

Product	Plant source	Uses
Artemisin	*Artemisia* spp.	Antimalarial
Azadirachtin	*Azadirachta indica* (Neem)	Insecticidal
Berberine	*Coptis japonica*	Antibacterial, Antiinflammatory
Capsaicin	*Capsicum annuum* (chilli)	Reumatic pain treatment
Codeine	*Papaver* spp.	Analgesic
Digoxin	*Digitalis lanata*	Cardiac tonic
Diosgenin	*Dioscorea deltoidea*	Antifertility
Scopolamine	*Datura stramonium*	Antihypertensive
Quinine	*Cinchona officinalis*	Antimalarial
Shikonin	*Lithospermum erythrorhizon*	Antimicrobial; Red pigment used in lipstics & dye for silk
Taxol	*Taxus* spp.	Anticarcinogenic
Vincristine	*Cathranthus roseus*	Anticarcinogenic

The indiscriminative use of such valuable medicinal and other plants has brought them to near extinction. A possible solution in this direction is provided by cell and root cultures. Certain plant products such as shikonin are being produced commercially in large-scale using bioreactors.

Somaclonal variation

It has been observed that the long-term callus and cell suspension culture and plants regenerated from such cultures are often associated with chromosomal variations **(somaclonal variation)**. It is this property of cultured cells that finds potential application in the crop improvement and in the production of mutants (e.g., disease resistance in potato).

Larkin and Scowcroft (1981) proposed the term **'somaclones'** for plant variants obtained from tissue cultures of somatic tissues. Similarly, if the tissue from which the variants have been obtained is having gametophytic origin such as pollen or egg cell, it is known as **'gametoclonal variation.**

In vitro plant germplasm conservation

Germplasm refers to the sum total of all the genes present in a crop and its related species. The availability of a wide diversity (which includes land races, cultivars and primitive wild species of crop plants) and its conservation is utmost important as they are invaluable for future breeding programmes. However, the existence of primitive cultivars and wild relatives of crop plants has been endangered by the extensive use of newly introduced cultivars and hybrids, and this has posed a great concern to avoid the erosion of plant genetic resources. Therefore, attempts have been made to preserve the genetic resources by conventional methods (e.g. seeds, vegetative propagules, etc. and it is known as ***in vivo* gene banks**) as well as non-conventional methods, i.e. cell and tissue culture methods (known as ***in vitro* gene banks**). This will ensure the availability of valuable germplasm to breeder, to develop new and improved varieties.

The germplasm conservation through the conventional methods has several limitations such as, the short-lived seeds, seed dormancy, seed-borne diseases and high inputs of cost and labour. On the other hand, the biotechnological approaches for the conservation of plant germplasm can surpass many of these problems, and it can be done by the following approaches.

- **Freezing storage or Cryopreservation** - this utilizes the long-term preservation of cells and tissues (e.g. shoot tips, axillary buds, meristems, somatic embryos, etc.) at ultra-low temperature (-196°C, i.e. in liquid nitrogen) for indefinite time by using cryoprotectants (e.g. dimethylsulfoxide, glycerol, proline and mannitol). The cells and tissues can be recovered after thawing, and can be used for regeneration of plants.
- **Cold storage** - this uses the short- or medium-term storage of germplasm by using shoot tip, nodal or meristem explant cultures. The storage in done under conditions that impose slow growth such as low temperature (4°C or 15°C), nutrient limitation or the addition of growth retardants (e.g. absisic acid) in medium.

6.2.4. Gene transfer methods in plants

For achieving genetic transformation in plants, the basic pre-requisite is the construction of a vector (genetic vehicle) which carrys the genes of interest flanked by the necessary controlling sequences, i.e. promoter and terminator, and deliver the genes into the host plant. There are two kinds of gene transfer methods in plants as discussed below.

Vector-mediated or indirect gene transfer

Among the various vectors used in plant transformation, the Ti-plasmid of *Agrobacterium tumefaciens* has been used extensively. This bacterium contains large size plasmid, known as Ti-plasmid (tumour-inducing plasmid) and portion of this plasmid referred as **T-DNA** (transferred DNA) is transferred to plant genome in the infected cells and cause plant tumours (**crown galls - Fig. 7A**). This means that *A. tumefaciens* has natural ability to transfer T-DNA of its plasmid into plant genome. (plant chromosomes) upon infection of cells at the wound site, and therefore this bacterium is known as '**natural genetic engineer of plants**'. Because of this unique property, Ti-plasmid can be used as gene vectors for delivering useful foreign genes into plant cells and tissues. The foreign genes (**transgenes**), i.e., the gene of interest (e.g. Bt gene for insect resistance) and plant selection marker gene, usually an antibiotic gene like *nptII* which confer resistance to kanamycin are cloned in the T-DNA region of Ti-plasmid in place of unwanted DNA sequences **(Fig. 7B)**.

The general strategy for transforming plants is to collect leaf discs (in case of dicots) or embryogenic callus (in case of monocots like cereals) and then infect the tissue with Agrobacterium carrying recombinant disarmed Ti-plasmid vector **(Fig. 7B)**. The infected tissue can then be cultured (**co-cultivation**) on shoot regeneration medium for 2-3 days during which time the transfer of T-DNA along with foreign genes takes place. Later, the transformed tissues (leaf discs/calli) are transferred onto selection cum plant regeneration medium supplemented with usually lethal concentration of an antibiotic (e.g. kanamycin) to selectively eliminate non-transformed tissues. This **selection medium** also contain a bacteriostatic agent, i.e. an antibiotic like cefotaxime which suppresses or kills the Agrobacterium present with the transformed tissues and is no longer needed as the transfer of foreign genes has already taken place during co-cultivation. After 3-5 weeks, the regenerated shoots (from leaf discs) are transferred to root-inducing medium, and after another 3-4 weeks, complete plants can be transferred to soil following the hardening (acclimatisation) of regenerated plants. The presence of foreign genes in the putative transgenic plants can be confirmed by molecular techniques like PCR and Southern blot hybridisation.

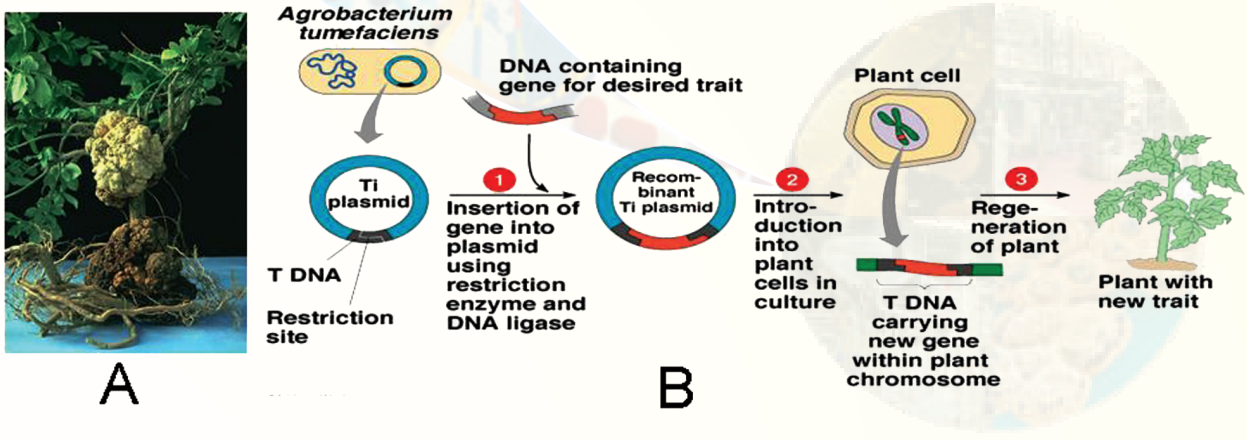


Fig. 7. Induction of crown gall on the wounded stem induced by wild-type virulent *Agrobacterium tumefaciens* (A), and the schematic diagram showing the cloning of the gene of interest in Ti-plasmid of Agrobacterium and its transfer to plant cells in culture to produce transgenic plants with desirable traits (B).

Vectorless or direct gene transfer

In the direct gene transfer methods, the foreign gene of interest is delivered into the host plant cell without the help of a vector. The following are some of the common methods of direct gene transfer in plants.

Chemical mediated gene transfer: Certain chemicals like polyethylene glycol (PEG) and dextran sulphate induce DNA uptake into plant protoplasts.

Microinjection: Here, the DNA is directly injected into plant protoplasts (specifically into the nucleus) using fine tipped (0.5-10 micrometer diameter) glass needle or micropipette to transform plant cells. The protoplasts are immobilized on a solid support (e.g. agarose on a microscopic slide) or held with a holding pipette under suction, and the DNA is injected into the protoplasts with the help of a fine microscope.

Electroporation: In this case, a pulse of high voltage is applied to protoplasts/cells/tissues, which makes transient (temporary) pores in the plasma-membrane which facilitates the uptake of foreign DNA. The DNA is mixed with the plant protoplasts in a specially made cuvettes before the application of the electric field.

Particle gun: A popular and widely used direct gene transfer method for delivering foreign genes into virtually any tissues and cells or even intact seedlings. In this method, the foreign DNA (containing the genes of interest) is coated (precipitated) onto the surface of minute gold or tungsten particles (1-3 micrometers) and bombarded (shot) onto the target tissue or cells using a particle gun (also called as gene gun/shotgun/microprojectile gun). Then, the bombarded cells or tissues are cultured on selection medium to regenerate plants from the transformed cells **(Fig. 8)**.

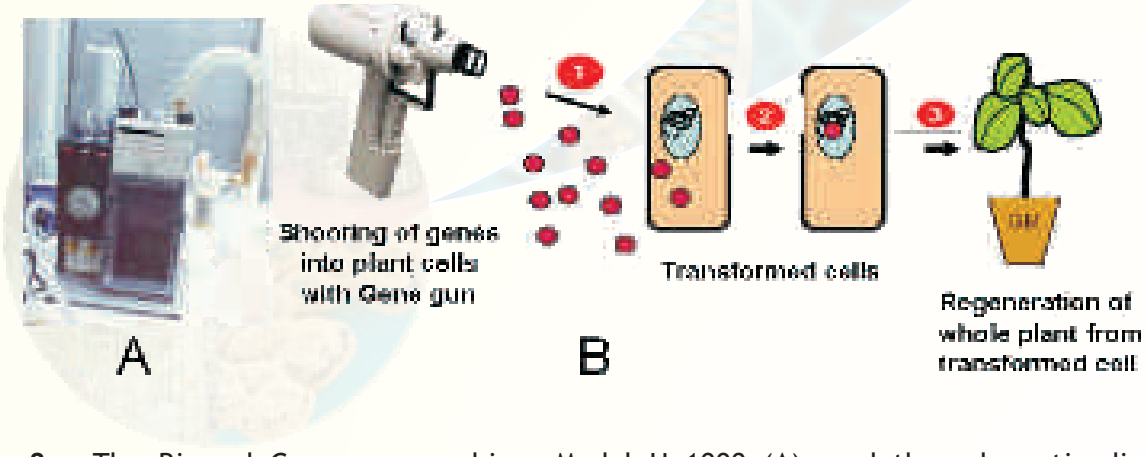


Fig. 8. The Bio-rad Gene gun machine, Model He1000 (A), and the schematic diagram showing the bombardment of gold particles containing the DNA (gene of interest) on their surface onto the plant cells in culture to produce transgenic plants with desirable traits (B).

Transgene analysis

The selection of transformed plant cells from untransformed cells is an important step in the plant genetic engineering. For selection, the gene of interest is transferred along with a genetic marker gene (usually antibiotic resistant genes such as *npt*II that confer resistance to kanamycin), so that only the transformed cells can be selected and regenerated on the selection medium containing the selection agent (e.g. kanamycin). The untransformed cells are eliminated on the selection medium as they are susceptible to the antibiotic (due to the absence of antibiotic resistance gene). The segregation and stability of the transgene integration and expression in the subsequent generations can be studied by genetic and molecular analyses (PCR, Southern, Northern and Western blot analysis). (Refer to earlier chapters for details of these techniques).

6.2.5. Transgenic plants with beneficial traits

During the last couple of decades, considerable progress has been made to understand the function of genes, isolation of novel genes and promoters as well as the utilization of these genes for the development of transgenic or genetically modified (GM) crops with improved and new characters. There are many potential applications of plant genetic engineering and some important examples of transgenic crops, approved by the U.S Food and Drug administration are summarised in **Table 2.**

Table 2. Few Examples of transgenic crops approved by the U.S Food and Drug administration

Gene(s) introduced	New/Improved Character	Developer
Thioesterase	High laurate oil Weed	Calgene
EPSP synthase PAT & Barnase/	Control Weed Control &	Monsanto
Barstar	Hybrid production	AgrEvo
EPSP synthase	Weed control	Monsanto Monsanto,
Bt CrylA(b)	Insect resistance	Ciba-Geigy Northrup King
Bt CrylA(c)	Insect Resistance	Dekalb Genetics
Acetolactate synthase	Weed Control	DuPont
Nitrilase	Weed Control	Calgene
EPSP synthase	Weed Control	Monsanto
Bt CrylA(c)	Insect Control	Monsanto
Coat protein	Virus Resistance	Univ. Hawaii & Cornell Univ.
Bt CryIIIA & Coat protein	Insect & virus control	Monsanto
EPSP synthase	Weed control	Monsanto
Gmfad2-1	Improved Oil	DuPont
Bt CrylA(c)	Insect control	Monsanto Calgene
Antisense PG	Delayed ripening	

Stress tolerance

Crop plants are very productive under ideal cultural conditions, but ideal growing conditions rarely occur. Moreover, plants encounter both biotic (viral, bacterial, fungal pathogens, nematodes, insect pests and weeds) and abiotic (salinity, drought, extreme temperatures, nutrient deficiency, etc.) stresses and these stresses cause a colossal loss of crop yield and quality. The application of chemical and biological pesticides as well as the use of resistant varieties is only partial success and these have certain limitations. Therefore, newer and effective technologies are essential to meet the demand. In this context, biotechnological strategies can be used to create transgenic plants with increased resistance to diseases and pests as well as abiotic stresses.

Biotic stress tolerance

Herbicide tolerance: Weeds (plants growing where they are not wanted, e.g. Striga) decrease crop yields and quality primarily by competing with crop plants for light, water and nutrients. Farmers apply herbicides/weedicides (e.g. glyphosate) for the eradication of weeds in the fields, but the main problem with this is the development of herbicide tolerance by weeds. Newer techniques, based on biotechnological tools, have been developed which are quite effective for weed management as well as in increasing the yields and income. There are several biotechnological strategies for weed control, but the most commonly employed approach is the over-production of herbicide target enzyme (usually in the chloroplast) in the plant, so that it becomes insensitive to the herbicide. The popular example for such an approach is the introduction of a modified gene from an Agrobacterium species that encodes for a resistant form of the herbicide target enzyme into crop plants for tolerance against the most extensively used herbicide glyphosate (sold as Roundup) and is effective against many weeds. Roundup Ready GM crop plants such as canola **(Fig. 9A)**, soybean, corn and cotton tolerant to glyphosate has already been commercialised.

Pest resistance: All crop plants are affected by a variety of insects and nematodes, and significantly reduce their yield and quality. To minimize these losses (both food and money), farmers use the synthetic pesticides extensively which cause severe effects on human health and environment. The transgenic technology provides an alternative and innovative method to improve pest control management which are eco-friendly, effective, sustainable and beneficial in terms of yield. The first genes available for genetic engineering of crop plants for pest resistance were *Cry* genes (popularly known as Bt genes) from a bacterium *Bacillus thuringiensis*.

These are specific to particular group of insect pests, and are not harmful to useful insects like butter flies and silk warms. Transgenic crops (e.g. cotton, rice, maize, potato, tomato, brinjal, cauliflower, cabbage, etc.) with Bt genes have been developed for and such transgenic varieties proved effective in controlling the insect pests and it has been claimed worldwide that it has led to significant increase in yields, and dramatic reduction in pesticides' use. Bt crops have already been commercialised in several countries. The most notable example is Bt cotton (which contains *CrylAc* gene) that is resistant to a notorious insect pest Bollworm and in the year 2002, Bt cotton was adopted in India **(Fig. 9B)**.

Disease resistance: Pathogens (viruses, fungi and bacteria) infect crop plants and drastically reduce their yield and quality. Globally, several diseases have caused havoc at several points of time in several countries. For instance, the great Irish famine resulted in great damage of potato crop due to the late blight disease caused by fungal pathogen. India has also experienced a famine in the form of Bengal famine due to destruction of rice caused by fungal pathogen. Thus, there is a great concern about the control of plant diseases. Traditionally, farmers apply chemical pesticides or use resistant crop varieties developed by the breeders, but these methods have certain limitations.The alternative and useful strategy is the creation of disease resistant transgenic crop plants by transfer of resistance genes from varied sources.

Virus resistance: There are several strategies for engineering plants for viral resistance, and these utilizes the genes from virus itself (e.g. the viral coat protein gene). The virus-derived resistance has given promising results in a number of crop plants such as tobacco, tomato, potato, alfalfa and papaya. Some viral resistance transgenic plants like papaya resistant to papaya ring spot virus **(Fig. 9C)** have been commercialised in some countries.

Fungi and bacteria: Plants respond to pathogens by inducing a variety of defence responses like pathogenesis-related proteins (PR proteins), enzymes that degrade/destroy fungal cell wall, antifungal proteins and compounds, phytoalexins, etc. Several transgenic crop plants showing increased resistance to fungal pathogens are being raised with genes coding for the different compounds mentioned above.

Some of these strategies, particularly the genes that encode for phytoalexins and cell wall degrading enzymes were also used for producing bacterial resistant transgenic plants.

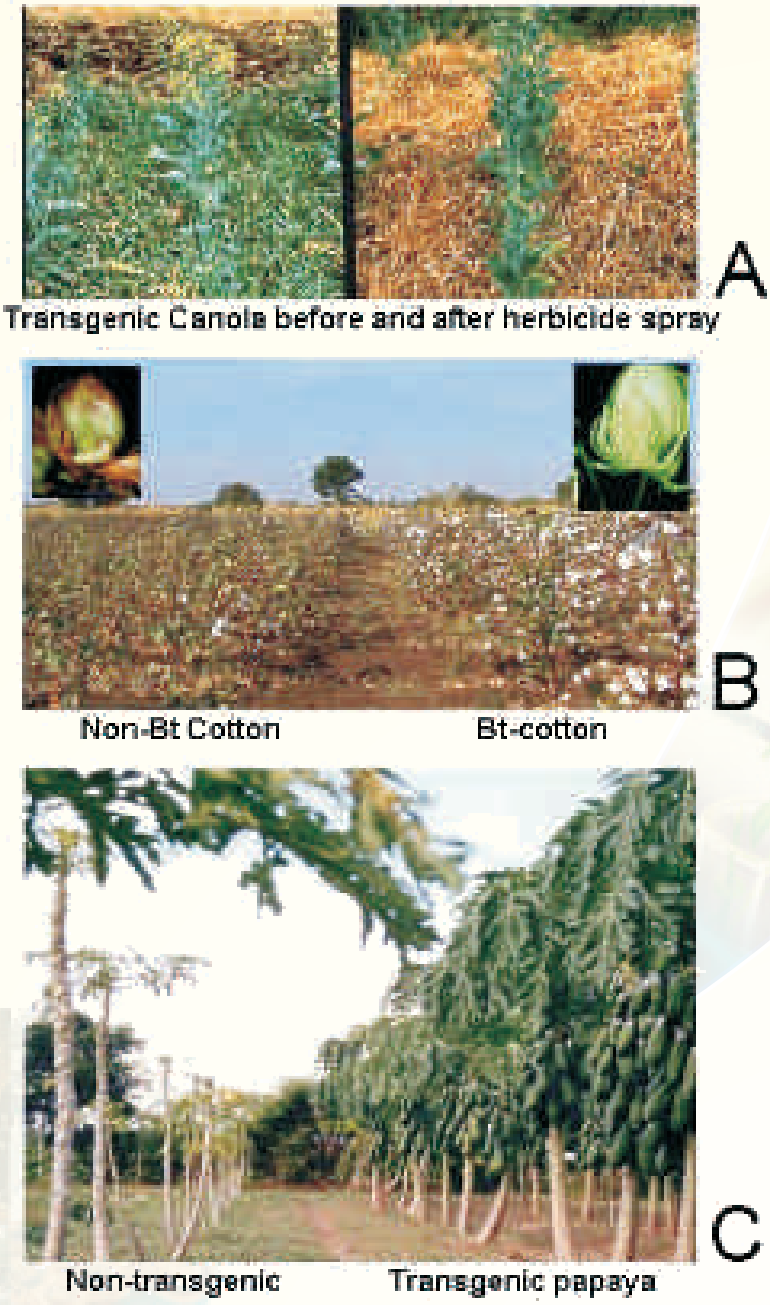


Fig.9. Genetically engineered crops with desirable traits. Transgenic canola for herbicide (glyphosate) tolerance before and after herbicide spray in the field (A); Bt cotton resistant to bollworm *(Helicoverpa armigera)* with healthy bolls (inserted photo) and non-Bt cotton with infested insect larvae (inserted photo) (B); Transgenic papaya resistant to papaya ringspot virus (c).

Abiotic stress tolerance

Plant growth and productivity are greatly affected by various environmental stresses/ abiotic stresses like high salinity and drought. Plant breeding efforts to produce abiotic stress tolerant plants while retaining high production is not very successful.

Plants have evolved many types of adaptations to cope with abiotic stress conditions like the production of the stress-related osmolytes like sugars (e.g. trihalose and fructans), sugar alcohols (e.g. mannitol) and amino acids (e.g. proline), glycine betaine, and certain proteins (e.g. antifreeze proteins). Transgenic plants have been developed which over-express the genes for one or more of the above mentioned compounds. Such plants have shown increased tolerance to environmental stresses.

Delayed fruit ripening

The gas hormone, ethylene is involved in the regulation of fruit ripening. Therefore, ripening can be slowed down by blocking or reducing ethylene production. This can be achieved by introducing ethylene forming gene(s) in a way that will suppress its own expression in the crop plant. Fruits from such plants ripen very slowly (however, they can be ripen by ethylene application) and are very important for export to longer distances without spoilage as they show longer-shelf life due to slow ripening. The notable example of this kind is the ***'Flavr Savr'*** transgenic tomatoes which were commercialized in U.S.

Male sterility

Male sterile plants are very important to prevent unnecessary pollination and to eliminate the process of emasculation during the production of hybrid plants. These are created by introducing a bacterial gene from *Bacillus amyloliquefaciens* that encode an enzyme **barnase**, which is an RNA hydrolyzing enzyme that inhibits pollen formation if, the expression of this gene specifically in the tapetal cells of anther using **tapetal-specific promoter** (e.g. TA29) to restrict its activity only to the cells involved in pollen production. Male fertility can be restored by introducing another gene from the same bacterium under the control of TA29, whose product **barstar** (protein) tightly bind with RNase, so that the normal pollen are formed. This **barnase/barstar** system was successfully utilized in producing male sterile/restorer lines in number of crops, particularly mustard for hybrid production.

Transgenic plants as bioreactors (Molecular farming)

Plants are amazing and cheap chemical factories that need only water, minerals, sun light and carbon dioxide to produce thousands of sophisticated chemical molecules with different structures. Given the right genes, plants can serve as bioreactors to modified or new compounds such as amino acids, proteins, vitamins, plastics, pharmaceuticals (peptides and proteins), drugs, enzymes for food industry and so on. Some of the potential and remarkable examples of this kind are described here.

Nutrient quality

Plants are the principle source of human nutrition. A small number of crop plants such as cereals, legumes, vegetables and root crops supply most of the energy and nutrients (in the form of fats, carbohydrates, proteins, vitamins and micronutrients) in the human diet. It is estimated that about 800 million people, mostly in developing countries are malnourished (particularly children) and suffering with several diseases due to the deficiency of nutrients. Therefore, the improvement of nutritional quality of crop plants is extremely important. Transgenic crops with improved nutritional quality have already been produced by introducing genes involved in the metabolism of vitamins, minerals and amino acids. Few examples of genetic modification of nutrition quality are described below.

Vitamin A: The source of vitamin A is either directly from animal food (e.g. milk, cheese and meat) or indirectly from green leafy vegetables (e.g. carrots, spinach, tomatoes, and chillies) and fruits (e.g. mango, melon and papaya), which contain carotenoids (beta-carotene - the provitamin A) that are converted to vitamin A in the body. Vitamin A deficiency can lead to night blindness, permanent blindness and skin disorder, among others. About 124 million children worldwide are deficient in vitamin A and a quarter of a million go blind each year due to vitamin A deficiency.

The staple food rice is extremely low in vitamin A, and therefore the improvement of vitamin A content is very important. In a remarkable example of genetic engineering, Prof. Ingo Potrykus and Dr. Peter Beyer developed genetically engineered rice (popularly known as **'Golden Rice'**), which is enriched in pro-vitamin A (beta-carotenoids) by introducing three genes involved in the biosynthetic pathway for carotenoid under the control of endosperm-specific promoter, so that gene products (enzymes) are synthesized in the rice endosperm. The seeds of Golden Rice are yellow in colour because of pro-vitamin A is produced in the entire grain **(Fig. 10A)**. Interestingly, they have also further engineered this Golden Rice by introducing three more genes from different organisms for iron source and its absorption. Similarly, transgenic crop plants are also being produced to raise the level of other vitamins, particularly vitamin E (which is an essential antioxidant in humans) and vitamin K (which is involved in bone formation).

Seed protein quality: The nutritional quality of cereals and legumes are limited because of deficiency of the essential amino acids, i.e. lysine in cereals, and methionine and tryptophan in pulses. Two genetic engineering approaches have been used to improve the seed protein quality. In the first case, a transgene (e.g. gene for protein containing sulphur rich amino acids was introduced into pea plant (which is deficient in methionine and cysteine, but rich in lysine) under the control of seed-specific promoter. In the second approach, the endogenous genes are modified, so as to increase the essential amino acids like lysine in the seed proteins of cereals.

Diagnostic and therapeutic proteins

Transgenic plants can also produce a variety of proteins used in diagnostics for detecting human diseases and therapeutics for curing human and animal diseases in large-scale with low cost. The monoclonal antibodies, blood plasma proteins, peptide hormones and cytokinins are being produced in transgenic plants and their parts such as tobacco (in leaves), potato (in tubers), sugarcane (in stems) and maize (in seed endosperm).

Edible vaccines

Crop plants offer a cost-effective bioreactor to express antigens which can be used as edible vaccines. Some of the antigenic determinants on the surface of viruses and bacteria are proteins made by the pathogen. The genes encoding these antigenic proteins can be isolated from the pathogens and expressed in plants, and such transgenic plants or their tissues producing antigens can be eaten for vaccination/immunisation **(edible vaccines)**. The expression of such antigenic proteins in crops like banana and tomato are useful for immunisation of humans since banana and tomato fruits can be eaten raw. In case of animals, such genes can be expressed in crops like alfalfa and other forage/fodder crops, which are suitable for vaccination. The edible vaccines that are produced in transgenic plants have great advantages like the alleviation of storage problems, easy delivery system by feeding and low cost as compared to the recombinant vaccines produced by bacterial fermentation. Vaccinating people against dreadful diseases like cholera and hepatitis B by feeding them banana/tomato, and vaccinating animals against important diseases such as foot and mouth disease by feeding them sugar beets could be a reality in the near future.

Biodegradable plastics

The biodegradable plastic, e.g. polyhydroxybutyrate (PHB) is being produced commercially by fermentation with the bacterium *Alcaligenes eutrophus*. The main drawback of bacterial PHB is its high production cost, making it substantially very expensive than synthetic plastics. Alternatively, transgenic plants can be used as factories to produce PHB. The genetically engineered Arabidopisis plants with the three genes involved in PHB synthesis from *A. eutrophus* produced PHB globules exclusively in their chloroplasts without effecting plant growth and development **(Fig. 10B)**. The large-scale production of PHB may be easily achieved in tree plants like populus, where PHB can be extracted from leaves. Industry has already begun to explore the production of biodegradable plastics from transgenic plants.

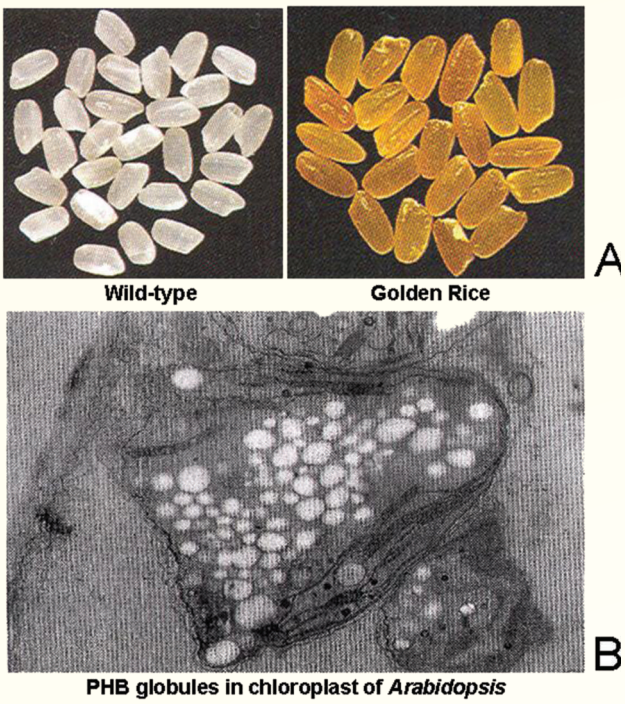


Fig. 10. Golden rice (A) and electron microscopic picture of chloroplast of transgenic Arabidopsis showing PHB globules (B).

Metabolic engineering and secondary products

Plant biotechnology will lead to improved plant sources for the production of valuable secondary metabolites mentioned in previous section on cell culture products. Biosynthetic pathway which lead to the production of such valuable products are being engineered for their over-production to reduce the cost. The over-expression of the gene, which encode for the first enzyme in a pathway generally results in higher levels of the desired end product, and this has been successfully done in the enhancement of taxol production from the transformed tissue cultures of *Taxus* sp. Another strategy involves the use of *Agrobacterium rhizogenes* to induce the excessive formation of secondary roots **(hairy roots)** in plants that normally produce useful secondary metabolites in this region.

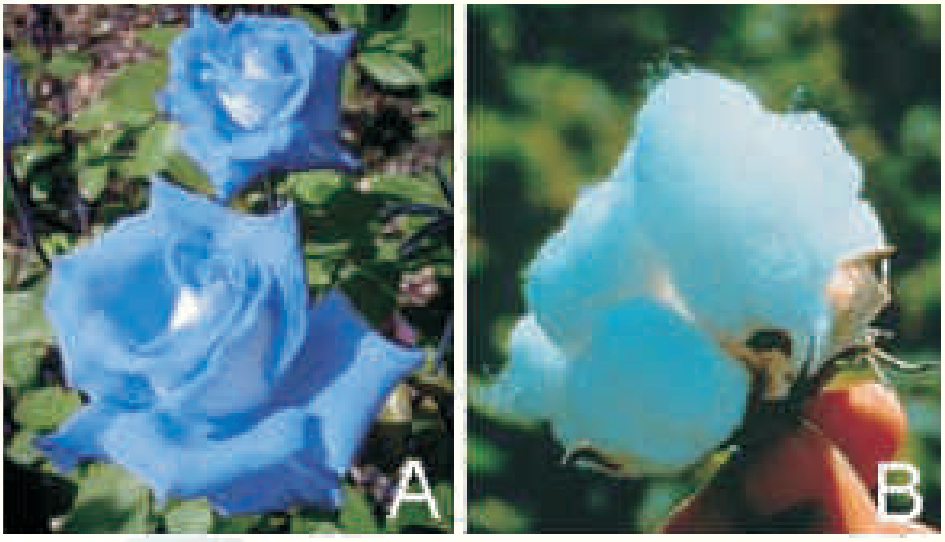
Other applications

There are many other applications of plant genetic engineering, which are listed below:

- Production of healthy oils with altered fatty acid profiles.
- Modification of starch properties for specific uses.
- Favourable change of grain storage products and their chemical composition to improve the processing of bread making with wheat flour, malting of barley and brewing of beer.

- Removal of undesirable toxic compounds in certain plants.
- Development of blue roses **(Fig. 11A)**, which is otherwise not possible by conventional plant breeding because of the absence of blue pigment in roses.
- Development of blue coloured cotton **(Fig. 11B)** and also with other colours.
- Development of tear-less onions, caffeine-free coffee and low nicotine tobacco.

Undoubtedly, there will be many more exciting applications of plant genetic engineering in the future.



Blue Rose **Blue Colore Cotton**

Fig. 12. Genetically modified blue rose (A) and blue coloured cotton (B).

6.2.6. Biosafety in Plant Genetic Engineering

The GM crops are fast becoming a part of agriculture throughout the world because of their contribution to the increased crop productivity and to global food, feed and fibre security, besides their use in health-care and industry. However, the constraints associated with public acceptance of transgenic crops continue to be important challenges facing the global community. The following are the major concerns about GM crops and GM foods:

- The safety of GM food for human and animal consumption (e.g. GM food may cause allergenicity).
- The effect of GM crops on biodiversity and environment.
- The effect of GM crops on non-target and beneficial insects/microbes.
- Transgenes may escape through pollen to related plant species (gene pollution) and may lead to the development of super weeds.

- The GM crops may change the fundamental vegetable nature of plants as the genes from animals (e.g. fish or mouse) are being introduced into crop plants.
- The antibiotic resistance marker genes used to produce transgenic crops may horizontally transfer into microbes and thus exacerbate problem of antibiotic resistance in human and animal pathogens (i.e. transgenes may move from plants to gut microflora of humans and animals).
- The GM crops may lead to the change in the evolutionary pattern.

Unfortunately, the public debate over the benefits and hazards of plant gene technology suffers from an astounding array of misinformation, misunderstanding and manipulation. In fact, with the continuing accumulation of evidence of safety and efficiency, and the complete absence of any evidence of harm to the humans and animals as well as the environment, more and more consumers are becoming comfortable because of the increased awareness about the potential of the plant genetic engineering. Further, the transgenic crops (e.g. cotton, tomato, corn and soybean) which have already entered the market place (which carry a label, i.e., GM crop or GM food in several countries were subjected for the extensive field trials for environmental safety related to wild species and competitive performance of transgenic and toxic effects as per the standard regulatory policies before they were approved for commercialisation. In fact, in 2009, a record of 14 million farmers from 25 countries cultivated 134 million hectares (330 million acres) with the crops that were genetically engineered for herbicide, insect resistance, delayed fruit ripening and improved oil quality. The global area of biotech crops planted from 1996 to 2009 is shown in **Fig. 12**.

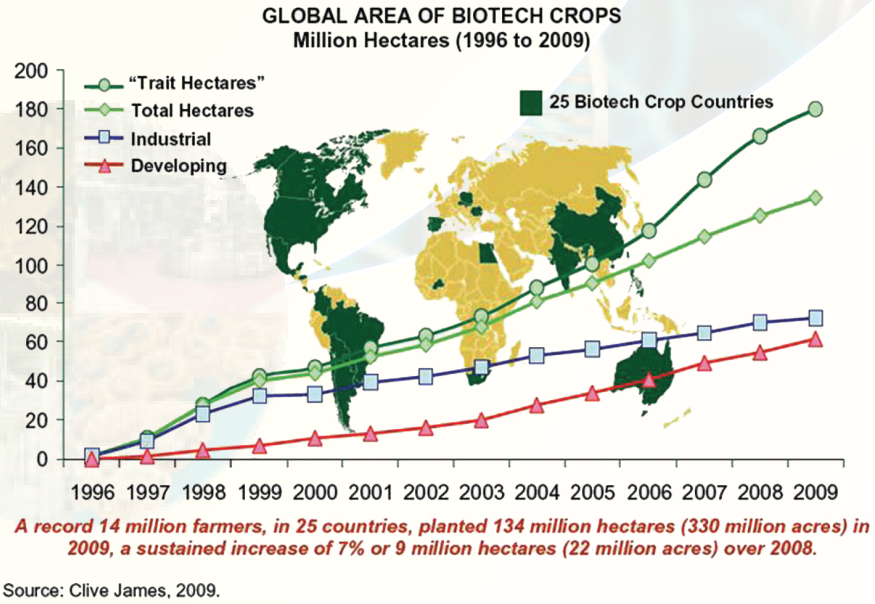


Fig. 12. Global area of biotech crops from 1996 to 2009 (in million hectares).

Nevertheless, the thorough assessment of the risks associated with transgenics for plants, animals and humans is important before they are released. Foods or food ingredients derived from GMOs must be shown to be as safe as or safer than their traditional counterpart before they can be recommended for public use. In general, many scientists believe that plant genetic engineering is the 'technology' that can solve various human problems, especially hunger and disease.

Review Questions

1. What is meant by plant tissue culture? List the various steps involved in this technique?
2. What is plant regeneration? Give the different pathways of plant regeneration.
3. Name a few useful secondary metabolites that can be produced through plant cell culture technology.
4. List the various applications of plant cell and tissue culture technology.
5. Differentiate:
   i) Cell culture and protoplast culture
   ii) Direct gene transfer and indirect gene transfer
6. Explain how embryo rescue can be used to produce novel hybrids.
7. Write short note on artificial seeds.
8. How are plant cells converted to protoplasts? Why are protoplasts more amenable to produce somatic cell hybrids and cybrids?
9. What is genetic engineering? Add a note on the various steps involved in this technology.
10. Why is *Agrobacterium* described as 'natural genetic engineer of plants'?
11. Explain how gene gun functions to deliver genes into plant cells?
12. What are the proposed benefits of genetic engineering in crop improvement?
13. What are the genetic engineering strategies to create the following traits in transgenic crops:
    a) Herbicide tolerance
    b) Abiotic stress tolerance
    c) Insect resistance
    d) Virus resistance
14. What is the common strategy to produce transgenic crops with delayed ripening and longer shelf life of fruits?

15. What is meant by 'Golden Rice'? In what way it is different from the normal rice?

16. Write briefly the benefits of biodegradable plastics that are produced from GM plants.

17. What are the transgenic crops that are commercialised globally?

18. Explain the social, economical and environmental implications of genetic engineering techniques.

19. Define:

Explant	Hairy roots
Clone	Roundup
Callus	PR proteins
Micropropagation	Bt genes
Encapsulation	Male sterility
Embryo rescue	*Flavr Savr*
Somatic hybrids	Molecular farming
Cybrids	PHB
In vitro gene bank	Edible vaccines
T-DNA	Metabolic engineering
Crown galls	

20. Fill in the blanks:

I) Rapid multiplication of plants by tissue culture techniques is referred to as _____.

ii) Gottlieb Haberlandt is known as father of _____.

iii) The most commonly employed gene transfer method in plants is _____.

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CHAPTER 3

ANIMAL CELL CULTURE AND APPLICATIONS

6.3.1. Introduction

Animal cells, just as plant cells, will continue to grow outside the living organism if supplied with the appropriate nutrients and growth conditions. The process of growing of cells under laboratory conditions is called **Cell Culture**. It is carried out ***in vitro*** ('within glass') as opposed to ***in vivo*** ('within the living'). The advantage and limitations of animal cell culture are given in **Table 1**. A homogenous population of cells derived from a single parental cell is called a **clone**. Therefore, all cells within a clonal population are genetically identical. The growth rate of animal cells is relatively slow and usually require 18 to 24 hour to divide. This makes the animal cell culture vulnerable to contamination, as a small number of bacteria would soon outgrow a larger population of animal cells. The animal cell culture became a routine laboratory technique in 1950s after **George Gay** established the first human cell line (HeLa) from cervix cancer that led to several important discoveries in medical sciences. The need for cell culture, especially at large scales, became apparent with the need for viral vaccines.

Table 1. Advantages and limitations of animal cell culture.

Advantages	Disadvantages
• Homogenous genetic population. • Controlled physico-chemical environment. • Easy to add genes (Transfection) or regulate protein levels (RNAi). • Available in adequate numbers to do chemical study. • Easy production of biopharmaceuticals. • No ethical clearance required. • Cost effective screening assays.	• Small size (high sensitive techniques to detect changes). • Scale-up is challenging. • May not represent *in vivo* phenotype/genotype.

Advent of recombinant DNA technologies in the 1970s, enabled the expression of mammalian genes in bacteria. However, soon it was realized that large proteins or glycoproteins of therapeutic value could not be produced in bacteria. This prompted the usage of animal cell lines for large-scale production of therapeutic proteins. The development of hybridoma technology allowed us to produce large quantities of monoclonal antibodies of immense diagnostic and therapeutic values. Further, recent advances in the area of stem cells, tissue engineering and gene therapy are likely to open new treatment regimens.

Before you learn about the details of animal cell culture technology, it is instructive to know some essential features of animal cell growth in culture.

6.3.2. Animal Cell Culture Techniques

Features of animal cell growth in culture

Animal cells can be grown in glass or plastic vessels, in the presence of nutritive media that need to be is periodically replenished. However, depending on the tissue they have been isolated from, they can be grown only for limited generations even in the best nutritive media. There is a mortality associated with all normal animal cells. Another important feature of animal cells is that they divide and fill the surface of the culture vessel and then stop growing. Relate this to what happens in the animal body. The infant animal grows only to adulthood and not any further. Cells comprising tissues and organs such as the liver grow only to a certain size after which they cease to grow. This phenomenon which occurs in the normal body is observed also in cell culture and is termed **"contact inhibition"**. This means that when cells grow and reach the walls of the container (i.e., reach confluency) they stop growing further. Another important feature of cell growth in culture is that their environment is different from that *in vivo*. These differences affect the adherence of cells to culture vessels, their shape and rate of proliferation. It is of interest to know that in culture, cancer cells appear very different from normal cells. Cancer cells loose contact inhibition and pile on each other due to uncontrolled growth and among other features, appear more rounded in shape. Such differences in growth patterns in normal versus cancer cells are utilized by Oncologists (cancer biologists) to determine whether tumors are cancerous or not using **`Colony formation assay'**. Let us now learn about various types of cell cultures and the technology associated with it.

Primary Cell Cultures

Cells are dissociated from the parental tissue (such as kidney, liver) by mechanical or enzymatic methods and maintained in suitable culture medium and vessels. The most frequently used enzymes for separating cells from a given tissue (dispersion) are crude preparations of trypsin and collagenase that cleave the proteinaceous cementing material between cells in a tissue. The maintenance of growth of such cells under laboratory conditions is known as primary cell culture.

The characteristics of cells in culture usually depend on their original source within the animal.

Cells can be grown as **adherent** (anchorage-dependent) or **suspension cultures** (anchorage-independent). Adherent cells are usually derived from tissues of organs such as kidney where they are not mobile and are embedded in connective tissue. They grow adhering to the cell culture vessel. On the other hand, suspension cells do not attach to the surface of the culture vessel. Virtually all suspension cultures are derived from cells of the blood system. This is because, these cells (e.g., lymphocytes) are also suspended in plasma *in vivo*. The drawbacks of primary culture are that they are time consuming and require the use of live animals or fresh tissue. There can be considerable variation from one preparation to another particularly if prepared by different people. These difficulties can be overcome by the use of **secondary cell cultures** or **cell lines**.

Secondary Cell Cultures and Cell Lines

Once the primary culture is subcultured, it is known as secondary culture or cell line. Subculturing or "splitting cells," is required to periodically provide fresh nutrients and growing space for continuously growing cell lines. The frequency of subculture or density of cells to be plated, depends on the characteristics of each cell type. If cells are split too frequently or at too low a density, the line may be lost. If cells are not split frequently enough, the cells may exhaust the medium and die. Sub-culturing involves: removing the growth media, washing the plate, disassociating the adhered cells, usually enzymatically (e.g. with trypsin), although some cells may be removed by repeated pipeting or gentle scraping), and diluting the cell suspension into fresh media. **Rous** and **Jones** were first to introduce proteolytic enzyme trypsin for the subculture of adherent cells. Such cultures may be called **secondary cultures**. Sometimes, certain cells of these secondary cell cultures can spontaneously become altered (transformed) and give rise to continuous cell lines which show immortality, as they can grow indefinitely without dying in culture. These cultures can contain mixed cell types or can consist predominantly of a single cell type.

Types of Cell Lines

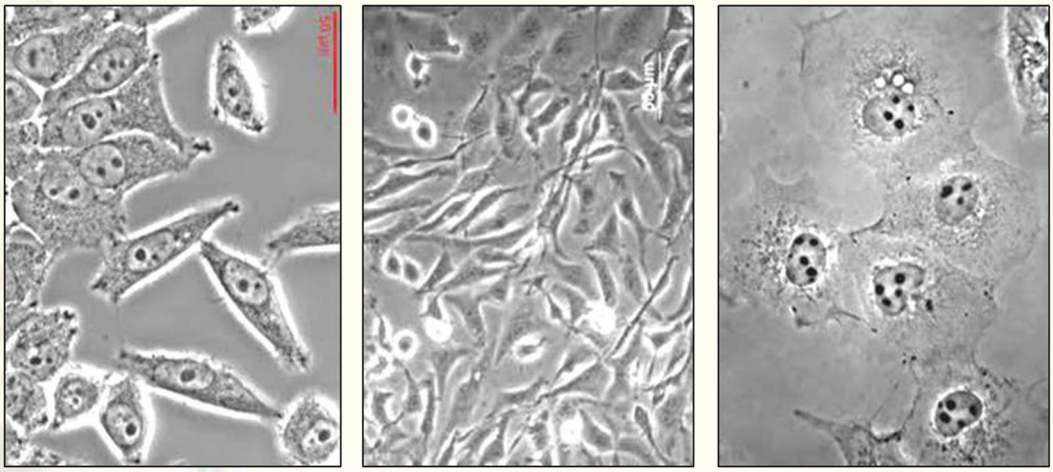
The various types of cell lines are categorized into two types, i.e., finite cell line and continuous cell line.

Finite Cell Lines

Finite cell lines are those cell lines which have a limited life span and they grow through a limited number of cell generations. Finite cell lines show the property of contact inhibition, density limitation and anchorage dependence. The mode of growth is in the monolayer form. The growth rate is slow and doubling time is around 24 to 96 hours.

Continuous Cell Lines

Cell lines transformed under *in vitro* culture conditions give rise to continuous cell lines **(Fig. 1)**. The various properties associated with continuous cell lines are: the **ploidy** (change in basic number of chromosomes), no contact inhibition and no anchorage dependence. The mode of growth is either monolayer or suspension form. The growth rate is rapid and doubling time is between 12 to 24 hours. The density limitation is reduced or lost.



HeLa cell line from human cervical carcinoma

CHO continuous cell line from Chinese hamster ovary

Cos-1 cell line from monkey kidney

Fig. 1. Different mammalian cell lines.

Physical environment for culturing Animal Cells

The culturing of animal cells under *in vitro* condition involves creation of appropriate physical, nutritional and hormonal environments in which the cells can grow. The physical environment includes controlling the temperature, pH, osmolality and gaseous environment by providing a supporting surface and protecting the cells from chemical, physical and mechanical stresses.

Temperature

The mammalian cells are grown in incubators maintained at 37°C. This temperature is chosen because it is the core body temperature of *Homo sapiens*. Further, it has been observed that most cells derived from the warm blooded animals will grow at this temperature.

pH

The regulation of extra-cellular and intra-cellular pH is essential for the survival of mammalian cells. The correct pH is not only important for maintaining the appropriate ion balance but also for maintaining optimal function of cellular enzymes and for optimal binding of hormones and

growth factors to cell surface receptors. Even transient changes in pH can alter cell metabolism which can lead to cell death. Most media strive to achieve and maintain the pH between 7 and 7.4. The regulation of pH is done using a variety of buffering systems. Most media use the bicarbonate - CO_2 buffering system. The interaction of CO_2 derived from cells or atmosphere with water leads to a drop in pH described by the equation below:

$$H_2O + CO_2 \leftrightarrows H_2CO_3 \leftrightarrows H^+ + HCO_3^-$$

The bicarbonate content of the medium neutralizes the effect of increased CO_2 according to the following equation: $NAHCO_3 \rightarrow Na^+ + HCO_3^-$. The increased HCO^-_3 ion drives the equation above to the left until the equilibrium is reached at pH 7.4. This kind of system is called an open system.

Osmolality

The osmolality of the culture medium also has a significant bearing on cell growth and function. It preserves the membrane integrity of cells. If the outside osmotic pressure becomes higher or lower than that which must be maintained inside the cell, it will shrink or swell accordingly. The osmolality of the medium used is determined by the media formulation. Salt and glucose are the major contributors to the osmolality of the medium, although amino acids may also contribute significantly. Almost all commercial media are formulated to have a final osmolality of around 300 mOsm. Osmolality can be checked directly with an osmometer.

Medium

The most commonly varied factor in culture systems is the growth medium. Medium is a mixture of inorganic salts and other nutrients capable of sustaining cell survival *in vitro*. Having the correct nutrient mixture can often be the determining factor in failure or success in cell culture. The medium provides essential nutrients that are incorporated into dividing cells, such as, amino acids, fatty acids, sugars, ions, trace elements, vitamins, cofactors, and ions necessary to maintain the proper chemical environment for the cell. Some components may perform both roles; for example, the sodium bicarbonate may be used as a carbonate source but also may play an important role in maintaining the appropriate pH and osmolality. All media contain an energy source, usually glucose. Many of the media contain phenol red as a pH indicator. This is very helpful in monitoring the pH of the culture medium in an incubator. Highly acidic conditions turn the phenol red into yellow while highly alkaline conditions turns the phenol red into pink color.

Serum and antibiotics

Serum is one of the most important components of animal cell culture, as it supports cell proliferation and their attachment to culture vessels. The peptide hormones or hormone-like growth factors that promote healthy growth are often derived from animal blood, such as **foetal**

bovine serum (FBS). Serum is also a source of various amino acids, hormones, lipids, vitamins, polyamines and salts containing ions such as calcium, chloride, ferrous, ferric, potassium etc. Current practice is to minimize using blood-based supplements and switch to **serum-free medium** due to some complications of FBS usage. Although not required for cell growth, **antibiotics** such as penicillin and streptomycin are often used in culture medium to control the growth of bacterial and fungal contaminants.

Vessels and Equipments required for Animal Cell Culture

Cultures should be examined daily for their morphology, colour of the medium and density of cells. The animal cells are usually grown and maintained in Petri dishes, Culture flasks or Multi-well plates of various shapes and sizes **(Fig. 2)** at an appropriate temperature and gas mixture (typically, 37°C, 5% CO_2 for mammalian cells) in an incubator. Culture conditions vary widely for each cell type, and variation of conditions for a particular cell type can result in different phenotypes being expressed.

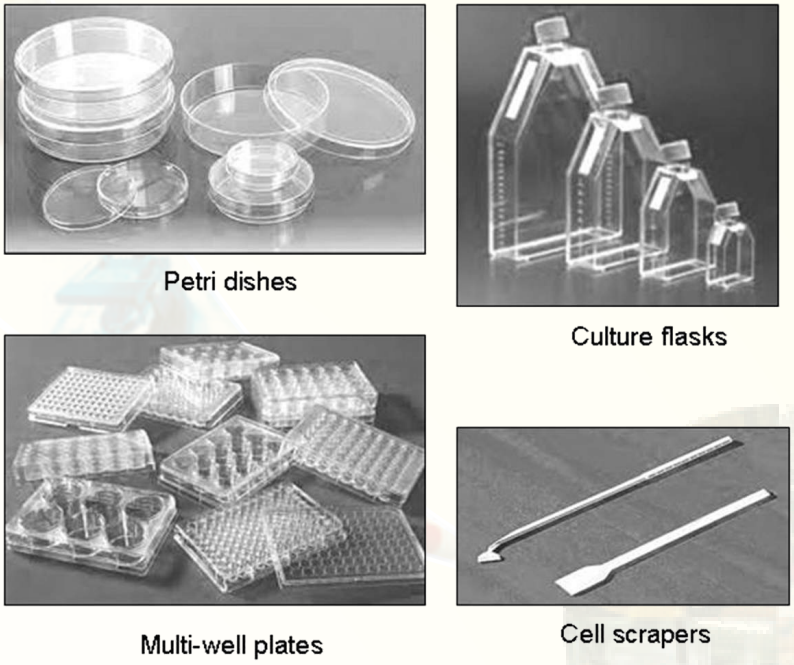


Fig. 2. Vessels and accessories for animal cell culture.

A cell culture laboratory should have equipments like tissue culture hood, CO_2 incubator, inverted microscope, Centrifuge etc. for doing animal cell culture work.

Tissue Culture Hood

All tissue/cell culture manipulations must be performed asceptically, i.e., without any bacterial or fungal contamination. Otherwise, animal cell culture media can easily get contaminated with

bacteria or fungi which will outgrow animal cells. The Laminar Air Flow (LAF) hoods allow the work area to be free of such contamination. A LAF hood essentially performs two functions:

1. Protects the tissue culture from the operator (by providing a sterile environment).
2. Protects the operator from the tissue culture (from possible infection risk).

Depending on the nature of cells/tissue being handled (especially infective agents), the biology safety cabinets are designated as Class I to class III. The LAF hoods have continuous displacement of air that passes through a high efficiency particle air **(HEPA)** filter that removes particulates from the air. The hoods are equipped with a short-wave UV light source that can be turned on for a few minutes to sterilize the surfaces of the hood just before use.

CO_2 Incubator

The CO_2 incubator is designed to reproduce as closely as possible the environmental conditions of the living cells. The essential functions of the incubator are to maintain, the sterility of the chamber, a constant temperature, an atmosphere with a fixed level of CO_2 and high relative humidity. A pan of water is kept at all times in the incubator chamber to maintain high relative humidity and prevent desiccation of the culture medium and maintain the correct osmolarity **(Fig. 3A)**. The animal cells are grown in an atmosphere of 5-10% CO_2 because the medium used is buffered with sodium bicarbonate/carbonic acid and the pH must be strictly maintained.

A. CO_2 Incubator B. Centrifuge C. Inverted Microscope

Fig. 3. Equipments for animal cell culture.

Centrifuge

For most cell culture only low-speed centrifuges are required **(Fig. 3B)**. A gentle braking action helps prevent disruption of the separated bands of cells. In most cases cells should be centrifuged at 20°C; nevertheless low operation temperature is useful to avoid exposing cells to uncontrolled higher temperatures.

Inverted Microscope

In tissue culture vessels, for example a petri dish, the cells are present at the bottom with the culture medium above. The inverted microscope allows the cells at the bottom to be visualized because the optical system is at the bottom with the light source on top **(Fig. 3C)**. Observation of cultures in this way will give an immediate idea of the health and growth of cells. Microscopes should be kept covered to protect from dust and the lights turned down when not in use.

6.3.3. Characterization of Cell Lines

In order to analyze the growth characteristics of a particular cell type or cell line, a growth curve can be established from which one can obtain a population doubling time, a lag time, and a saturation density. A growth curve generally will show the cell population's lag phase, that is, the time it takes for the cells to recover from subculture, attach, and spread; the log phase, in which the cell number begins to increase exponentially and a plateau phase, in which the growth rate slows or stops due to depletion of growth factors and nutrients. An increase in cell number is also a frequently used method of assessing the effect of hormones, nutrients, and so forth on a specific cell type. The culture doubling time, allows prediction of the likely cell concentration at any time in the future **(Fig. 4)**.

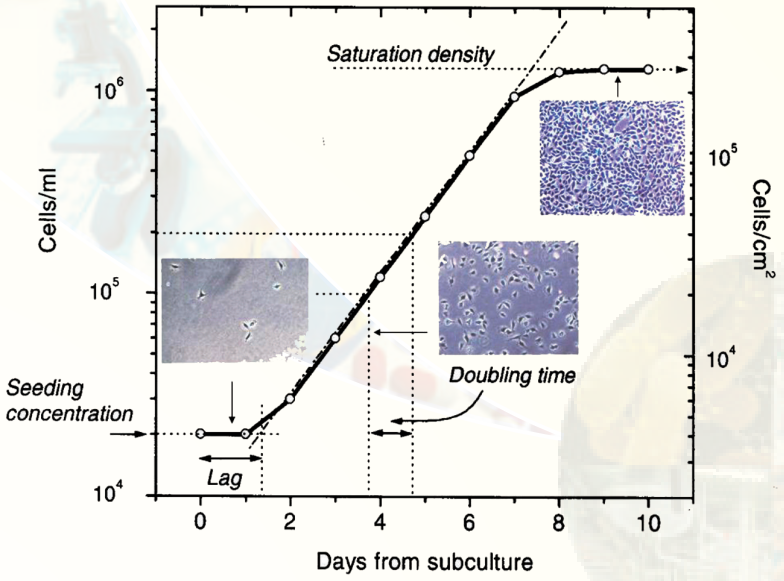


Fig. 4. Normal growth curve of animal cells.

There are laboratories and institutions which maintain various cell lines for scientists to use (for example American Type Culture Collection or ATCC, Virginia, USA). The advantage of using established cell lines is that their growth characteristics, media requirements and responses to selected reagents are established and therefore, convenient for the scientist to use. However, it

is important to check the identity of any newly acquired cell line for species of origin, tissue of origin and the maintenance of specific properties.

Once a cell line has been established, its karyotype has to be determined. This will confirm the species of origin, at least for those cells karyotyped, and determine the extent of gross chromosomal changes in the line. Karyotypes may vary from being near normal (i.e., the vast majority of cells in the culture have normal karyotypes) to being aneuploid. While a normal karyotype is desirable, the presence of an abnormal karyotype does not preclude using the cells for *in vitro* studies, especially if it has been demonstrated that the cells retain the normal function expected of them. If a normal karyotype is required (e.g., cell lines to be used to create transgenic animals), then special care must be taken in handling the cells to minimize chromosomal changes. The stability of the karyotype depends on the species from which the cell lines was derived, the growth conditions used, the way in which the cells are sub-cultured and whether or not the cells are frozen.

Storage and revival of cells

Liquid nitrogen is used for storing cells at very low temperature (-180°C to -196°C). Cells are stored at liquid nitrogen temperatures because the growth of ice crystals is retarded below -130°C. Freezing can be lethal to cells due to the effects of damage by ice crystals, alterations in electrolyte concentration, dehydration and changes in pH. To minimize the effects of freezing, a **cryoprotective agent** such as glycerol or DMSO, is added. A typical freezing medium is 90% serum, 10% DMSO. Further, it is desirable to use healthy cells that are growing in log phase and to replace the medium 24 hours before freezing. Also, the cells should be slowly cooled from room temperature to -80°C to allow the water to move out of the cells before it freezes.

Revival

Frozen cells should be thawed as rapidly as possible by placing the vial containing them into a 37°C water bath with moderate shaking. This is to minimize ice crystal formation, which may damage cells. After thawing the cells can be transferred directly into a tissue culture vessel containing suitable media for further growth.

6.3.4. Methods of Gene Delivery into Cells

A number of methods have been developed over the years for efficient transfer of genes in cell culture. Some common methods of plasmid **DNA transfection** are outlined below:

Calcium phosphate

Here HEPES-buffered saline solution is mixed with a calcium chloride solution containing DNA for transfection to form a fine precipitate of calcium phosphate with DNA. The suspension of the precipitate is then added to the monolayer of cells. The cells take up the calcium-phosphate- DNA complexes by endocytosis and express genes.

Lipofection

In this case, gene is transferred with the help of tiny vesicles of bipolar phospholipids that fuse with the cell membrane, releasing the DNA into the cytoplasm.

Microinjection

It is the most efficient method of gene delivery into cells. Here, DNA is directly injected into the nucleus using a fine glass capillary under a microscope. However this method acquire a great effort as each and every cell has to be injected individually.

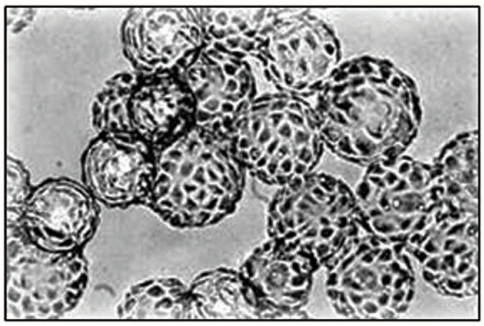
Electroporation

In this method, cells are mixed with the DNA and placed in a small chamber with electrodes connected to a specialized power supply. A brief electric pulse is applied, which is thought to 'punch holes' in the cell membrane, enabling the cell to take up DNA.

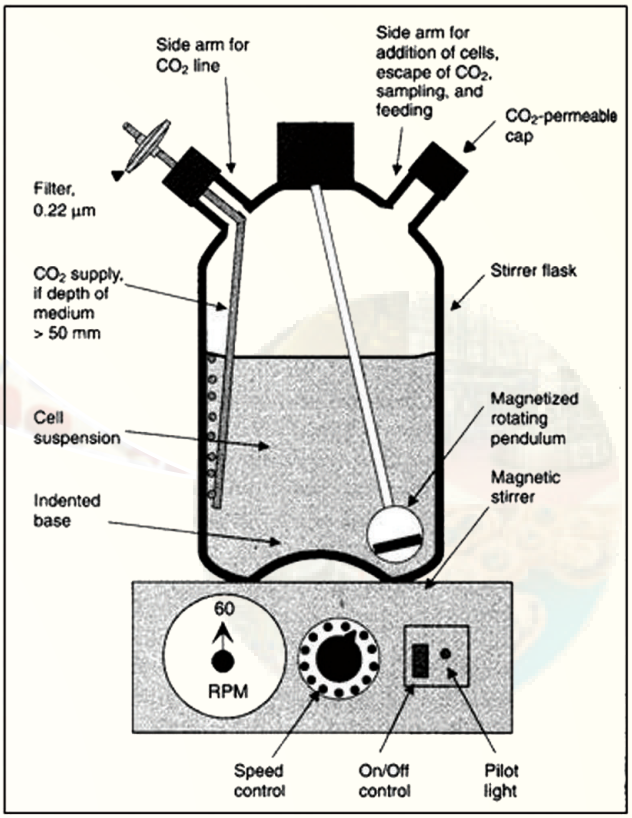
6.3.5. Scale-up of Animal Culture Process

The various scale-up methods include roller bottles with micro carrier beads for adherent cell cultures and spinner flasks for suspension cultures **(Fig. 5).**

A. Roller bottle



B. Microcarrier beads



C. Spinner cell culture

Fig. 5. Tools for scale-up of animal culture process.

Roller bottles

In roller bottles, the cells adhere to the total curved surface area of the micro carrier beads, thereby markedly increasing the available space for growth. These tissue culture bottles can be used in specialized CO_2 incubators with attachments that rotate the bottles along the long axis. After each complete rotation of the bottle, the entire cell monolayer has transiently been exposed to the medium. The volume of medium need only be sufficient to provide a shallow covering over the monolayer **(Fig. 5A)**.

Micro carrier beads

These beads are used to increase the number of adherent cells per flask and are either dextran or glass-based and come in a range of densities and sizes. The beads are buoyant and therefore, can be used with spinner culture flasks. The surface area available for cell growth on these beads is huge **(Fig. 5B)**. Microcarrier beads when re-suspended at the recommended concentration provide an area of 0.24 m^2 for every 100 ml of culture flasks. Under these conditions, adherent cells can be grown to very high densities before crowding becomes a problem. Cells growing at such high densities will rapidly exhaust the medium, which may need replacing the medium during culture.

Spinner cultures

Spinner cultures are used for scaling up the production of suspension cells. They consist of a flat surface glass flask with a suspended central teflon paddle that turns and agitates the medium when placed on a magnetic stirrer. Commercial versions incorporate one or more side arms for sampling and/or decantation. The cells are not allowed to settle to the bottom of the flask and thus cell crowding occurs only at very high densities. Stirring the medium improves gas exchange **(Fig. 5C)**.

6.3.6. Applications of Animal Cell culture

Several medically important protein pharmacueticals have been produced using animal cell culture and recombinant DNA technology. The important ones are listed in **Table 2.**

Table:2

Proteins	Animal Cell Line used	Therapeutic use
Erythropoietin (EPO)	CHO cells	Anemia
Factor VIII	CHO cells	Hemophilia A
Factor IX	CHO cells	Hemophilia B
Follicle Stimulating Hormone (FSH)	CHO cells	Infertility
Human Growth Hormone (hGH)	CHO cells	GH deficiency
Interleukin 2 (IL2)	CHO cells	Cancer therapy
Tissue Plasminogen Activator (t-PA)	CHO cells	Stroke
Monoclonal antibodies (mAbs)	Hybridoma cells	Cancer therapy & Autoimmune diseases

Erythropoietin

Erythropoietin (EPO) is a glycoprotein hormone that is involved in RBC production (erythropoiesis) and wound healing. EPO stimulates the bone marrow to produce more red cells and thereby increase the oxygen-carrying capacity of the blood. It is produced in response to hypoxia (shortage of oxygen) or anoxia (lack of oxygen) caused by anaemia. EPO is useful in the treatment of certain types of anemia such as anemia due to cancer, chronic renal failure and treatment of AIDS. Recombinant human EPO (r-HuEPO) has been produced using Chinese Hamster Ovary (CHO) cell lines. The use of r-HuEPO is advantageous over blood transfusion as it does not require donors or transfusion facilities, and there is no risk of transfusion-associated disease.

Factor VIII

Haemophilia A is a common heritable genetic disorder where the body lacks the ability to produce Factor VIII required for blood clotting. Like EPO, factor VIII is also a glycoprotein and has been produced in CHO cells due to its large structure.

Factor IX

Hemophilia B or Christmas disease is the second most common type of bleeding disorder due to deficiency of factor IX. Recombinant Factor IX produced in CHO cells is used to treat haemophilia B.

Tissue Plasminogen Activator (tPA)

tPA is a serine protease that catalyzes the conversion of plasminogen to plasmin which is responsible for dissolving blood clots. It is approved for use in certain patients having a heart attack or stroke. tPA is the first drug to be produced through mammalian cell culture **(Fig. 6)**.

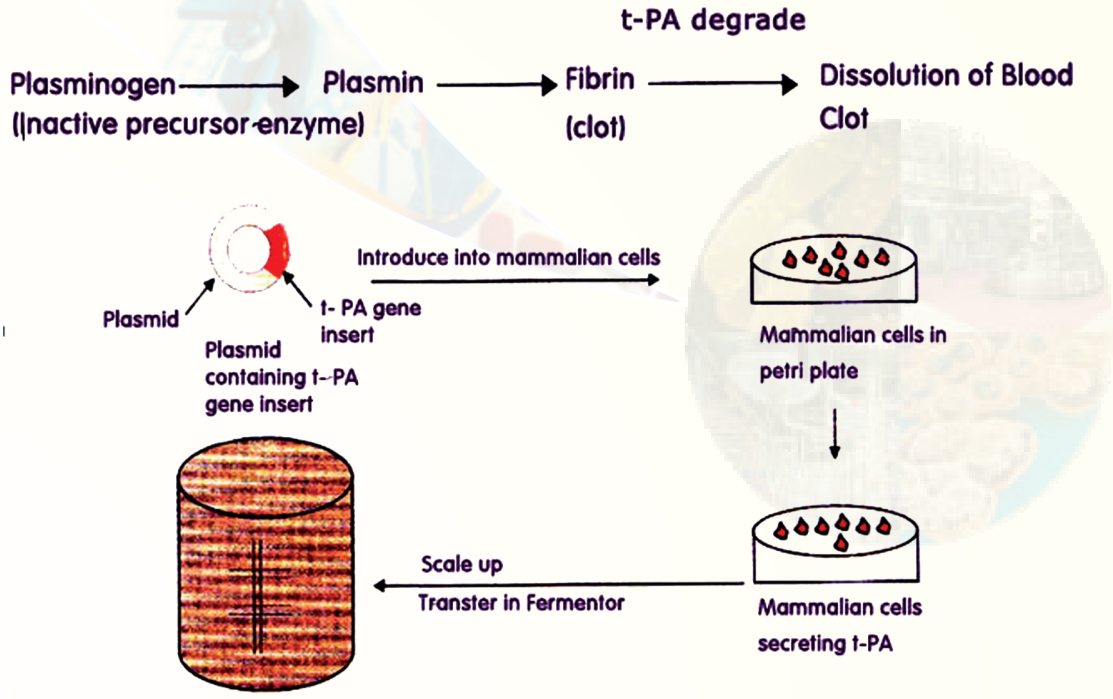


Fig. 6. Production and mode of action of tPA.

Hybridoma Technology for Monoclonal Antibody Production

It has been observed that antibodies bind to specific domains of macromolecules **(antigens)** known as **epitopes**. Antibodies present in serum are a heterologous population released by different populations of B-lymphocytes and therefore are known as polyclonal antibodies. Monoclonal antibodies (mAbs), on the other hand bind specifically to an epitope on an antigen and therefore are useful in detecting specific antigens **(diagnostics)** or blocking their binding by other molecules. mAbs are produced by antigen-activated B lymphocytes that have been immortalised by hybridising (fusing) them with a myeloma cell (cancerous lymphocyte). **Ceasar Milstein** and **George Kohler** (Nobel Prize winners) developed **hybridoma technology** by fusing antibody producing B cells with myeloma cells using polyethylene glycol. The hybrid cells retain the ability of B cells to secrete antibody and the ability of myeloma cells to grow indefinitely. The hybrid clones when grown in culture produces epitope-specific mAb **(Fig. 7)**. This technology has revolutionised the area of diagnostics and antibody-based therapies. The availability of monoclonal antibodies has helped in early detection of many infectious diseases like hepatitis and AIDS.

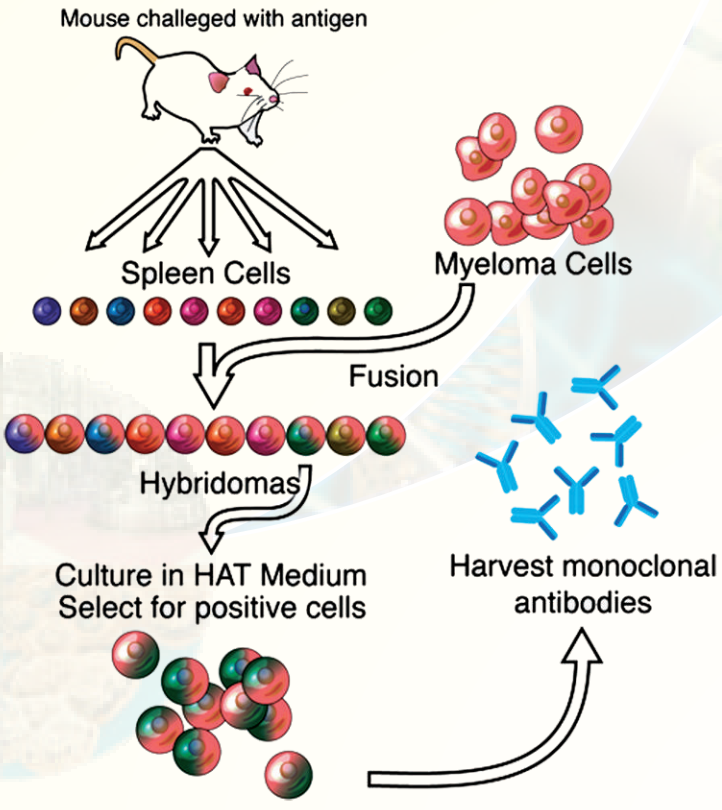


Fig. 7. Production of monoclonal antibodies.

Therapeutic mAb - OKT3

OKT-3 is monab-CD3, an immunosuppressant drug given intravenously to reverse acute rejection of transplanted organs such as heart, kidney and liver. OKT3 is the first monoclonal antibody to be used for the treatment of patients. OKT3 acts by blocking the function of T cells which play a major role in acute graft rejection **(Fig. 8)**. OKT3 binds and blocks the function of a cell surface molecule called CD3 in T cells. The binding of OKT3 to T cells results is followed by blocking of their functions. After OKT3 therapy is over, T cell function usually returns to normal within a week.

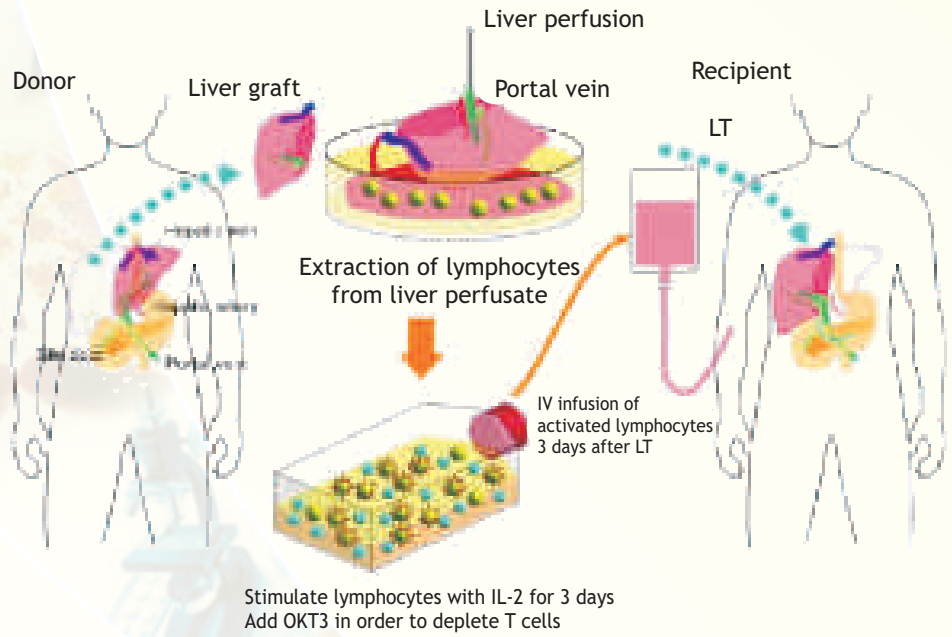


Fig. 8. Adoptive immunotherapy with OKT3 during liver transplantation (LT).

Therapeutic mAb - Herceptin

Herceptin (trastuzumab) is a monoclonal antibody approved for therapy of early-stage breast cancer that is Human Epidermal growth factor Receptor 2-positive (HER2+). These cell surface receptors receive signals that help cells to grow and multiply. Herceptin works by attaching itself to HER2 receptors by blocking them from receiving growth signals. The result is impaired growth of breast cancer.

6.3.7. Stem Cell Technology

Stem cells are characterized by their ability to renew themselves through mitotic cell division and differentiate into a diverse range of specialized cell types. Stem cells are found in all multi cellular organisms. Stem cells are like good shares in the stock market which can either be multiplied **(self renewal)** by getting bonus shares or sold to buy goods **(differentiate)**. Tissues like skin, blood and intestinal epithelium are subject to continuous renewal throughout life and

must maintain an adequate number of cells (stem cells) that retain the potential to proliferate to make good such losses. The most well studied process has been the formation of blood cells (haematopoiesis). It was known in case of mouse that haematopoiesis occurs in the spleen and bone marrow. In human being about 100,000 haematopoietic stem cells produce one billion RBC, one billion platelets, one million T cells, one million B cells per Kg body weight per day. The field of stem cell research was established in 1960s by **Ernest McCulloch** and **James Till** at the University of Toronto.

The two broad types of mammalian stem cells are: **embryonic stem (ES) cells** that are isolated from the inner cell mass of blastocysts, and **adult stem cells** that are found in adult tissues. The ES cells are **pleuripotent** and can differentiate into all types of specialized tissues **(Fig. 9)**. The adult stem cells are **multipotent** (lineage restricted) and act as a repair system for the body by maintaining the normal turnover of regenerative organs, such as, blood, skin, or intestinal tissues **(Fig. 10)**. Stem cells are now routinely grown and transformed into specialized cells such as, muscles or nerves through cell culture and used in medical therapies. The stem cells are useful in many medical conditions where cells are either dead or injured or abnormal, such as:

- Leukemia (cancerous blood cells).
- Heart disease, heart attack (cardiac tissue damage).
- Paralysis (spinal cord injury).
- Alzheimer's, Parkinson's, Huntington's (dead brain cells).
- Burns (damaged skin cells).

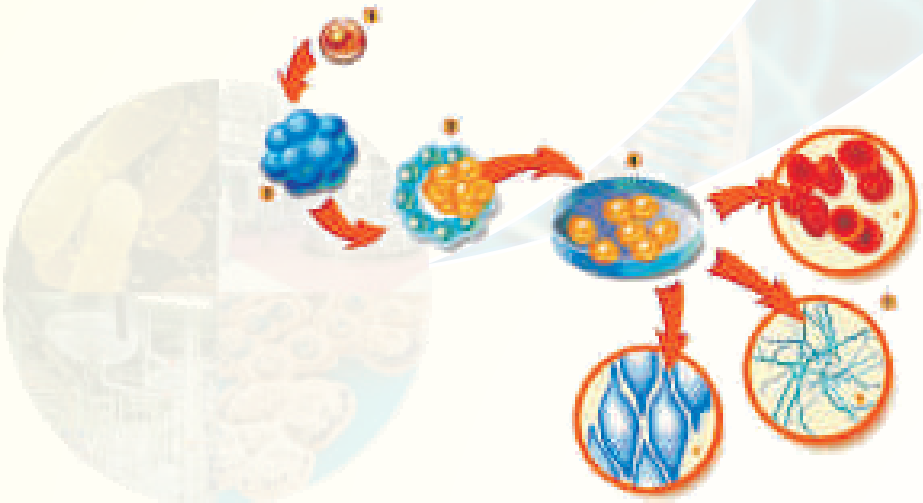


Fig. 9. Cultivation of Embryonic Stem cells. 1, *In vitro* fertilized eggs; 2, Morula; 3, Blastula with inner stem cell mass; 4, Cultured undifferentiated stem cells; 5, Differentiated cells - (a) blood, (b) neural, and (c) muscle cells

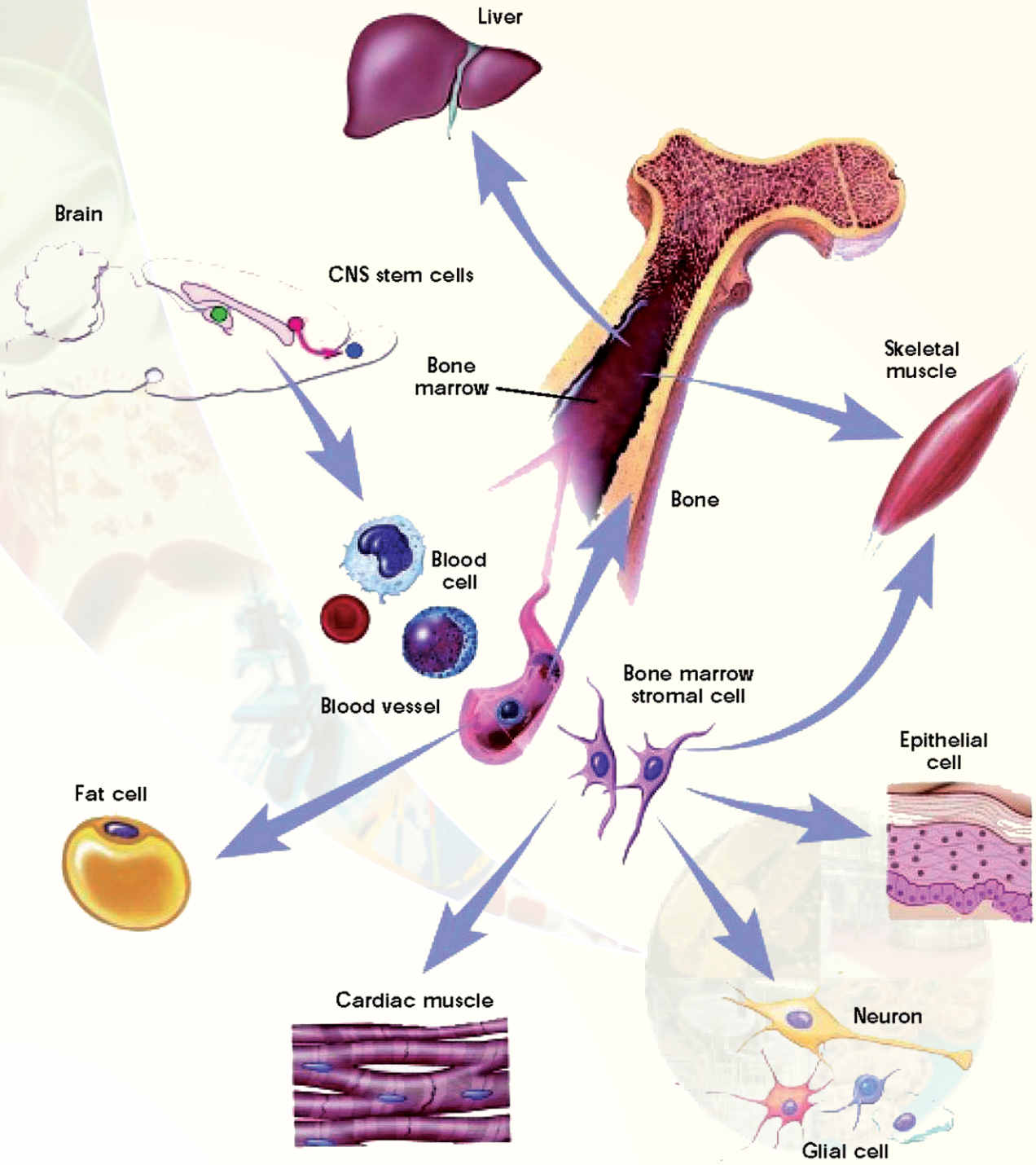


Fig. 10. Cultivation of adult stem cells from bone marrow and their differentiation into specialized cells.

ES Cell culture and its applications

The ES cells are cell lines derived directly from the inner cell mass of growing embryos without use of immortalising or transforming agents. The **inner cell mass** (ICM) of embryos can be maintained in cell culture in the presence of irradiated fibroblast cells. The stem cells:

a. retain the characteristics of founder cells, even after prolonged culture and extensive manipulation.

b. reintegrate fully into embryogenesis if transferred.

c. could be used to create chimeric mice by taking ES cells from a black mouse and implant it into the embryo of an albino mouse (white). The progeny so developed had skin color of black and white (a chimera **Fig. 11**).

d. could maintain a stable euploid karyotype.

e. could self renew without differentiating in culture.

Now it is possible to selectively remove a gene **(gene knock outs)** and make other precise genetic modifications in the mouse ES cells and create mouse models of human diseases. Such mouse models have been extremely useful not only in understanding the genetic basis of a disease but also in search for new diagnostic and therapeutic modalities.

In 1998, **James Thomson** developed a technique to isolate and grow human ES cells in culture. The human ES cells can be derived from the inner cell mass of blastocyst or from human germ cells before they initiate meiosis and cultured in a petri dish. Specialised cells can be grown in the presence of specific growth factors such as fibroblast growth factor and platelet-derived growth factor. The human ES cells have opened new possibilities for stem cell therapy in clinics.

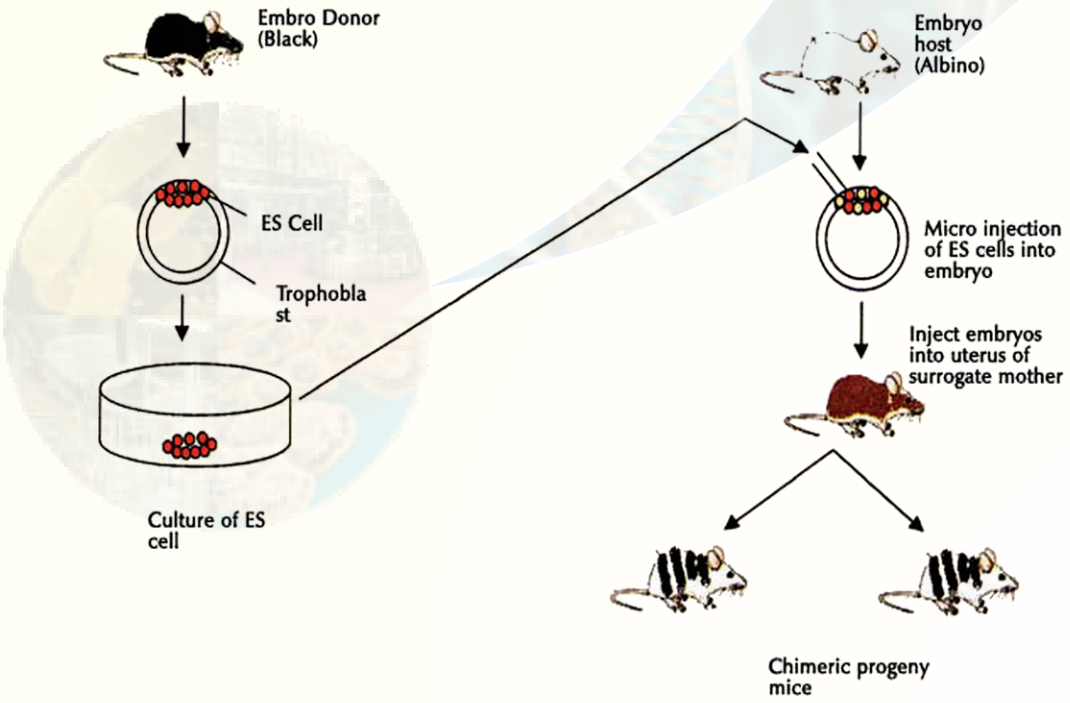


Fig. 11. Creation of chimeric mice using ES cells

6.3.8. Tissue engineering

Recent advances in the fields of cell biology, biomedical engineering and materials science have given rise to the inter-disciplinary field of tissue engineering. The aim of tissue engineering is to supply body parts for repair of damaged tissue and organs, without causing an immune response or infection or mutilating other parts of the body. Tissue engineering potentially offers dramatic improvements in low-cost medical care for hundreds of thousands of patients annually. Large-scale culturing of human or animal cells-including skin, muscle, cartilage, bone, marrow, endothelial and stem cells-may provide substitutes to replace damaged components in humans. Naturally derived or synthetic materials may be engineered into "scaffolds" that when implanted in the body could provide a template that allows the body's own cells to grow and form new tissues **(Fig. 12)**. Such implants could function like neo-organs in patients without triggering immune responses. **Genetically-modified animals** may also provide a source of cells, tissues, and organs for xenografts.

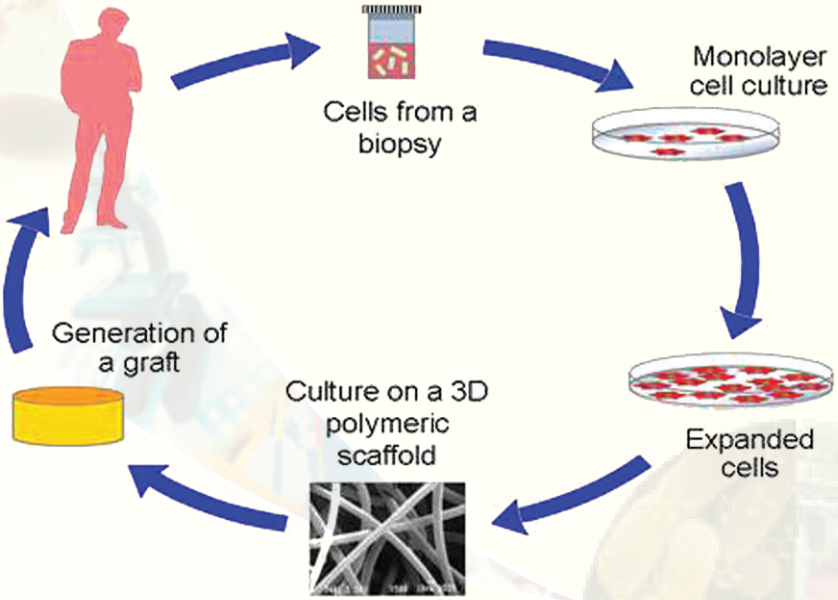


Fig. 12. Basic principle of Tissue engineering.

Review Questions

1. Name two important products from Animal Cell Culture Technology. What are their functions?
2. Write two important features of cultured animal cells. Differentiate between primary and secondary cell cultures.
3. What are cell lines? How the growth characteristics of cell lines determined?

4. What is the importance of pH while culturing animal cells? How is the pH maintained in culture media?
5. What are some of the characteristic features of normal and transformed cells?
6. What is the role of serum for culturing animal cells?
7. Why are $C0_2$ incubators required for animal cell culture?
8. Differentiate between roller bottle and spinner cultures?
9. How are animal cells cryopreserved? Give two examples of cryopreservatives.
10. How is erythropoietin produced by animal cell culture? Write down the procedure involved.
11. What is the mode of action of tPA? How is it produced by animal cell culture technology?
12. How are monoclonal antibodies different from polyclonal antibodies? Write one therapeutic application of monoclonal antibody.
13. What are stem cells? Describe the application of embryonic stem cell technology.
14. What is gene knock out? How is this useful in generating genetic models of human disease?
15. What is meant by tissue engineering? Discuss some important medical applications of tissue engineering.

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