UNIT-12: APPLIED BIOLOGY

A. Microbial fermentation and production of small and macro molecules.

Fermentation has always been an important part of our lives: foods can be spoiled by microbial fermentations, foods can be made by microbial fermentations, and muscle cells use fermentation to provide us with quick responses. Fermentation could be called the staff of life because it gives us the basic food, bread. But how fermentation actually works was not understood until the work of Louis Pasteur in the latter par t of the nineteenth century and the research which followed. Fermentation is the process that produces alcoholic beverages or acidic dairy products. For a cell, fermentation is a way of getting energy without using oxygen. In general, fermentation involves the breaking down of complex organic substances into simpler ones. The microbial or animal cell obtains energy through glycolysis, splitting a sugar molecule and removing electrons from the molecule. The electrons are then passed to an organic molecule such as pyruvic acid. This results in the formation of a waste product that is excreted from the cell. Waste products formed in this way include ethyl alcohol, butyl alcohol, lactic acid, and acetone-the substances vital to our utilization of fermentation

Role of Fermentation in Industry

In industry, as well as other areas, the uses of fermentation progressed rapidly after Pasteur's discoveries. Between 1900 and 1930, ethyl alcohol and butyl alcohol were the most important industrial fermentations in the world. But by the 1960s, chemical synthesis of alcohols and other solvents were less expensive and interest in fermentations diminished. Questions can be raised about chemical synthesis, however. Chemical manufacture of organic molecules such as alcohols and acetone rely on starting materials made from petroleum. Petroleum is a nonrenewable resource; dependence on such resources could be considered short-sighted. Additionally, the use of petroleum has associated environmental and political problems.

The worldwide interest in microbial fermentations is once again growing especially with reference to renewable resources and microbial biocatalysts. Plant starch, cellulose from agricultural waste, and whey from cheese manufacture are abundant and renewable sources of fermentable carbohydrates. Additionally these materials, not utilized, represent solid waste that must be buried in dumps or treated with waste water.

Other Benefits Microbial Fermentations

Microbial fermentations have several other benefits. For one, they don't use toxic reagents or require the addition of intermediate reagents. Microbiologists are now looking for naturally occurring microbes that produce desired chemicals. In addition, they are now capable of engineering microbes to enhance production of these chemicals. In recent years, microbial fermentations have been revolutionized by the application of genetically-engineered organisms. Many fermentations use bacteria but a growing number involve culturing mammalian cells. Some examples of products currently produced by fermentation are listed in Tables 1 and 2 .

Table 1.1 Fermentations by Naturally-Occurring Organisms

PRODUCT	APPLICATION	ORGANISM
Bacitracin	Antiobiotic	Bacillus subtilis (bacterium)
Chloramphenicol	Antiobiotic	Streptomyces venezuelae (bacterium)
Citric acid	Food flavoring	Aspergillus niger (fungus)
Erythromycin	Antibiotic	Streptomyces erythaeus (bacterium)
Invertase	Candy	Saccharomyces cerevisiae (fungi)
Lactase	Digestive aid	Escherichia coli (bacterium)
Neomycin	Antibiotic	Streptomyces fradiae (bacterium)
Pectinase	Fruit juice	Aspergillus niger (fungus)
Penicillin	Antibiotic	Penicillium notatum (fungus)
Riboflavin	Vitamin	Ashbya gossypii (fungus)
Streptomycin	Antibiotic	Streptomyces griseus (bacterium)
Subtilisins	Laundry detergent	Bacillus subtilis (bacterium)
Tetracycline	Antibiotic	Streptomyces aureofaciens (bacterium)

Table 1. 2 Fermentations by Genetically Engineered Organisms

PRODUCT	APPLICATION	ORGANISM
B. growth hormone	Milk production(cows)	*Escherichia coli* (E. coli)
Cellulase	Cellulose	*E. coli*
H. growth hormone	Growth deficiencies	*E.coli*
Human insulin	Diabetics	*E. coli*
Monoclonal antibodies	Therapeutics	Mammalian cell culture
Ice-minus	Prevents ice on plants	*Pseudomonas syringae*
Sno-max	Makes snow	*Pseudomonas syringae*
t-PA	Blood clots	Mammalian cell culture
Tumor necrosis factor	Dissolves tumor cells	*E.coli*

Fermentation Work in Biotechnology

In the pharmaceutical and biotechnology industries, fermentation is any large-scale cultivation of microbes or other single cells, occurring with or without air. In the teaching lab or at the research bench, fermentation is often demonstrated in a test tube, flask, or bottle-in volumes from a few milliliters to two liters. At the production and manufacturing level, large vessels called fermentors or bioreactors are used. A bioreactor may hold several liters to several thousand liters.

Bioreactors are equipped with aeration devices as well as nutrients, stirrers, and pH and temperature controls.

A2. Antibiotics and Pharmaceuticals

In the beginning of 20th century, the idea of growth inhibition of one micro-organism present in the vicinity of other one came into existence. Later, it was demonstrated that growth inhibitor of the former micro-organism was mediated by secretion of toxic metabolites by the latter. This toxic metabolite was termed as 'antibiotic' and the phenomenon of act of growth inhibition by antibiotics as 'antibiosis'. The antibiotics are defined as "the complex chemical substances, the secondary metabolites which are produced by microorganisms and act against other microorganisms".

In nature, there is universal distribution of antibiosis among the micro-organisms owing to which they are involved in antagonism. Those micro-organisms which have capacity to produce more antibiotics can survive for longer time than the others producing antibiotics in less amount.

However, antibiotics produced by micro-organisms have been very useful for the Cure certain human diseases caused by bacteria, fungi and protozoa. Due to continuous endeavor, made in this field, the antibiotics discovered at present are about 5,500. Total world production of antibiotics is more than one million tonne per annum. This success has been possible only due to continuous researches made during the last 4 decades. A list of micro-organisms prodcing antibiotics and their applications are given in Table1.

Table 1: Antibiotics produced by microorganisms

Micro-organism	***Antibiotics ****	***Applications***
Bacteria :		
Bacillus brevis	Tyrothricin (G^+, G^-)	Mouth and throat infection
B. polymyxa	Polymyxin B (AT)	UTI, gastroentritis
B. subtilis	Bacitracin (G^+)	Dermatitis, superficial pyogenic, infection
Streptococcus cremoris	Nistin(G^+)	In cheese, food preservation (non-medical use)
Actinomycetes:		
Micromonospora purpurea	Gentamicin (G^+, G^-)	UTI, abscess
Nocardia mediterranei	Rifamycin (My)	Meningitis
Streptomyces griseus	Streptomycin(G^+, G^-, My)	Tuberculosis
S. *aureofaciens*	Tetracyclines *(G^+, G')	Cholera, tetanus, UTI
S. *erythreus*	Erythromycin (G^+)	Cholera, tetanus, arthritis
S. *noursei*	Nystatin (AF)	Skin lesions
S. *spheroids*	Novobiocin (G^+)	Abscess
Fungi:		
Cephalosporium acremonium	Cephalosporins (G^+, G^-)	UTI, pneumonia, meningitis
Penicillium chrysogenum	Penicillin (G^+)	Pneumonia, pharyngitis
P. griseofulvum	Griseofulvin (AF)	Skin and hair lesions
P. notatum	Penicillin (G^+)	Fever, pneumonia, genital infections

UTI, Urinary tract infection; * Antibiotic spectrum; G^+, Gram positive bacteria; G^-, Gram negative bacteria, Mycobacteria, AF, Antifungal; AT, antitumour.

Among the antibiotics discovered so far, there are 4 major groups which are most extensively used throughout the world: the penicillins, cephalosporins, tetracyclines and erythromycins.

It has been already mentioned that antibiotics are produced in culture medium during idiophase due to depletion caused by one or more nutrient (s) in the medium. Perlman (1979) has described that the biosynthesis of antibiotics may be regarded as a result of a series of in-born errors of metabolisms. These errors may be exaggerated by subjecting the original microorganims to mutagenjc substances. However, high yield of commercially important antibiotics owe much to the selection of such mutant stains as improvement of strain of *Penicillium chrysogenum* to yield benzylpenicillin about 20 mglml over the normal rate 720 µg/ml.

Moreover, researches done on this aspect have shown (in U.K.) that the synthesis of some of the antibiotics in Streptomyces was mediated not by a plasmid. Therefore, there is possibility to produce new antibiotics by transfer of plasmids into a single cell of Streptomyces.

At present, there are thousands of antibiotics produced by micro-organisms of which only a few hundreds have been marketed so far. Among these the penicillins, cephalosporins and tetracyclines have been commercialized. In this context penicillin production is described in detail.

Penicillin

Penicillin, because of the impetus of World War II, was the first antibiotic to be produced on a large scale, and it still is one of the best antibiotics available. It is active against many Grain positive bacteria, *Nocardia,* and *Actinomyces,* but not against most Gram negative forms except at higher dosage levels. It interferes with cell-wall synthesis of sensitive organisms and is active only against growing cells. In addition, it presents the favorable characteristic of being almost nontoxic to mammals, except for certain allergic reactions that develop with a small percentage of individuals.

There actually are several penicillins, all closely relate in structure and in activity against sensitive microorganisms. These penicillins have a common chemical nucleus and differ principally in the chemical structure of a side chain attached to this nucleus. The various penicillin fermentations also are unusual in that various compounds resembling the side chains can be added as precursors to the fermentation medium, and these compounds, through microbial action, are directly incorporated into the penicillin molecule. Also, the side chain can be enzymatically removed, liberating the penicillin nucleus so that unnatural side chains can be chemically added to the nucleus in order to create new penicillins. Several different fungi are able to produce one or more of the penicillins, although this activity resides chiefly with the aspergilli and penicillia. Today, however, the principal organisms for commercial penicillin production are highly mutated strains of *Penicillium chrysogenum*.

1. Biochemistry of Penicillin molecule and precursors

Whereas in present day commercial terms penicillin is regarded as penicillin G, penicillin also is a generic name applied to a group of compounds having the same nucleus and approximately similar antibiotic activity characteristics against sensitive microorganisms. The various penicillins differ primarily in the nature of their "R" side chains, which are attached by an amido linkage to the chemical nucleus of the molecule. Study of the R side chain in relation to the use of precursors has been highly profitable to the development of high-yielding penicillin fermentations.

Fleming's original *Penicillium notatum* strain, when grown on his medium, produced largely penicillin F, also known as 2-pentenyl penicillin, in part because he did not utilize precursors in his studies. However, the particular type or types of penicillin produced without added precursors are, to some extent, also a function of the particular mold strain being employed. Thus, descendants of *Penicillium chrysogenum* Q-176 in the absence of precursors produced largely penicillin K with smaller amounts of dihydro penicillin F. As is evident to this point, the medium constituents have a profound effect on penicillin yields. The corn steep liquor provides peptides, amino acids, and amines which are deaminated to provide the ammonia required in the early stages of the fermentation, as well as some of the carbon nutrients. The glucose is rapidly utilized to provide mycelial growth but allows very little penicillin production. The lactose, however, is only slowly degraded to glucose plus galactose, and it is this slow glucose availability from the lactose that allows the starvation conditions required for penicillin production. In fact, a series of publications by Johnson and his coworkers have shown that penicillin yields equivalent to those with lactose can be obtained from glucose alone, if the glucose is added only slowly to the fermentation as required by the mold. In this regard, it is probable that with the relatively lower cost of commercial glucose today, a carbohydrate regimen somewhat similar to that described above, in fact, may be commercially employed in penicillin production. Lipid nutrients also are utilized by the fungus during penicillin production, and fatty oils, such as lard oil, soybean oil, and linseed oil, and fatty acids of greater than 14 carbon chain lengths and their esters are especially effective. Some of the oil is added as antifoam, and the rest is purposely added directly to the medium. These nutrients increase both the amounts of mycelium and yields, but high levels can be deleterious in both early and late stages of the fermentation. Also, these nutrients can provide too great an acidity, but this is usually neutralized by the calcium carbonate of the medium. These oils probably are degraded by the fungus to the two-carbon acetate or similar compound level before being used in formation of mycelium and penicillin.

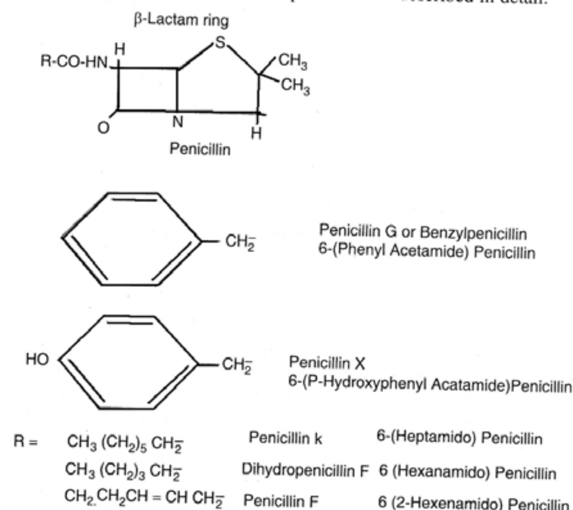
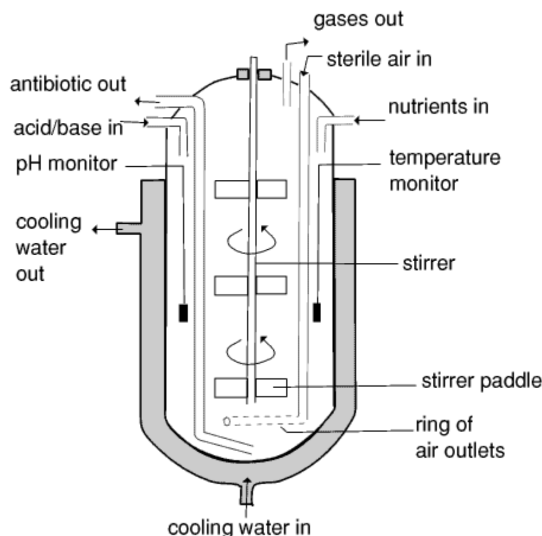


Fig. Chemical structure of penicillins.

Various synthetic media have been developed for penicillin production, and it has been claimed that these media provide penicillin yields equivalent to those from a medium containing cornsteep liquor. Obviously, such media are far too expensive for their industrial use in the production of penicillin, but they have been of value in studies on the mechanisms and factors involved in penicillin production.



2. Recovery of penicillin recovered from the fermented broth

Penicillin in the acid (anion) form is solvent extractable, and the antibiotic, as dissolved in an organic solvent, can be backextracted as a salt into aqueous solution. These considerations, in general, are made use of for the recovery and purification of penicillin from harvested culture broths, although the exact procedures to be used depend somewhat on the particular production medium employed and on the final penicillin yields in this medium. Obviously, high yields in conjunction with a medium that does not interfere with recovery and purification greatly simplify the procedures required to obtain a pure product.

To be more specific, at harvest, the completed penicillin fermentation culture is filtered on rotary vacuum filter to remove the mycelium and other solids although, under the right conditions, this may not even be required. Phosphoric or sulfuric acids are added to lower the pH to 2 to 2.5 in order to convert the penicillin to the anionic form, and the broth is immediately extracted in a Podbielniak countercurrent solvent extractor, with an organic solvent such as amyl acetate, methyl isobutyl ketone, or butyl acetate. The penicillin is then backextracted into water from the organic solvent by adding enough potassium or sodium hydroxide to form a salt of the penicillin, and the resulting aqueous solution is again acidified and reextracted with methyl isobutyl ketone. These shifts between water and solvent aid in purification of the penicillin. The solvent extract finally is carefully back-extracted with aqueous potassium or sodium hydroxide, but more often with sodium hydroxide, and from this aqueous solution various procedures are utilized to convert the penicillin to crystallize as sodium or potassium penicillin. The resulting crystalline penicillin salt then is washed and dried, and the final product must pass rigorous government standards. Spent solvents resulting from the above procedures are recovered for reuse.

3. Production of semisynthetic penicillins and Cephalosporins:

As we have seen, the objective in semisynthetic penicillin production is to generate compounds with improved properties, e.g. acid stability, resistance to enzymatic degradation, broader spectrum of activity, etc.. It involves removal of the side chain of the base penicillin to form 6-APA. This is achieved by passage through a column of immobilized penicillin acylase, usually obtained from *Escherichia coli,* at neutral pH. Penicillin G, for example, is converted to 6-APA and phenylacetic acid. The 6-APA is then chemically acylated with an appropriate side chain to produce semisynthetic penicillin. Yields of cephalosporins from direct fermentations are much lower than those for penicillins. Consequently, as 6-APA can also serve as a precursor of cephalosporins, it is often used as the starting material for their semisynthetic production. A base natural penicillin is converted to 6-APA, as described above, followed by its conversion to the preferred precursor, 7- amino deacetoxycephalosporic acid (7-ADCA), by ring expansion. A suitable side chain can then be readily attached.

4. Future of antibiotic Fermentation:

Antibiotics have found use in medical and veterinary applications, treatment of plant diseases, as an aid in animal nutrition when mixed with feeds or water, and in the preservation of food and other materials. In recent years, however, relatively few new antibiotics have come into commercial production and, in fact, the search for new antibiotic products has been somewhat curtailed. This is due largely to the fact that extensive screening programs have turned up only a few commercially usable antibiotics over those discovered in the 1940's and 1950's, and because any new antibiotic must be better than those already in commercial usage. This is not to say, however, that new antibiotics of great value will not be discovered in the future, although it may be that the approach to discovering valuable new antibiotics may lie in a slightly different direction. Thus, there are many potentially good antibiotics already known which, because of moderate toxicity or some other feature, are not presently usable, and manipulation of the genetic characteristics of the organism, changes in fermentation conditions, or even the use of chemical reactions to alter the structures of the antibiotic molecules might provide a change in the antibiotic that would allow its commercial acceptability. Such altered chemical structures conceivably could provide an additional valuable feature in that sensitive organisms might be less likely to acquire the antibiotic resistance that is characteristic of some antibiotics after long exposure of the cells.

5. Other important health care products:

Ergot alkaloids: Alkaloids are a diverse group of small nitrogen containing organic compounds produced by certain plants and microorganisms.

Many are toxic, but some have various therapeutic properties. Species of the filamentous fungus *Claviceps,* which are pathogens of grasses, produce a range of alkaloids. Some of the best known are the ergot alkaloids. These compounds are produced within the sclerotia (fruiting bodies) of *Claviceps purpurea* that develop naturally when this organism infects developing cereal grains. Infected grains become black and are referred to as ergots. These structures contain indole alkaloids, derived from a tetra cyclic ergo line ring system, which are classified into two groups. Members of the first group are based on clavin and contain no peptide component. Clavin-based alkaloids are also produced by other groups of fungi, including species of *Aspergillus* and *Penicillium.* Some possess antibiotic and antitumour activity, but few are produced commercially. The second group, based on lysergic acid (LSA) and containing a tripeptide or an amino alcohol are found only in *Claviceps* species. Examples include ergometrine, ergocristine, ergosine and ergotamine. Ergotamine, for example, is a structural analogue of serotonin (a neurotransmitter) and is formed from LSA by the action of a peptide synthetase that adds alanine, proline and phenylalanine.

These LSA-based alkaloids have medical roles as analgesics in migraine therapy, as hallucinogens and for treating circulation problems. Others have particular applications in obstetrics, for inducing the smooth muscle of the uterus to contract during labour and after childbirth.

Previously, the alkaloids were extracted from ergots that developed within infected cereal crops, usually rye, or by chemical synthesis. Most are now produced by fermentation of *Claviceps fusiformis, C. paspali* or *C. purpurea* in surface, submerged or immobilized cell culture. Inoculum for the production fermentor may be developed by mycelium or conidiospores. The production medium contains an organic acid of the tricarboxylic acid (TCA) cycle and a carbohydrate, such as citrate and sucrose, the specific combination depending upon the target alkaloid. In later stages, the organic acid stimulates the necessary metabolic change from the TCA cycle to the glyoxylate cycle. Alkaloid production, like that of many secondary metabolites, exhibits phosphate regulation. The synthesis is delayed until the medium phosphate has been utilized during the trophophase and the culture enters the idiophase. However, phosphate inhibition can be overcome by addition of tryptophan or a tryptophan analogue, which act as inducers and precursors.

A3. Microbial Production of Organic Acid

3.1 Citric Acid

Citric acid or 2-hydroxy-1,2,3-propanetricarboxylic acid was first isolated from lemon juice by Scheele (1784). Although it occurs in rather high concentrations in citrus fruits, citric acid is ubiquitous in nature forming as it does an intermediate in the citric acid (Krebs) cycle whereby carbohydrates are oxidized to carbon dioxide. The widespread presence of citric acid in the animal and plant kingdoms is an assurance of its non-toxic nature and it has long been used as an acidulent in the manufacture of soft drinks, as an aid to the setting of jams and in other ways in the confectionery industry.

Production Processes -fermentation

From among the historically used processes for the production of citric acid the following are still important:

a. ***A niger***

- Surface fermentation using beet molasses;
- submerged fermentation using beet or cane molasses or glucose syrup Submerged processes using sucrose as carbohydrate source are also believed to be running in areas where sugar is cheap.

b. Yeast: Submerged fermentation using beet molasses or glucose syrup

Fermentation Media: The use of cane molasses is not practicable in the surface mode because very low yields are obtained both beet and cane molasses are very variable in quality both from season to season and from refinery to refinery. In spite of many investigations no clear reason for this has emerged. probably because the composition of molasses is so complex. It is necessary therefore to make a selection of available molasses on the basis of performance. In order to obtain good yields of citric acid particularly when *A. niger* is used it is especially important to keep available levels of heavy metals including iron and manganese below certain critical levels. This is done in molasses media by making additions of sodium or potassium ferrocyanide. Other inorganic nutrients are supplemented where necessary but most inorganic nutrients are already present in molasses. Where glucose syrup is employed as carbohydrate source heavy metals are removed by ion exchange. Pretreatment of sucrose based media by additions of ferrocyanide have also been proposed. In processes where pure glucose or sucrose is used as the substrate, additions of nitrogen. phosphate and other essential nutrients are made.

Yields: The yield of citric acid in the fermentation is expressed as kg citric acid monohydrate per 100 kg carbohydrate supplied. Yields in the range 70-90%, on this basis have been reported. It should be mentioned that the theoretical yield of citric acid monohydrate from sucrose assuming no carbon is diverted to biomass, carbon dioxide or other by-products, is 123% and that from anhydrous glucose is 117%. Thus up to about three quarters of the supplied carbon is converted to citric acid in good fermentation.

Surface Ferementation with *A. Niger*

Media: The surface fermentation using *A.niger* with beet molasses as raw material is still extensively employed by major manufacturers. Although somewhat labour intensive, the power requirements are less than in the submerged fermentation. Beet molasses is diluted with water to a suitable sugar concentration, e.g. 150 kg m-3, and the pH adjusted. An initial pH of 5 to 7 is usually employed because *A. niger* will not germinate at higher hydrogen ion concentrations. This effect was unknown when media based on sucrose were being used and starting pH values as low as 2 were in some cases employed. The lack of germination in molasses at low pH is ascribed to the presence of acetic acid, which is a normal constituent of molasses. It appears that unionized acetic acid is the species that prevents the germination, acetate being harmless in this respect which explains the effect of pH.

Additional nutrients and alkali ferrocyanide are then added, and whole boiled or otherwise sterilized. After cooling, the prepared medium is run down into a series of trays supported on racks in a ventilated chamber. The trays. which are usually made of very high purity aluminum, are filled to a depth of between 0.05 and 0.20 m.

Inoculum: Spores of *A. niger* are obtained by growing a selected strain on a sporulation medium. The spores are collected and distributed over the surface of the medium in the trays.

Physical Conditions: Sterile air is sup-plied to the fermentation chamber. The air performs the dual function of supplying oxygen and carrying away fermentation heat and the rate of flow of the air is regulated accordingly. A temperature in the region of 30 °C is often employed.

- The mycelium forms a coherent film on the surface of the liquid becoming progressively more convoluted. The removal of the heavy metals by the ferrocyanide severely restricts sporulation.
- After a period of 7 to 15 days the trays are emptied and the mycelium separated from the fermented liquor.
- The liquors are pumped forward to the recovery section.
- Unwanted by-products of the process are gluconic and oxalic acids. In many processes oxalic , acid production is minimized by careful strain selection.

Submerged Fermentations with *A. niger*

Cultivation of A. niger in Carbohydrate Media: Fermentation layout of a possible citric acid plant, consists of

a. medium preparation section.
b. fermenter section
c. section for separating the fermented liquor from the organism

The medium preparation (suitable in this case for molasses) shows an in-line sterilization step but sterilization of the medium in the fermenters is a possible alternative. Where inline sterilization is used the fermenters are sterilized separately.

In the plant shown a vegetative inoculum stage is used where spores of *A. niger* are allowed to germinate in inoculum medium before being transferred to the main fermentation medium in a larger vessel. In some processes the spores arc introduced directly into the main fermentation but in all cases the inoculum stage or the initial-growth stage in the main fermenter is of the utmost importance to the success of the fermentation. The morphology of the mycelium at this point is crucial according to many reports not only in relation to the shape of the hyphae them-selves but also in the aggregation of the growth into small spherical pellets. Thus the hyphae should be abnormally short stubby, forked and bulbous. This state of affairs is brought about by a deficiency of manganese in the medium or the obviously related additions of ferrocyanide ion.

The mycelial pellets should be small (0.2 to 0.5 mm) with a hard surface. Factors leading to the production of such pellets are correct ferro-cyanide level, an iron concentration of less than 1p.p.m, adjustment of pH, adjustment of aeration and agitation, concentration of manganese and amount of spore inoculum. Whether the aggregation of the deformed hyphae into pellets is really necessary is doubtful, especially when stirred fermenters is being used, but the pellet form does give a broth that is more readily mixed Where a separate inoculum stage is employed, a suspension of spores of *A. niger,* usually grown on a solid medium, is introduced into sterilized medium in the inoculum fermenter.

The medium is aerated and, in some processes, agitated and the mould allowed to grow at a tempera-ture of about 30°C for a period of from 18 to 30 hours as judged by pH level reached or in other ways. The fermentation medium is prepared and transferred to the main fermenter and the grown inoculum incorporated at the rate of about 1 m^3 inoculum to 10 m^3 fermentation medium. Where the inoculum or fermentation medium is based on molasses the initial pH is normally in the range 5 to 7. As mentioned above, most A. niger strains do not germinate or grow at lower pH values in this type of medium. On the other hand lower initial pH values can be tolerated in glucose- or sucrose-based media. The fermentation is conducted at about 30°C

Two types of fermenter are in common use, namely the
a. stirred, aerated, baffled tank
b. aerated tower fermenter which has a much higher aspect ratio than the former and often contains an internal draught tube to promote circulation.

Both types are constructed of high grade stain-less steel and have provision for cooling. Both designs are sparged from the base with sterile air, although extra oxygen is sometimes used in the tower type. Sometimes super atmospheric pressure in the fermenter is used to increase the oxygen solution rate. A growth phase is followed by a citric acid producing phase during which only a small amount of growth occurs. When the rate of increase in citric acid concentration has reached a point where it is uneconomic to proceed the fermentation is discontinued and the broth pumped to a centrifuge where the mould is separated from the liquor which is passed forward to the recovery section.

A continuous fermentation with a mould of the *A. niger* group has been studied on the laboratory scale but no commercial production by this type of process is known. Because the accumulation of citric acid is only partly growth associated, and because for economic reasons the substrate sugar must be almost completely utilized. it is necessary to use several fermenters in series which greatly reduces the potential savings of this method.

The Classical Citric Acid Recovery Process

The classical citric acid recovery process, which is particularly suitable for use with the very impure liquors derived from molasses is to heat the fermented liquor and add lime. The insoluble calcium citrate tetrahydrate is precipitated. The washed precipitate is treated in aqueous suspension with H2SO4 yielding an aqueous solution of citric acid and a precipitate of by-product $CaSO_4$ (gypsum). This set of operations has the effect of removing most of the impur-ities either those derived from the substrate or those generated in the fermentation. As mentioned above, the conditions of the concentration/crystallization steps can he varied to produce either the anhydrous acid or the monohydrate.

Solvent Extraction: Solvent extraction is a possible alternative to the classical method but, because the available solvents tend to extract some of the impurities contained in molasses-derived liquors, it is easier to apply to the products from glucose or alkane-based substrates. The advantage of the solvent extraction method is that it avoids the use of lime and H_2SO_4 and the concomitant problem of gypsum disposal. Les Usines de Melle (1961) proposed the use of butan-2-ol as an extractant. Later it was claimed that tributyl phosphate diluted with a minor amount of kerosene could be used. In this process a better recovery could be realized by extracting the citric acid into the solvent at a low temperature, subsequently stripping the solvent with hot water. Another variant of the solvent extraction process is the ion pair extraction system in which the extractant consists of secondary or tertiary amines having in total at least 20 carbon atoms dissolved in a water immiscible solvent. Again the extraction of the citric acid from the fermented liquor is done at a lower temperature (</ 20°C) and the stripping stage at a higher (</ 80 °C). It is believed that this process has been licensed to Miles Laboratories Inc. who have secured FDA approval for the use of a mixture of tridodecylamine, octyl alcohol and isoalkanes. A further development of this process is the use of N-substituted alkyl amides as extractants (Alter and Blumberg, 1981).In fermentations with n-alkanes and in particular where sodium hydroxide is used for pH con-trol, monosodium or trisodium citrate can be directly crystallized from the clarified fermented liquor (Ferrara et al.. 1977). In this case citric acid may be produced from the sodium citrate by electrodialysis.

Effluent Disposal Reference has already been made to the production of gypsum in the calcium citrate precipi-tation method of citric acid recovery. The disposal of this solid waste can pose a problem. A more serious problem is the disposal of the filtrate from the calcium citrate precipitation, especially where molasses is used as the starting material. This waste although non-toxic, has a very high oxygen demand which makes it unacceptable in rivers without treatment. Braun et al. (1979) have proposed the cultivation of yeasts on the effluent producing a material suitable for animal feed. Another possibility is to evaporate the effluent to produce a concentrated molasses-like material usually called condensed molasses solubles (CMS). This material can be used in feedstuff formulations. Another method of treatment is anaerobic digestion which has the advantage of producing a fuel gas.

3.2. Lactic Acid:

Lactic acid (2-hydroxypropanoic acid, 2-hydroxypropionic acid) is organic hydroxy acids which has a widespread natural occurrence. The production history of lactic acid dates back to 1881.

Use of Lactic Acid:
Lactic acid is sold in technical, food and pharmaceutical grades, although most lactic acid meets the food and pharmaceutical requirements. The most common concentrations are 88 and 50% lactic acid. Lactic acid concentrations above 90% are difficult to pump and handle. The higher quality grades have lower concentrations of contaminants such as sugar, metals, chloride, sulfate and ash. Fermentation grade acid generally contains some residual sugars and other impurities from the carbohydrates and nitrogenous nutrients used in the fermentation. Fermen-tation lactic acid generally has a yellow color which is darkest for the concentrated technical grade and a pale yellow for the food grade. The corrosiveness and liquid form of lactic acid may present a handling problem for some users. Thus, some manufacturers supply a powdered form consisting of lactic acid on a calcium lactate base for uses that require a solid form. Aqueous solu-tions of lactic acid are sold in plastic-lined tank cars and drums, and in plastic carboys.

In Medicine: Lactic acid finds medical applications as an intermediate for pharmaceutical manufacture, for adjusting the pH of preparations, and in topical wart medications. Biodegradable plastic made of poly(lactic acid) is used for sutures that do not need to be removed surgically, and has been eva-luated for use as a biodegradable implant for the repair of fractures and other injuries.

In Food: The largest single use of high quality 'heat stable' food or phar-maceutical grade lactic acid is for the production of stearoyl-2-lactylates. Most of this acid is produced synthetically, because residual sugar from the fermentation causes a caramelization color, odor and flavor during the manufacture of the stear-oyl-2-lactylates. Calcium stearoyl-2- lactylate (CSL) is used mostly in baking. CSL acts as a 'dough conditioner' by combining with the gluten in the dough, making it more tolerant to mixing and processing conditions as well as allowing a wider variation of bread ingredients. It also acts as a 'crumb softener' by complexing the starch in the flour which produces baked products with a softer texture. Sodium stearoyl-2-lactylate (SSL) behaves similarly to CSL and also acts as an emulsifier as well. Both CSL and SSL help extend the shelf life of baked products. Stearoyl-2-lactylates are also used as starch conditioners in other food products such as dehydrated potatoes, and as emulsifiers in cosmetics and food products. Stearoyl-2-lactic acid is hard to handle and the lactylate moiety depolymerizes easily; however, a small amount is used in prepared food mixes. Lactylated fatty acid esters of mono- and diglycerides such as glycerol lactopalmitate and glycerollactostearate are used as emulsifiers in cake mixes, bakery products, liquid shortenings and cosmetics. Stearoyl- 2-lactylates and lactylated fatty esters compete with other emulsifiers such as ethoxylated mono- and di-glycerides and succinylated monoglycerides. Lactic acid is mainly used directly as a food ingredient. Perhaps more than 50% of all lactic acid is used for this purpose. Both synthetic and fermentation derived acid are used for this purpose. Lactic acid is used as a food acidulent because it naturally occurs in many foodstuffs, has a mild acid taste, and has no strong flavors or odors of its own. Lactic acid is also used as a preservative, sometimes in combination with other food acids such as propanoic and acetic acid. Lactic acid is generally more expensive to use than other food acids, but it is sometimes preferred because it adds less of its own flavor to the food. Lactic acid is used in brines for processing and packaging foods such as olives, pickles and sauerkraut. Many cheese products such as cheese spreads, cold pack cheese, process cheese and cheese food contain lactic acid. Creamy salad dressings in both liquid and powder form often contain lactic acid. Some powder mixes for preparation of dips, sour cream and cheese cake contain lactic acid. A few meat products such as salami contain lactic acid. Lactic acid's use in soft drinks has been largely replaced by citric acid, phosphoric acid and other food acids in the US. It is still found in a few formulations such as peppertype soft drinks. Lactic acid is used directly in the production of some rye and sourdough breads. It is also used in a few bakery products for its qualities as a preservative. The use of lactic acid in jams, jellies, pie fillings and packaged pectin ppwders has been largely replaced by citric, malic and fumaric acid in the US, although it is still used to some extent in Europe. Lactic acid competes with phosphoric acid for use

Immobilization of Cell

Lactic acid bacteria have been immobilized in gel supports by several investigators.

- Compere and Griffith (1976) used a 3 m high, 5 cm wide column with 0.64 cm berl saddles upon which a mixed culture of yeast and lactobacilli were immobilized in a glutaraldehyde cross-linked gelatin coating. Sour whey was fermented from 1.4% lactic acid to 2.1 % lactic acid with a superficial resi-dence time of 10-20 h.
- Stenroqs etal. (1982) have immobilized L. delbreuckii in calcium alginate beads and used them in continuous flow column reactors. A maximum yield of 97% lactic acid from 4.8% glucose was obtained-with a residence time of 18 hours. Solid calcium carbonate was used as a buffer and the effluent pH was 5.5-5.7. Vick Roy et al. (1982) have immobilized L. del-breuckii ina hollow fiber fermenter. Reactor productivities were as high as 100 kg lactic acid m^{-3} h^{-1}. Excessive growth of the organisms reduced the long term operation of the reactor system.

Recovery Processes

- Lactic acid is sold in three major grades: technical, food (FCC) and pharmaceutical (USP). The grades are listed in order of increasing purity and differ in their recovery processes. More elaborate recovery processes are needed to produce the higher quality material.
- The recov-ery of lactic acid or"lactate salts from the fermentation broth is a large part of the total cost of manufacture.
- Synthetically made lactic acid, may be purified with less effort and thus in the past has been preferred for uses where heat stability is needed.Materials of construction for fermentation and recovery equipment are limited by the very cor-rosive nature of lactic acid, and contribute significantly to the product's final cost.
- Iron, copper, copper alloys, steel, chrome steel is unsatisfactory.
- Silver and tanta-lum are suitable, but too expensive for general use.
- In addition to equipment failure, corrosion increases the number of metal

ions in the product which must be removed for some end uses.

- Wood, especially cypress and pitch pine, are satisfactory for dilute solutions but become dried out when exposed to concentrated solutions.
- Rubber is suitable for low temperature applications.
- Glass and ceramics are resistant, but their brittleness and poor heat transfer properties limit their usefulness.
- Some plastics are softened by warm concentrated lactic acid; however, heresite-lined, saran-lined, teflon-lined and polyester materials have been used. Plasticizers and other additives in plastic and rubber materials may be extracted or decomposed by lactic acid under some con-ditions.
- Advances in plastic, rubber, ceramic, composite materials and metal alloys may provide some new materials choices with more attractive cost and acid resistance.

Steps in Recovery Process

- The first step in all recovery processes is to raise the fermentation liquor's temperature to 80-100 °C and increase the pH to 10 or 11. This procedure kills the organisms, coagulates the pro-teins, solubilizes the calcium lactate, and degrades some of the residual sugars.
- The liquid is then decanted or filtered.
- For some purposes, acidification of this liquor yields a usable product; how-ever, for most applications further processing by one of the following methods is required. It should also be noted that use of cheap but impure raw materials must be weighed against higher purification costs.

Processing of the Filtrate

Filtration, Carbon Treatment and Evaporation

One of the methods for commercially producing lactic acid relies on the fermentation of rela-tively pure sugars with minimal amounts of nitrogenous nutrients. Thus, by using a pure feed-stock the recovery process is simplified.

- After the fermenter broth is filtered, activated vegetable carbon is used to bleach the calcium lac-tate for production of food grade acid. No carbon treatment is used for the technical grade.
- Next, the calcium lactate is evaporated to a 37% concentration at 70°C and 0.57 atm.
- The concentrated lactate is then acidified with 63% sulfuric acid, and the calcium sulfate precipitate is removed by a continuous filter and sent back to the first filter which treats the fermenter liquor.
- The filtered acid is then treated with activated carbon from the filter cakes of the first, third and fourth carbontreatments.
- The carbon from this step is discarded.
- The lactic acid is then evaporated from 8 to 52% or 82% in stainless steel evaporators.
- Technical grade acid is then diluted to 50% 'or 80% and treated with sodium sulfide to remove heavy metals if needed.
- Edible grade acid is diluted to 50 or 80%, bleached with activated carbon for a third time, and treated with sodium sulfide to remove heavy metals.
- It is then bleached a fourth time with carbon before packaging.
- Heavy metals could be removed by ion exchange which may also remove some of the amino acids present. Heavy metals can also be removed by the stoichiometric addition of calcium or sodium ferrocyanide to form insoluble ferrocyanide salts of the heavy metals.

Calcium Lactate Crystallization

Lactic acid may be recovered from fermentations utilizing cruder raw materials such as whey or molasses.

- The filtered liquor from the fermenters was treated with carbon first under slightly alkaline and then under slightly acidic conditions.
- The crude cal-cium lactate liquor was then evaporated under vacuum to a density of about 1.12 kgm $^{-3}$.
- Technical grade acid was made from this liquor after evaporation, acidification, filtration of the precipitated calcium sulfate, carbon treatment and heavy metals precipitation.
- To make higher grades of product the liquor was cooled, crystallized and washed.
- The mother liquor and wash water were also cooled, crystallized and washed.
- The crystals were redissolved and similarly recrystallized as in earlier steps to create purer grades.
- Acids of different purity were made from the different grades of crystals by dissolution in water, acidification, calcium sulfate precipitation, filtration, evaporation, carbon treatment and heavy metals precipitation.

Crystallization is presently performed in a single unit and a stainless steel double effect evaporator is used. Impurities are removed by filtration and carbon treat-ments. The hydration of the final product is controlled in the drying stage.

Liquid-liquid Extraction

The extraction of lactic acid into an immiscible solvent phase has been researched by many investigators. Lactic acid can be purified in this

way from fermentations using crude raw materials. In all such processes the acid must first be extracted from the crude liquor by the sol-vent, and then recovered from the solvent by some means such as back extraction into water or distillation of the solvent-lactic acid mixture.

The extraction solvent should have a low water solubility, a high distribution coefficient for lactic acid, and a low distribution coefficient for impurities such as the residual sugars. The distribution coefficient is defined as the concentration of lactic acid in the solvent phase divided by the concentration of lactic acid in the water phase.

Croda-Bowmans Chemicals Ltd. (England) has used a countercurrent extraction with isopropyl ether as the solvent. The fermentation liquor is filtered and then acidified with sulfuric acid; the calcium sulfate is filtered off. Next, the crude lactic acid is decolorized with acti-vated carbon and then heavy metals, calcium and amino acids are removed by ion exchange. The acid is then evaporated under vacuum before it enters the countercurrent extraction columns. The acid is recovered from the solvent by countercurrent extraction into water. Next, the acid is given additional activated carbon and ion exchange treatments as needed. Lastly, the acid is eva-porated to its final concentration.

The solubility of isopropyl ether in water is low and the loss of solvent is tolerable. Lactic acid refined by liquid-liquid extraction is substantially free from ash, but contains other impurities from the raw materials and needs additional treatment by activated carbon, oxidation and other means.

Distillation of Lactate Esters

High quality lactic acid, substantially free from residual sugars and other impurities, can be prepared by the esterification of lactic acid with a low molecular weight alcohol, distillation of the lactic ester, hydrolysis of the distilled lactate ester to yield the alcohol and lactic acid, and distilla-tion of the alcohol from the regenerated lactic acid.

Dietz et ai. (1947) esterified lactic acid to form an alkyl lactate and then extracted the ester into a solvent such as 1,2- dichloroethane. Filachione and Costello (1952) give details for a procedure to make lactic acid esters directly from ammonium lactate which is the crude fermentation product if ammonia or one of its salts is used to neutralize the acid during the fermentation. Schopmeyer (1954) dis-cusses the operation of a commercial unit that was used to continuously refine lactic acid by the distillation of methyl lactate. The product obtained was ash free and low in all other impurities. Corrosion of the stainless steel columns contaminated the product with iron. Ceramic equipment was found to be unsuitable because of frequent temperature changes and the strong acid. Problems were also encountered with gasket materials.

Other Recovery Processes

Lactic acid may be recovered by the adsorption of lactic acid on solid adsorbents or by the adsorption of lactate on ion exchange resins. Sugimoto et al. (1976) patented a process for the production of L(+)-lactic acid in which strongly acidic and alkaline ion exchange resins were used to separate the acid from the broth.

Zinc or magnesium lactate may be recrystallized and dissolved in water. The zinc may be precipi-tated with hydrogen sulfide and magnesium may be precipitated as magnesium sulfate. An edible grade can be produced by partial oxidation of the crude liquor containing the free acid or lactate salts. Various distillation schemes utilizing steam, hot air, inert gases and vacuum have been unsuccessfully tried. Krumphanzel and Dyr (1964) have examined the use of electrodialysis for the continuous removal of lactic acid from fermentation. Other mobile ionic species in the fer-mentation broth pose a problem for this method.

3.3. ACETIC ACID

Vinegar that is the aqueous solution of acetic acid is a household product. Earlier acetic acid was obtained from natural carbohydrates by biochemical oxidation of carbohydrate and destructive distillation of wood. Today acetic acid is one of the fastest growing commercial organic chemical amongst the important aliphatic intermediates. Microbial production of acetic acid was started to cope up with the increasing industrial demand.

Aerobic Process of Production

The Producer Organism: The empirical approach to the manufacture of vinegar from an alcoholic mash has been known for a long time. Vinegar had been produced by natural fermentation in the Orleans region of France long before the discovery of acetic acid bacteria. The biological nature of ‘mother of vin-egar’ was first suggested by Boerhaave (1732) and in 1822 Persoon first reported a bacterial study of ‘mother of vinegar’ and named the film appearing on the liquid layer ‘Mycoderma’. Kutzing (1837) recognized that minute organisms of the mother liquor were responsible for acetification of alcohol. Pasteur (1868) confirmed Kutzing’s findings and reported that the conversion of wine to vinegar was brought about by one species of bacteria which he named ‘Mycoderma aceti’.

The acetic acid bacteria belong to the family Pseudomonadaceae. The cells are rod-shaped, but elongated, swollen or branched forms may occur. They may be motile or non-motile; they do not form endospores. The species which do not convert ethanol to acetic acid are either peritrichous flagellate (Acetobacter aceti) or non-flagellate (Acetobacter rancens). The young cells are Gram-

negative in character while the older ones are Gram-positive (Asai, 1968).

The 'slow' Process: Vinegar used to be produced by keeping wine in open, partiallyfilled containers (Allgeier, 1960). Later it was found that addition of some vinegar to the partially filled casks speeded up the reaction. This concept was already incorporated into the process known as the 'Orleans process' (1670), the 'French method' or the 'slow process'.

In this process, wooden barrels are partially filled with good quality vinegar which acts as a source of inoculum and wine is added at weekly intervals for four weeks. After five weeks, a portion of vinegar is withdrawn and the same amount of wine is introduced and the process is repeated resulting in a very slow continuous process. Air is admitted through holes at a height just above the surface of the liquid. The acetic acid bacteria grow on the surface of the liquid and a gelatinous zoogloeal mat known as the 'mother of vinegar' is formed; this contains a large number of bacteria. This process was modified by immobilization of the cells on a floating light wooden grating on the surface of the medium so that addition of alcoholic solution did not break the mat. The costly 'slow process' was used extensively for a long time and
was eventually replaced by the 'quick pro-cess'.

The 'quick' Process: The idea that vinegar can be produced. rapidly by trickling wine through packed pumice was discovered by Boerheave in the early part of the nineteenth century. It was improved by Schiizen-bach (1823) to make it the 'quick process', also known as the 'German process', which is the basis for modern methods of manufacture of vinegar using a generator. The generator consists of a wooden or metal-coated tank packed with beech wood shavings on which cells are allowed to grow.

The feed trickles from the top through the wood shavings. A large volume of air is sparged into the tank through perforations in the bottom. Employing 12% (v/v) alcohol, 98% conversion into acetic acid is attained in 5 days by this process (Conner and Allgeier, 1970). This process has been used for the commercial production of vinegar for about a century.

Frings Process:

Major improvements in the quick process took place in 1929 when forced aeration and temper-ature control were introduced and the trickling generator widely used today emerged.

The significant advantages of this process include the following:
1. the cost is low, it is relatively simple and easy to control
2. higher ace-tic acid concentrations are obtained
3. the tank occupies less space
4. evaporation losses are low

The Submerged Culture Process: The application of submerged fermentation to the oxidation of ethanol to acetic acid by Hro-matka and Ebner (1949) was the next technical advance in the commercial production of vinegar. They noted that Acetabacter species in submerged conditions were very sensitive to oxygen-deficiency and that the fermentation stopped when the level of oxygen in the gas phase was less than 5% (Hromatka and Ebner, 1950). The success of this process depends largely on the efficiency of the aeration of the broth. The acetic acid bacteria during submerged exponential fermentation have an average oxygen uptake rate of 7.75 l O_2 per gram cell per hour.

The advantages of submerged cultivation over the trickling generator are:
1. the submerged cultivation permits 30 times faster oxidation of alcohol
2. a smaller reactor volume is needed (about 16% of the trickling generator) to produce an equivalent amount of
vinegar
3. greater efficiency is achieved; yields are 5-8% higher and more than 90% of the theoretical yield is obtained
4. the process can be highly automated
5. increased economy owing to the elimination of clogging by shavings, interruptions, etc. The ratio of productivity to capital invest-ment is much higher in the case of submerged cultivation.

The role of metabolites

The.importance of the addition of yeast extract to the medium with regard to higher ethanol tolerance and higher productivity has already been discussed. It has another important effect, viz. shortening of the lag phase in acetic acid fermentation. Lactic acid, pyruvic acid, glycerol, glycenic acid, α-glycerophosphate, alanine, succinic acid and fumaric acid are reported to exercise such effects; decreased lag effect is accompanied by an increase in the ratio of acid produced to cell growth implying that the flow of metabolism in cells is changed in favour of acetic acid production rather than cell growth. A remarkable acceleration in acetic acid fermentation has been observed by supplying low-priced malt sprouts to the medium

Tetracoccus soyae, a homofermentative lactic acid bacterium, can utilize glycerol as an energy source only under aerobic conditions and both growing and resting cells produce acetic acid as the end product from glycerol (Kawasaki et al., 1975). The acid yield is about 70% and its concentration reaches 12 g l-l. Glycerol can be completely dissimilated to acetic acid; lactic acid is produced initially but it readily undergoes oxidative degradation to acetic acid. Glycerol dissimilation (Figure 15) is catalyzed by inducible enzymes and cells possess high levels of catalase and cytochromes. H2O2 produced in the oxidation of L-glycerol 3-phosphate is decomposed by catalase.

Anaerobic Fermentations

Producer Organism:

The mixed flora popularly known as acid-formers, which are responsible for the conversion of cellulose to volatile fatty acids, comprise two distinct groups of bacterial species: cellulolytic and non-cellulolytic. The interaction between these two groups in acidogenesis is very complex. Bio-synthesis and extracellular cellulase activities are constrained by non-cellulolytic bacteria as om-petition exists between these two groups for the soluble products of hydrolysis. It is likely that the non-cellulolytic organisms provide some nutritional inputs such as vitamins, growth factors and branched chain fatty acids necessary for cellulolytic species.

The first step in acidogenesis is the solubilization of cellulose and hemicellulose. The most active cellulolytic species have been shown to be Gram-negative, short rods (Bacteroides sp.) and cocci (Ruminococcus sp).

Recovery and Purification: The physical methods of separation of acetic acid from water include:

- Fractional distillation
- Azeotropic dehydration distillation
- Solvent extraction
- Extractive distillation
- Caron adsorption

In extractive distillation counter current washing of mixed vapors in a distillation column takes place via descending stream of high boiling point liquid, which is a preferential solvent for one of the components.

The most economic method for the recovery of acetic acid from a dilute aqueous stream is by extraction with a hydrocarbon followed by distillation. To avoid product inhibition in the reactor, a concurrent extraction process is required which will remove acetic acid continuously to keep its level blow the critical point in the fermenter. The extraction methods suggested are ion-exchange resins, solvent extraction and membrane separation. A membrane system has been developed having a hollow fiber membrane device consisting of silicone rubber tubing. This system is resistant to biological attack and does not suffer mechanical fatigue.

Comparative Merits of Aerobic and anaerobic Processes

Advantages of Acidogenic Fermentations over Acetic Acid Fermentations

Acidogenic fermentations have several advantages. Since acidogenesis is a non-sterile fermentation, fermentation costs are less, operation is relatively simple and contamination problems are avoided. Aeration costs are avoided and there is a reduction in equipment design constraints. As it is a mixed flora system, it can utilize cellulose, hemicellulose and other carbohydrates, proteins and lipids to produce volatile fatty acids. A maximum of 3 mol of acetic acid can be obtained from 1 mol of glucose, whereas in the case of an aerobic process only 2 mol of ethanol can be obtained from 1 mol of glucose; two carbon atoms of the glucose molecule are lost as CO_2, so the theoretical yield based on glucose is 1.0 in the case of acidogenesis, whereas for aerobic processes it is 0.66. Conversion of carbon sources to cellular material is minimized. The primary products are volatile fatty acids, a single class of compounds which can be decarboxylated to produce volatile liquid fuel and/or chemical feedstock. The energy requirement for the productin of acids by anaerobiosis is less than that required in conventional chemical processes, so this can be an effec-tive means to supply volatile fatty acids as industrial chemical feedstock.

Advantages of Aerobic Acetic Acid Fermentations over Acidogenesis: Aerobic fermentations have the following advantages over acidogenesis. In acidogenesis it is necessary to suppress methanogenesis so that organic acids are the principal products. Acido-genesis is slower-than the aerobic process. Vinegar is produced only by the aerobic process because in an anaerobic process with acetic acid, propionic and butyric acids are also produced in significant quantities. To comply with the definition of vinegar it is necessary to adopt the aerobic process for vinegar production. Further processing of the mixture of volatile fatty acids is necess-ary to make useful volatile combustible products. A higher concentration of acetic acid (up to 11 %) is obtained in an aerobic process.

A4. Microbial Production of Vitamins

In a bioreactor micro-organisms grow at optimum temperature and increase their biomass with utilisation of carbon and nitrogen sources long with other growth factors. When microbial cells enter in the log phase, they utilise nutrients very fast with subsequent production of primary metabolites. With gradual depletion of nutrients of growth medium, their growth retards and secondary metabolites are secreted.

Primary metabolites are the microbial metabolites which are secreted during trophophase (active growth phase i.e. log phase). Hence, these are called growth-dependent metabolites. It has earlier been mentioned that primary, metabolites, secreted by micro-organism, in turn are required by micro-organisms.

Vitamins

All phototrophic micro-organisms are capable of synthesizing vitamins and other growth stimulating compounds for their vegetative growth by using the chemical constituents from the culture medium. When synthesis of these compounds exceeds beyond their requirement, it accumulates in

cultures; there from it is recovered. Now a-days, commercial exploitation of such micro-organisms is being done which synthesize vitamins on large scale under different cultural conditions. Micro-biologically produced some vitamins are: carotene, precursor of vitamin A (*Blakeslea trispora*), riboflavin (*Ashbya gossypii*), L-sarbose in vitamin C synthesis (*Gluconobacter oxidans*), and vitamin B_{12} (*Bacillus coagulans, B. megaterium, Pseudomonas denitrificans* and *Streptomyces olivaceous*).

VitaminB_{12} (Cyanocobalamine)

In nature, vitamin B_{12} is synthesized by micro-organisms. For industrial production of vitamin a number of bacteria and streptomycetes are used. The amount of vitamin B_{12} produced by them has been estimated about 20 mg/litre. It is used in medicine and feed supplements, and is most essential for human growth. Daily reqirement of vitamin B12 is about 0.001 mg.

(a) Chemical Structure: Vitamin B 12 (cyanocobalamin) contains a molecule of cobinamide linked to a nucleotide which has 5,6-dimethyl benziminazole as its base, instead of a purine or pyrimidine base. The cobinamide molecule has a central atom of cobalt linked to a cyanide group and surrounded by four reduced pyrol rings joined to form a macroring. A number carbon atoms carry methyl or other substituent group (Riviere, 1977).

(b) Commercial Production: Commercially vitamin B 12 is produced in a continuous culture, are two fermenters are used in a series. In each fermenter culture is kept for about 60 hours. Precaution taken is that the first fermenter should be anaerobic, while the second aerobic one. In second fermenter 5,6-dimethyl benziminazol is added continuously.

Sterilized culture medium, containing glucose, com steep, betain (5%), cobalt (5 ppm) and pH 7.5 in fermenter, is inoculated with *Propionibacterium freudenreichii* and allowed to anaerobic fermentation for about 70 hours. During this period cobinamide is produced which accumulates in the broth. Thereafter, 5,6-dimethyl benziminazole (0.1 %) is added to it. The fermenter is then kept further for 50 hours for aerobic fermentation. During this period nucleotide is synthesized and linked with cobinamide molecule to yield about 20 ppm cobalamin. Culture is acidified to pH 2.0 to 3.0, gently heated to 100°C and filtered to remove cell debris. Finally potassium cyanide (5ppm) is added to filtrate to give cyanocobalamin. Generally sodium sulphite is mixed with the solution so that cyanocobalamin could not be oxidized.

Using genetic engineering techniques, it has become possible to obtain mutants of micro-organisms, producing more vitamins than the natural ones. *P. denitrificans* is able to produce 50,000 times more vitamin BI2 than its parental strain (Sasson, 1984).

A5. Production of Amino Acids

Glutamic acid (L-glutamate) was first time discovered in 1908 by K. Ikeda while working on flavouring components of kelp, after acid hydrolysis and fractionltion of kelp and neutralisation with caustic soda. These treatments enhanced the taste of kelp. This was the birth of the use of monosodium glutamate (MSG) as a flavour-enhancing compound. Soon the production of MSG was commercialised. Later on in 1957, S. Udaka and S. Kinoshita isolated a specific bacterium that excreted glutamic acid on a mineral salt medium. It could also be discovered that the isolated bacterium required biotin for secretion of glutamic acid. This bacterium was identified as Corynebacterium glutamicum which is a Gram-positive bacterium. Therefore, glutamic acid production by using C. glutamicum was boosted up. Table 1 shows some of the important amino acids commercially produced for a variety of purposes.

Table 1. Production and main uses of amino acids.

Amino acid	Production method	Uses
L-Glutamate	Fermentation	Flavour enchancer
L-Lysine	Fermentation	Feed additive
D/L -Methionine	Chemical synthesis	Feed additive
L -Asparate	Chemical synthesis	Feed additive
L –Phenyl alanine	Fermentation	Aspartama
L -Threonine	Fermentation	Feed additive
Glycine	Chemical synthesis	Feed additive, sweetner
L -Cysteine	Reduction of cysteine	Food additive
L-Arginine	Fermentation, extraction	Pharmaceutical
L -Leucine	Fermentation, extraction	Pharmaceutical
L -Valine	Fermentation, extraction	Pesticides, Pharmaceutical
L -Tryptophan	Whole cell process	Pharmaceutical
L -Isoleucine	Fermentation, extraction	Pharmaceutical

2.1. Production of L-Glutamate

It has earlier been mentioned that L-glutamate was the first amino acid to be produced and commercialised. *E.coli* and *Bacillus subtilis* are also reported to secrete L-glutamate. However, *C.glutamicum* is still used for its successful production.

2.1.1 Metabolic Pathway of L-glutamate Production: The generation of precursors or metabolites and reduced pyridine nucleotides, C. glutamicum utilises glycolysis, pentose phosphate pathway and citric acid cycle (CAC). But in the anaplerotic reaction of CAC, this bacterium shows a special feature. Glutamic acid is directly derived from α-ketoglutric acid. Hence, a high capability for replenishing the CAC is a prerequisite for production of glutamic acid in high amount.

C. glutamicum secretes phyruvate dehydrogenase (PyrDH) which suffles zcetyl-CoA into CAe. But the two other enzymes (i.e. pyruvate carboxylase and phosphoenophyruvate carbocylase) supply oxaloacetate. Both carboxy lases replace each other and facilitate the conversion of glucose into oxaloacetate. Glutamate dehydrogenase catalyses the reactive ami nation of a-ketoglutrate to yield glutamic acid.

2.1.2 Production Strains: For biotechnological production of glutamic acid, the intracelIularly synthesised amino acid must be released from the celI; but it is not so. The charged glutamic acid is retained in the cytoplasmic membrane. If it is not retained in celI membrane, the celI will not remain viable. As it was mentioned earlier that glutamic acid is secreted outside when biotin is a limiting factor. This can be explained that:

(i) active excretion of glutamic acid is mediated by a carrier, and

(ii) its activity is triggered by the liquid environment of this carrier.

Triggering of active transport by the appropriate molecular environment of the cytoplasmic membrance is important it important. The means that trigger excretion of glutamate are: (i) growth under biotin limiting condition, (ii) addition of local anaesthetic, (iii) addition of penicillin, (iv)addition of surfactant, (v) use of oleic acid auxotrophs, and (vi) use of glycerol auxotrophs.

Biotin is cofactor of the acetyl-CoA carboxylase. Its activity gets decreased with limited supply. This results in diminishing of fatty acid synthesis. Under biotin-limiting condition, phospholipid content sharply declines from 32 to 17 nmol mg^{-1} dry weight. The amount of unsaturated oleic acid increases relave to the saturated palmitic acid by 45%. This shows a severe change in physical state of the lembrance which changes efflux of glutamic acid. In addition, use of oleic acid auxotrophs and Iycerol auxotrophs facilitates the production of MSG from biotin-rich substrate.

In *C. glutamicum* α-ketoglutarate dehydrogenase has a weak enzyme activity and, therefore, it is unstable. Its enzymatic activity is diminished or reduced to 10% when cells are treated with penicillin, surfactant or biotin-limitation. But the activity of glutamate dehydrogenase is not affected. Due to lowering down of α-ketoglutarate dehydrogenase, excess amount of a-ketoglutarate is not converted to succinyl -CoA. This favours the conversion of α-ketoglutarate to glutamate.

2.1.3 Commercial Production: A flow diagram of process is shown in Fig. 1. The production strains are grown in a fermentor in 500 m^3 capacity. The factors that affect glutamate synthesis are pH, dissolved O_2 and ammonium concentration. For conversion of sugar to glutamate, ammonium concentration is necessary, but at high concentration it is inhibitory. Therefore, in the start of fermentauon, low amount of ammonium is added during fermentation. The surfactant (Tween 80) is added to control the onset of excretion of glutamate. On the basis of glucose concentration, 60-70% glutamate is produced. When fermentation is over, broth contains glutamate in the form of ammonium salt. Glutamate is separated through downstream processing and MSG is separated by elution with NaOH solution. Then MSG is crystallised directly.

2.2 Production of Lysine

Lysine is an amino acid essential for animal and human nutrition. It occurs in plant proteins only in low concentrations; addition of lysine can therefore increase the quality of plant foods. The market for lysine is increasing. Lysine is produced today only by microbial processes and a variety of approaches for its production have been developed.

2.2.1. Lysine production via diaminopimelic acid: Lysine-histidine double auxotrophic mutants of Escherichia coli (A TCC 13002) produce diaminopimelic acid (DAP) on a molasses medium with a yield of 19-24 g/l. The entire fermentation solution, including the cell material, is subsequently incubated with Aerobacter aerogenes (ATCC 12409) at 35°C. After 20 hours, the DAP has been quantitatively decarboxylated to L-Iysine. One complication with this procedure is that the DAP formed during the fermentation is a mixture of meso- and LL-forms; since only meso-DAP can be decarboxylated into L-Iysine, the LL-DAP must be transformed into the meso form by racemization before the decarboxylation step. A 10% DL-amino caprolactam solution (pH 8.0) is added to 0.1 % (w/v) acetone-dried cells of Cryptococcus Laurentii and of Achromobacter obae. A conversion efficiency of 99.8% is obtained at .woe after 24 hours. The enzymatic process has been commercialized in Japan by the Toray Com-pany, which has a capacity of over 4000 tons per year. In the Toray process, the enzyme D-α-aminocaprolactam racemase from Achromobacter obae has been expressed in a lysine auxotrophic mutant of E. coli.

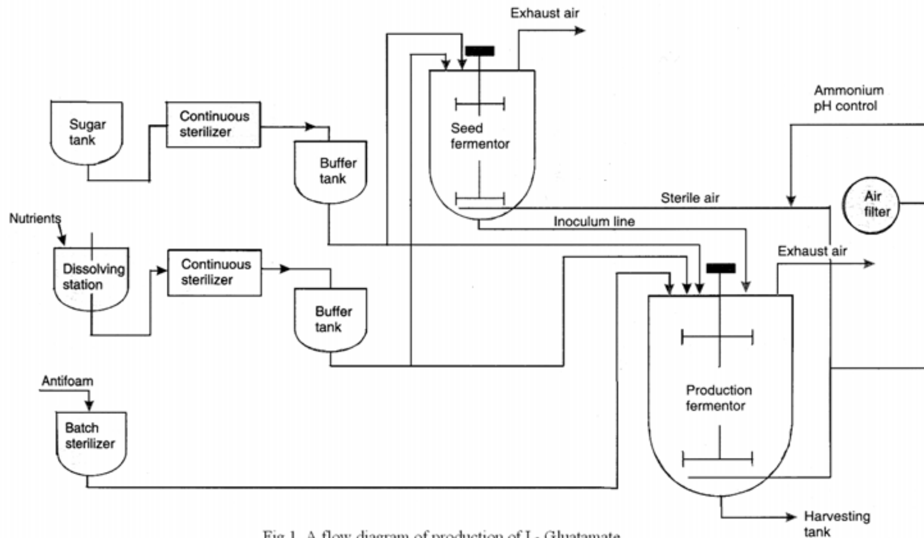


Fig 1. A flow diagram of production of L- Gluatamate

2.2.2. Direct fermentation: Direct fermentation processes are now used worldwide for the production of L-Iysine.

2.2.2.1 Production strains Efficient L-Iysine producers are found among glutamic-acid-producing mutants of Corynebacterium and Brevibacterium which are homoserine auxotrophs or among methionine-threonine double auxotrophs. High lysine producing strains are also found among organisms resistant to the lysine antimetabolite S-(β- aminoethyl)-L-cysteine (AEC). The development of high-yielding strains by mutation to auxotrophy and to antimetabolite resistance has been carried out with B. lactofermentum.

2.2.2.2 Protoplast fusion between high-yielding strains and wild strains of B. lactofermentum, Co-rynebacterium, and Brevibacterium mutants has led to strains with improved growth properties or higher efficiencies.

2.2.2.3 Cloning studies with E. coli, using plasmid pBR322 as a vector, have shown that only in transformed strains that contain the dapA gene does a significant increase in lysine production occur (6.5 g/l). The enzyme coded by dapA, dihydrodipicolinate synthase (DDPS) is therefore indicated as a rate-limiting step for lysine bio-synthesis. Studies have also been carried out on the transformation of C. glutamicum with plasmid pAC2 as vector for the DDPS-encoding gene. Yeasts, such as Candida periculosa, Saccharomyces cerevisiae or Saccharomycopsis lipolytica have been studied extensively for lysine production. Lysine accumulates intracellularly in these organisms at concentrations up to 20% of the dry weight.

However, these yeasts cannot be used successfully in industrial processes because lysine is not excreted into the medium. Biosynthesis and regulation Lysine is synthesized in microorganisms either via .the diaminopimelic acid pathway or the amino adipic acid pathway. However, in any single organism, only one of the two alternatives is used: Bacteria, actinomycetes, cyanobacteria (blue-green algae), some phycomycetes, and protozoa use the DAP pathway. Some phycomycetes, all ascomycetes, all basidiomycetes, and eukaryotic algae use the aminoadipic acid pathway.

Although two organisms may use-the same pathway, the manner in which this pathway is regulated may differ, as shown by the comparison of Escherichia coli and the lysine-producer Corynebacterium glutamicum. In Escherichia coli three distinct regulatory processes are involved:

- Two isoenzymes of homoserine dehydrogen-ase exist which are repressed by L-methio-nine or L-threonine.
- There are three isoenzymes of aspartokinase, one showing repression by L-methionine, the second showing multivalent repression by L--threonine and L-isoleucine in addition to feedback inhibition by L-threonine, and the third showing feedback inhibition and repression by L-lysine.
- Dihydrodipicolinate synthase, the first specific enzyme of lysine biosynthesis, shows feedback inhibition due to L-lysine.

For all three of these enzymatic reactions, regulatory mechanisms must be eliminated to obtain the overproduction of L-lysine which is necessary for its commercial preparation. In contrast to Escherichia coli, the regulatory mechanism for lysine-producing strains, such as Corynebacterium glutamicum or Brevibacterium flavum, is much simpler.

There is only one aspartokinase and one homoserine de-hydrogenase. Aspartokinase is regulated via multivalent feedback inhibition from L-threonine and L-lysine. In both organisms, feedback inhibition de to L-threonine and

repression due to L-methionine regulate the homoserine dehydrogenase. Biosynthesis from aspartate semialdehyde to L-lysine has already been accomplished in wild strains without regulatory control. Thus good lysine- producers are classified among three mutant types:

- The flux of aspartate semialdehyde to threonine is reduced in homoserine auxotrophs which have a block in the homoserine dehydrogenase. Because of the low threonine content in the cell, the multivalent feedback inhibition of aspartokinase is prevented de-spite increased lysine formation.
- The same effect is obtained with a mutant in which homoserine dehydrogenase is super-sensitive to feedback inhibition through threonine.
- In comparison to the wild type, the aspartokinase of AEC resistant mutants is insensitive to multivalent feedback inhibition. Excess aspartate semialdehyde flows into lysine biosynthesis, because the conversion to L-threonine is prevented through feedback inhibition. In this simply regulated system, strains with increased lysine formation can be developed in just a few mutation steps. Lysine excretion is accomplished through active transport. In some organisms, a loss of lysine through decarboxylation to cadaverine occurs, but this destruction does not occur in Corynebacterium or Brevibacterium strains.

2.2.3. Conditions for commercial production

1. Sugar cane molasses is primarily used as a carbon source in industrial production, acetate, ethanol or alkanes can also be used.
2. Gaseous ammonia or ammonium salts are used as nitrogen sources; urea is also used if the producing microorganisms has urease activity.
3. Growth factors L-homoserine or L-threonine and L-methionine must be added, but in suboptimal concentration to avoid undesirable regulatory effects.
4. Soy protein hydroly-sates or other inexpensive protein sources are frequently used. The biotin content in the medium must be over 30 μg/l for optimal lysine production. The biotin content in sugar cane molasses is usually high enough, but if sugar beet molasses or starch hydrolysates are used, biotin must be added.

The lysine yield is 44 g/l and conversion rate in relation to the sugar used approaches a maximum of 30-40%.

A6. Microbial Production of Proteins

The dried cells of micro-organisms (algae, bacteria, actinomycetes and fungi) used as food or feed are collectively known as '**microbial protein**'. Since the ancient times a number of micro organisms have been used as a part of diet. Fermented yeast (*Saccharomyces* sp.) was recovered as a leavening agent for bread as early as 2500 B.C. (Frey, 1930). Fermented milk and cheese produced by lactic acid bacteria (*Lactobacillus* and *Streptococcus*) was used by Egyptians and Greeks during 50-100 B.C. e.g. Lactobacillus and Streptococcus. Cultured dairy products contain 107 to 1010 lactic acid bacteria per gram of product (Pederson, 1971). During the first century B.C. the palatability of edible mushrooms was also realized in Rome. In 16th Century blue-green algae (e.g. Spirulina) was consumed as a major source of protein (Clement, 1968).

The term 'microbial protein' was replaced by a new term "single cell protein" (SCP) during the First International Conference on microbial protein held in 1967 at the Massachusetts Institute of Technology (MIT), Cambridge, Massachusetts, U.S.A. (Scrimshaw, 1968). Criteria for coining this term was the single celled habit of micro-organisms used as food and feed.

In 1973, when Second International Conference was convened at MIT, some actinomycetes and filamentous fungi were reported to produce protein from various substrates. Since then many filamentous fungi have been reported to produce protein. Therefore, the term SCP is not logical, if an organism produces filaments.

Since the 1920s, filamentous fungi have been used for the production of protein (Thatcher, 1954). For such fungi, the term 'fungal protein' has been used by many workers. Recently, the term 'mycoprotein' has been introduced by Ranks Hovis McDougall (RHM) in the United Kingdom for protein produced on glucose or starch substrates.

Importance of mass production of micro-organisms as a direct source of microbial protein was realized during World War I in Germany and consequently, baker's yeast *(S. cerevisiae*) was produced in an aerated molasses medium supplemented with ammonium salts. During World War II (1939-1945) the aerobic yeasts (e.g. *Candida utilis*) were produced for food and feed in Germany. Since World War II, considerable effort has been made to develop technologies for mass cultivation of SCP by formulating different types of growth media and improved culture of micro-organisms.

In the late 1950s, British Petroleum started producing the SCP from hydrocarbons since the crude oil contains 10-25 per cent n-alkanes (paraffins) and established a first large scale plant in Sardinia at the end of 1975. It had a capacity of 1,00,000 tonnes SCP per annum. Large scale production has been envisaged in England and

Rumania with the annual production of 60,000 tonnes bacterial mass in England. The erstwhile U.S.S.R. was the World's largest producer of SCP in 1980. The production was estimated to 1.1 million tonnes of SCP per annum (Carter, 1981).

In India, little attention has been paid on the production of SCP, though mushroom cultivation started in the early 1950s. However, work on mushroom culture at Solan (Himachal Pradesh) from 1970 onward has brought satisfactory results. Recently, National Botanical Research Institute (NBRI), Lucknow and Central Food Technological Research Institute (CFfRI), Mysore, have established Centres for mass production of SCP from cyanobacteria. At the NBRI, SCP is produced on sewage which is further utilized as animal feed (Anonymous, 1980).

Therefore, in the light of protein shortage, micro-organisms offer many possibilities for protein production. They can be used to replace totally or partially the valuable amount of conventional vegetable and animal protein feed. For this, development of technologies to utilize the waste products would play a major role for the production of SCP (Roth, 1982).

3.1. Advantages of producing microbial protein: Roth (1982) has described a number of advantages in the production of microbial protein, compared to protein problems of conventional crops used as food and feed. These include:

- Rapid succession of generations (algae, 2-6h; yeast, 1-3h; bacteria, 0.5-2h) ;
- Easily modifiable genetically (e.g. for composition of amino acids);
- High protein content of 43-85 per cent in the dry mass ;
- Broad spectrum of original raw material used for the production, which also includes waste products;
- Production in continuous cultures, consistent quality not dependent on climate in determinable amount, low land requirements, ecologically beneficial. Other advantages are : (a) high solar energy conversion efficiency per unit area (net production in cultivated land-290 gC/m^2/day, lakes and streams - 225 gC/m^2/day ; estuaries - 810 gC/m^2/day), (b) easy regulation of environmental factors e.g. physical, nutritional, etc. which maximize solar energy conversion efficiency and yield (c) cellular, molecular and genetic alterations and (d) algal culture in space, which is normally unused instead of competing for land.

3.2 Micro-organisms: Many groups of micro-organisms are used as sources of proteins. Some of the micro-organisms with their carbon and energy sources are given in Table 1.

Table 1: Single cell protein (SCP) and mycoprotein produced on the selected substrates.

Micro-organisms groups	Protein (% per l00g on dry weight basis)	Substrates
Algae		
Chlorella pyrenoidosa (36)[a]	-	CO_2 (10%), light
Scenedesmus acutus (20)[b]	-	CO_2, sunlight
Spirutina maxima (l5)[b]	53	CO_2 (5%), combustion gases, bicarbonate, sunlight (in pond)
Bacteria		
Achromobacter delvacvate	-	Diesel oil in fermenter
Bacillus megaterium	-	Collagen meat packing waste in fermenter
Cellumonas sp. (0.45)[c]	87	Bagasse
Methylomonas clara (0.5)[c]	13	Methanol
Pseudomonas sp. (1.0)[c]	-	n-alkanes fuel oil
Actinomycetes		
Nocardia sp. (0.98) [c]	-	n-alkanes
Thermomonospora fusca (O.4) [c]	5.6	Cellulose pulp
Fungi		
Yeasts		
Candida tipolytica (0.88) [c]	65-69	n-alkanes
C. utitis (0.39) [c]	-	Potato starch waste
B. utitis	54	Sulphite liquor
Saccharomyces cerevisiae (0.5) [c]	53	Molasses
Saccharomyces cerevisiae (0.5)[c]	45	Beer
S. fragitis	54	Milk whey
Rhodotorula glutinis	-	Domestic sewage
Torulopsis sp.	-	Methanol

Moulds		
Aspergillus niger	0	Molasses
Trichoderma viride	4	Straw, starch
Paecitomyces varioti	55	Sulphite waste liquor
Mushrooms		
Agaricus campestris	6-45	Glucose
Morchella crassipes	1	Glucose, cheese whey, liquor.

(a) yield g/day (on dry weight basis); (b) yield g/m^2/day (on dry weight basis); (c) yield dry weight basis (g/g substrate used). Values in parentheses denote yield of biomass.

3.3. Substrates used for production of SCP: A variety of substrates are used for SCP production. However, availability of necessary substrates is of considerable biological and economic importance for the production of SCP. Algae which contain chlorophylls, do not require organic wastes. They use free energy from sunlight and carbon-dioxide from air, while bacteria (except photoautotrophs) and fungi require organic wastes, as they do not contain chlorophylls.

The major components of substrates are the raw materials which contain sugars (sugarcane, sugarbeet and their processed products), starch (grains, tapioca, potato, and their by-products), lignocelluloses from woody plants and herbs having residues with nitrogen and phosphorus contents and other raw materials (whey and refuses from processed food). Organic wastes are also generated by certain industries and are rich in aromatic compounds or hydrocarbons. Recent price-increase in petroleum and refined petroleum products has made hydrocarbons and chemicals derived from them (such as methanol and ethanol) less attractive as raw materials for SCP production than renewable sources such as agricultural wastes or by-products.

3.4 Nutritional value of SCP: Nowadays, considerable information is available on the composition of microbial cells e.g. protein, amino acid, vitamin, and minerals. Commercial value of SCP depends on their nutritional performance and nevertheless, it has to be evaluated to the prevalent feed protein. SCPs either from alkanes or methanols, are characterized by good content and balance in essential amino acids.

Composition of growth medium governs the protein and lipid contents of micro-organisms. Yeasts, moulds and higher fungi have higher cellular lipid content and lower nitrogen and protein contents, when grown in media having high amount of available carbon as energy source and low nitrogen.

Ignoring a few extreme values, the mean crude protein in dry matter of algae and yeasts, on conventional substrates, lies between 50 and 60 per cent, for alkane yeasts between 55 and 65 per cent, and for bacteria about 80 per cent. A high content of nucleic acid free protein is extremely important for the economic efficiency of the procedure in SCP production. Because of high protein and fat contents, the contribution of carbohydrates to the nutritional value of SCP is not of prime importance.

The crude ash content is determined in particular by the nutrient salts of the fermentation medium. Estimation of crude protein is based on total nitrogen which is multiplied by the factor 6.25. The protein content of micro-organisms computed in this manner does not give the exact figure of protein content, as in the estimation of total nitrogen, the value of nucleic acid is also included which is somewhat erroneous.

The most important measure of nutritional value is the actual performance of SCP products as determined in feeding studies. The determinants of the utility of SCP product for application as food for human beings and feed for animals differ. For human beings, protein digestibility and protein efficiency ratio (PER), biological value or net protein utilization (NPU), determined in rats, are the parameters for food application, whereas for animals, metabolizable energy, protein digestibility and feed conversion ratio (weight of ration consumer/weight gain) are the measures or performance in broiler, chickens, swine and calves (and egg laying in hens).

Digestibility (D) is the percentage of total nitrogen consumed, which is absorbed through the alimentary tract. It is calculated as below:

$$\mathrm{D} = \frac{N_i - F_n}{N_i}$$

where,
N_i = nitrogen ingested from SCP
F_n = nitrogen content in faeces after feeding SCP

Biological value (BV) is the percentage of total nitrogen assimilated which is retained by the body, taking into account the simultaneous loss of endogenous nitrogen through excretion in urine.

This is expressed by the following formula:

$$\mathrm{BV} = \frac{N_i - (F_n + U_n)}{N_i - F_n} \times 100$$

where,

U_n = nitrogen content in urine after feeding SCP

Protein efficiency is the proportion of nitrogen retained when protein under test is fed compared with that retained when a reference protein (e.g. egg albumin) is fed.

However, there are certain problems which warrant the use of SCP products as human foods such as (i) high content of nucleic acid leading to development of kidney stone and gout if consumed in high quantity, (ii) possibility for the presence of toxic secondary metabolites and (iii) poor digestibility and stimulation of gastrointestinal and skin reactions.

3.5. Genetic improvements in microbial cells: At present, production of SCP by mass culture of micro-organisms is in its infancy. It needs much boost to solve the problem of starvation in the coming decades. One of the ways to enhance productivity and quality of SCP product is the genetic improvement of micro-organisms. At Sosa .Texcoco, Mexico, researches are in progress on production of genetically engineered cells, which can grow in alkaline environment even upto pH 8.0-10.0 and under the artificial conditions as well. Ciferri (1981) developed mutants of *S. platensis* , which had about 40 times more longer pools of certain amino acids than found in wild alga.

Moreover, transfer and expression of beneficial genes in the micro-organisms have opened a new era for the production of algal proteins and other compounds to be used in food and feed. Rochaix and Van Dillerviger (1982) have successfully introduced genes of S. cerevisiae into Chlamydomonas reinhardii cells and got expression of fungal genes in algal cell.

A7. Industrial Production of Ethanol by fermentation:

The preparation of distilled alcohol spirits was first described in the 12th century version of Mappae Clavicula (Key to Painting), with the first reported (nonbeverage) uses of spirits as an incendiary, a solvent and later medicinally. The preparation of absolute alcohol (by repeated distillation over potassium carbonate) was first reported in 1796. Extensive industrial use of ethyl alcohol began in the late 1800s with the growth of the synthetic chemical industry. Industrial ethanol end uses may be categorized into three classes: solvent, chemical intermediate and fuel.

Fermentation is a complex series of reactions, which convert carbohydrates, mainly sugars and starches, into ethanol and carbon dioxide. Several enzymes, such as zymase in yeasts, catalyse these reactions. Yeast is a living organism, and these are the products of anaerobic respiration.

7.1. Production Process: Raw materials for fermentative ethanol production can be divided into three classifications by carbohydrate type:

- saccharine materials
- starchy materials
- cellulosics.

Yeast (the primary organism used for ethanolic fermentation) will convert simple hexose sugar monomers (e.g. glucose and fructose) and some disaccharides (e.g. maltose and sucrose) to ethanol.

7.2. Substrate Selection

- The choice of raw material is critical as raw material costs typically make up 55-75% of the final alcohol selling price.
- Saccharine materials-with sugars available in fermentable form-require the least extensive preparation, but are
- generally the most expensive to obtain.
- Starch bearing materials are often cheaper but require processing to solubilize and convert the starch to fermentable sugars.
- Cellulosics can be available as cheap waste residue, but require the most extensive and costly preparation.

7.3. Fermentation Processes

7.3.1. Conventional Batch Fermentation: Batch fermentation begins with the production of an active yeast inoculum. This can be either by the conventional serial growth method or by the rapid semi aerobic method. Aseptic techniques are used throughout. In the serial growth method a pure culture inoculum from an agar slant is used to seed a laboratory shake flask. At the peak of growth (12-24 h) this culture is used to seed a succeeding culture 30-50 times larger. This is repeated, generally through three laboratory stages and two or three plant semiworks stages, to produce the final 2-5 vol % inoculum for the primary fermentation. The inoculum is grown on a medium similar to the final fermentation mash to minimize acclimatization time in the final fer-menter, but higher levels of yeast growth nutrients may be used to produce a high cell density (typically 150 billion cells l^{-1}). An inoculum 3-4 times more concentrated in yeast can be produced by the rapid semiaerobic method. Yeasts are grown in an aerated and agitated semiwork fermenter operated in fed batch mode. A large portion (20-25%) of the previous batch is retained to provide an inoculum. A high nutrient medium is added and pH and temperature are controlled. Sterile air is sparged at a rate of one-eighth volume of air per fermenter volume per minute. Aerobic metabolism is stimulated and a cell density of *500* billion cells l^{-1} reached in 5 h.

This high cell density allows the use of a proportionately smaller inoculum to the final fer-menter, and a smaller propagating fermenter can be used. However, aerobically grown yeast may require additional time to acclimatize to anaerobic fermentation conditions, resulting in an increased lag period between inoculation and rapid fermentation.

Cylindro-conical Nathan vessels are preferred for fermentation as these pro-mote better circulation and allow thorough drainage for cleaning. For very large plants, sloped bottom cone-roof tanks of up to 1 million liters volume are used and these large vessels are often agitated only by carbon dioxide evolution during fermentation.

After emptying and rinsing from the previous batch, mash at 13-17 wt% sugar is pumped to the fermenters. Once 20% full, the inoculum is added to allow growth during the remainder of the filling cycle, which can last 4-6 h. Fermentation temperature is regulated by circulating cooling water through submerged coils, circulating the mash through external heat exchanges, or simply spraying the vessel walls with cool water (adequate for small fermenters only). The feed is generally introduced at 25-30 ^{o}C, and the temperature allowed to gradually rise as heat is evolved. The temperature thus varies from 30-35 °C during the initial period (which is optimal for yeast growth). Cooling is then 'used to prevent the temperature from exceeding 35-38 °C, which is optimal for ethanol production.

These temperatures may be modified depending on the yeast strain used. Stillage backset provides excellent buffering. The pH is set initially at from 4.5-5.5 and decreases only slowly, generally holding at pH 4.0 or above. This is especially important for fermentation of grain mashes with simultaneous dextrin hydrolysis, as many amylase enzymes are rapidly denatured at lower pH (Stark, 1954). The ethanol production rate is the product of specific (per cell) productivity and the concentration of cells. Initially, the rate of alcohol production is quite low, but as the number of yeast cells increases the overall rate increases, and with rapid carbon dioxide evolution the beer appears to boil. After 20 hours a maximum in ethanol productivity is reached. The effects of reduced sugar concentration and ethanol inhibition then become important. The fermentation continues at a decreasing rate until, at 36 hours, 94% of the sugar is utilized and a final ethanol content of 69 g 1-1 is achieved. The average volumetric ethanol productivity over the course of the fermentation is 1.9 g $l^{-1}h^{-1}$.

Fermentation time will vary depending on yeast strain and substrate. Hawaiian and Cuban blackstrap molasses fermentation usually requires 36 h. Molasses from Java may require as long as 72 h. These times are reduced when molasses clarification is used. Grain fermentation requires 40-50 h to allow complete residual dextrin conversion.

After fermentation, the beer is pumped to a beer well to provide a continuous feed to distillation. The fermenters are then cleaned and prepared for another cycle. The fermentation rate can be increased 30-40% by improved agitation and temperature regulation (Aries, 1947). Turbine impellers have been ,used in smaller fermenters (100 000 l or less). For large fermenters, improved agitation and temperature control are achieved by rapidly circu-lating the beer through an external heat exchanger. The cooled beer is pumped back into the head of the fermenter tangentially and at a high velocity. The greatest portion of heat generation occurs during the 'boiling' fermentation period, creating a high peak cooling demand. Investment in cooling equipment can be reduced by teaming three or four fermenters to share a single bank of exchangers. The fermentations are then carefully scheduled to give a uniform heat load (Katzen, 1979). With improved agitation, continuous pH control can be used. Ammonium hydroxide or other base is added to the stirred fermenter or mixed with returning cycled beer. The pH can thus also be held at an optimum setpoint throughout the reaction period. Contamination by lactic acid bacteria is occasionally a problem and alcohol yield can be reduced by as much as 20% (Stark, 1954). Such contamination is more likely when stillage back-set is used, allowing contaminating organisms to accumulate and acclimatize to the fermentation conditions. Aseptic operation, with complete sterilization of the very large mash volume, was considered impractical until recently. Growth of organisms other than the seed yeast is generally restricted by the adverse conditions of low pH and high sugar or alcohol concentration, and the rapid growth of the yeast compared with contaminants was relied upon in place of aseptic techniques.

Highly efficient continuous media sterilization now makes aseptic operation quite practical (Pfeifer and Vojnovich, 1952). The medium is heated by steam injection to 135-140 °C and held in plug flow for 1-2 min, resulting in essentially complete sterilization. Cooling is by flashing to regenerate steam or by heat exchange to preheat incoming feed. The steam requirement is only 3.5 kg steam per 100 kg mash. Small spherical head fermenter vessels can be sterilized by pressurizing with steam. For very large fermenters this is impractical and antiseptic solutions such as ammonium bifluoride (Hodge and Hildebrandt, 1954), iodine (Katzen, 1979), sodium hypoch-lorite or formalin are automatically sprayed to rinse the vessel walls before filling. Heat exchangers and transfer lines can be steamed.

7.3.2. Simple Continuous Flow Fermentation: Continuous flow fermentation processes have been used in industrial sulfite waste liquor fermentation since the 1930s (McCarthy, 1954). The antiseptic qualities of sulfite liquor minimize the possibility of .adverse contamination and allow long continuous runs without shutdowns for cleaning. Early attempts at continuous fermentation of molasses and grain hydrolyzates on an industrial scale were unsuccessful due to contamination problems and these plants were retrofit for batch operation (Hough ct ai., 1977). With continuous media sterilization and aseptic plant techniques, the contamination problem has been overcome, as is illustrated by the success of continuous molasses fermentation plants in Europe (Rosen, 1978) and Japan (Karaki ct ai., 1972), and many continuous

beer brewing plants especially in New Zealand and Britain (Hough ct ai., 1977). Continuous culture techniques have been reviewed extensively (Yarovenko, 1978; Daw-son, 1976). Feed is pumped continuously into the fermenter, displacing beer which then overflows from the vessel. The pro-duct beer composition is the same as the composition in the fermenter vessel and thus, if a high ethanol product concentration is desired, the fermentation will be relatively slow as the entire course of the fermentation must take place under inhibitory high ethanol concentration conditions. The throughput rate must be adjusted to be slow enough to allow growth of new yeast in the fermenter to replace yeast washed out in the overflow and to allow essentially complete utilization of the sugar. Agitation by stirring or gas sparging was found to be especially important for successful continuous flow fermentation. With agitation, blackstrap molasses at 140 gl^{-1} concentration could be 95% utilized in a 21 h residence time compared with the batch fermentation time of 40 h. Without agitation, the continuous flow fermentation residence time for complete sugar utilization was 55 h. In laboratory tests with carefully optimized conditions of temperature, pH, agitation and flow rate, much higher productivities have been achieved. Molasses at 130 g sugar l^{-1} has been fer-mented to completion ina 7 h residence time (Bilford et ai., 1942), corresponding to a volumetric productivity of 8.3 g $l^{-1}h^{-1}$. A nutrient supplemented glucose medium of 130 g sugar l^{-1} could be completely utilized in 9.5 h residence time (Cysewski and Wilke, 1976a). This fermentation was continued for 60 days without decline in productivity.

7.3.3. Continuous Fermentation with Yeast Recycle: The productivity of continuous fermentations can be greatly enhanced by yeast cell recycle (DelRosario et ai., 1979). A centrifuge is used to separate yeast from the product flow and the concentrated yeast cream (up to 150 g yeast I-I) is recycled to the fermentation vessel. A small bleed of cells is required to maintain a viable culture (Pirt and Kurowski, 1970). In laboratory tests, a simple chilled settler was used to recycle cells, increasing the cell density four-fold to 50 g :-1 (Cysewski and Wilke, 1977; Ghose and Tyagei, 1979a). The residence time for complete con-version of a 100 g glucose 1-1 feed was reduced to 1. 6 h with a corresponding productivity of 30 g ethanol I-I h-I. Over a two-week total period the viable yeast fraction (96%) did not decrease and the specific ethanol productivity (per cell) was the same as in nonrecycle experiments. Capital costs for centrifuges are high and sealed sterilizable centrifuges have become available only recently. This has slowed the application of cell recycling in industrial fermentation. Attempts have been made to develop cheaper alternative cell recycle methods. Simple cell set-tling systems have been developed wherein the cells are thermally shocked *(to* temporarily halt CO2 evolution) and allowed to gravity settle. Very large settling vessels are required, however (Walsh and Bungay, 1979). Whirlpool separators have been used industrially in beer manufac-ture (Hudson, 1972). The fermenter broth is pumped tangentially into a verticle cylindrical vessel and flocculent yeast cells are deposited in a central cone for recycle. A very simple partial recycle fermenter has been developed and tested at pilot scale (Hough ct ai., 1962). The overflow is taken from a vertical pipe rising through the fermenter base and jacketed by a baffled sleeve. The region between overflow pipe and sleeve escapes agitation, allowing yeast to settle back and sep-arate from rising
beer. The cell density in the fermenter can be increased 2.5 times using floccu-lent rapid settling yeast strains with essentially no added equipment cost.

7.4. Alcohol Recovery: Alcohol product recovery is energy intensive, typically accounting for more than 50% of the total fermentative ethanol plant energy consumption. When heat from burning of raw material residues (such as bagasse) is not available, this constitutes a significant operating cost. Depending on recovery system design, recovery equipment cost generally makes up 6-12% of the plant total capital investment. Industrial alcohol is produced in various grades. The majority is 190 proof (95 vol% or 92.4 % minimum) alcohol used for solvent, pharmaceutical, cosmetic and chemical applications. Technical grade alcohol (containing up to 5% volatile organic aldehyde, esters and sometimes methanol) is used for industrial solvents and some chemical syntheses. A high-purity 200 proof anhydrous alcohol product (99.85 wt%) is produced for specialized chemical applications. For fuel use in mixtures with gasoline (gasohol), a nearly anhydrous (99.2 wt%,) alcohol, but with higher allowable levels of organic impurities, is used. Today, distillation is used almost exclusively as the means for ethanol recovery and purification and various designs are used to produce the different product grades. Ethanol distillation techno-logy was highly refined during the 1940s to reduce energy consumption to approximately 2.5 kg of steam per liter of anhydrous ethanol produced. Recent further refinements in distillation technique make possible marginal improvements with increased capital investment. Alternatives to distillation are under study to further reduce costs.

B1. Plant tissue culture Techniques

Plant tissue culture, also called **micropropagation**:

It is a practice used to propagate plants under sterile conditions, often to produce clones of a plant. Different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation, including:

- The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits.
- To quickly produce mature plants.
- The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds.
- The regeneration of whole plants from plant cells that have been genetically modified.
- The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.
- The production of plants from seeds that otherwise have very low chances of germinating and growing, i.e.: orchids and nepenthes.
- To clean particular plant of viral and other infections and to quickly multiplie these plants as 'cleaned stock' for horticulture and agriculture.

Plant tissue culture relies on the fact that many plant cells have the ability to regenerate a whole plant (totipotency). Single cells, plant cells without cell wall (protoplasts), pieces of leaves, or (less commonly) roots can often be used to generate a new plant on culture media given the required nutrients and plant hormones.

Plant propagation in tissue culture (micropropagation) is used to develop high-quality clonal standard plants. These plants are selected for unique horticultural trait, pest resistance, crop quality for environmental stress conditions. The main advantages are attributed to the potential of combining rapid, large-scale propagation of new genotypes, the use of small amount of original Germplasm. Micropropagation technology offers many unique advantages when compared with other conventional propagation methods. The main advantages are attributed to potential of combining rapid, large-scale propagation of new genotypes, the use of small amount of original germplasm and generation of pathogen-free propagules.

1 Stages of micropropagation

There are three major ways by which micropropagation can be achieved. These are (i) Enhancing axillary bud breaking, (ii) production of adventitious buds directly or indirectly on explant and (iii) somatic embryogenesis. Plant micropropagation *in vitro* is an integrated process by which the cells, tissues or organs of selected plants are isolated surface sterilized and incubated in growth promoting, sterile environment to produced many clonal plantlets. The physical and the chemical environmental factors effect the initiation of cultures, their growth, behavior and regeneration. Five operational stages are involved, three of them *in vitro* while one stage occur in green house environment. This classification of production stages has been suggested by Debergh and Maene, 1981.

Stage - O: **Explant source and mother plants**

The success of micropropagation *in vitro* is largely depends on the quality of source or mother plant. Effective selection and maintenance of source plants should provide occurrence that the plant is –

- horticultural true to type representative of desired species and cultivar.
- Free from infection and disease or can become pathogen-free using *in vitro* procedures.
- Viable and vigorous (i.e. potentially able to respond) to culture conditions that induce intensive cell division and regeneration.

With all these requirements, selected mother plants are often "pre-conditioned" by many specific growth regimes. These include nutrition and irrigation, light quality and temperatures, treatments with growth regulators, purning and pest control.

Stage – 1: Explant establishment in culture

For the establishment of cultures *in vitro*, the size of explant in range from 0.1 mm (e.g. meristems used for establishing virus free plants) to about 1-3 cm (e.g. bulb scales and stem). During this stage from 1 week to 2-3 months, the explant is established in culture, resulting in tissue activation and multiplication. This stage is usually carried out on agar-based media, but liquid medium can also be employed. The choice of basal media and growth regulators may very according to plant and tissue type and to the desired multiplication method. This stage is also used to screen for microbial contamination and horticultural fitness specific treatments of microbial and viral contamination can also be taken into consideration.

Stage – 2: **Rapid multiplication**

Primary explants that have successfully passed through stage-1 and transferred aseptically to stage 2 for generation of numerous clonal propagules. Masses of tissues are repeatedly manipulated by subculturing onto new culture media that encourage proliferation. The types of regeneration and proliferation are largely depends on growth

regulator combination. A high proportion of cytokinin usually stimulates continued multiplication of axillary or adventitious shoots, and a higher proportion of specific auxins is required for callus proliferation and somatic embryogenesis. The combined and balanced adjustment of growth regulator, basal media composition and environmental conditions are optimized to achieve maximal proliferation of quality, new plant propagules. Extensive experimentation may be necessary to reach commercially efficient multiplication with specific cultivars or varieties.

Stage – 3: Plantlet establishment, elongation and rooting

After repeated subcultures and screening the resulting plantlets are transferred to the final *in vitro* stage. This stage is designed to arrest rapid multiplication and to induce the establishment of fully developed plantlet. Shoot elongation, root formation, and when required, formation of storage organs (bulb, corms and tubers). This stage also provides the conditions for stimulations of photosynthesis and other physiological changes that are required for autrophic, *ex vitro* growth in the acclimatization stage. This is achieved by modifications in culture media (e.g. reduction of cytokinins concentrations, increased levels of auxins, reduced sugar levels etc.) and by modifying environmental conditions.

Stage-4: Acclimatization

The healthy plantlets that come out from the culture vessels are usually incapable of existence in natural field or green house conditions. 4-8 weeks in an acclimatization green house provide a weaning process. The first few days in green house are spent under low light, high temperature and high humidity conditions, often provided by fogging or an incubator for *ex vitro* plants. As cuticular waxes, stomatal function, and new functional roots develop; photosynthetic activity is increased and plant become ocytotrophic. Over the following few weeks light intensity is raised, and ambient temperature and humidity are regulated to natural environment or growing conditions. *In vitro* plants that are unable to survive this stage must be considered unsuccessful. Organs initiated in cultures are defined by the culture environment and they have the same characteristics as they did *in vitro* after transfer to green house. A gradual return to normal characteristics occurs during the acclimatization period in green house.

2 Applications of micropropagation:

Micropropagation offers significant advantages in quality, quantify and economics over conventional methods of vegetative propagation for many species. Some of these are –

- Production of a very large number of clonal propagules within a short time span – the techniques of plant tissue culture given an exponential increase of the propagation. Depending upon the multiplication rate, thousands and millions of plants can be rapidly produced *in vitro*.
- Production of disease-free plant material with the possibility of eliminating viral, bacterial and fungal contamination.
- Production of large stock of true-to-type clonal propagation material.
- The ability of air ship large quantities of plants material quickly, efficiently and relatively inexpensively.
- The possibility of bringing newly bred plants and selections to market quickly in large quantities.
- Elite selection
- Product uniformity
- Genetically engineered products

3. Techniques for Micropropagation:

3.1. Plant Cell Culture:

It was Haberlandt in 1890s, which put forth the pioneering attempt to culture mechanically isolated mesophyll cells. He succeeded in maintaining these cells alive for 10 days in culture but it did not divide. He failed to achieve division of isolated cells. Since then, the progress in the field of isolating single cell bur and culturing individual cells has been spectacular that it has become possible not only to culture isolated single cells but also induce divisions in such cells in complete isolation and finally , to regenerate whole plant from them. The most important aspect that deserves attention in the isolated cell culture technology is the initiation of division and proliferation of isolated singer cells which starts only when they are placed into conditioned medium. Isolated single plant cell resembles with animal cells in this respect. However, a conditioned medium is prepared by culturing high densities of cells of same or different species on same fresh medium for some days and then the cultured cells are removed by filter sterilization making he medium free of them. This medium now contains essential amino acids such as glutamine and serine as well as growth regulators such as cytokinins. When the isolated single cell are placed in the conditioned media in exactly the same way as microorganisms, they initiate the cell division and instead, of developing a colony as do the microorganism they proliferate to form a callus. The later is induced to regenerate a plant.

3. 2. Applications of cell culture:

1. **Resistant to diseases:** Tobacco protoplasts selected from resistance to methionine sulphoximine showed enhanced resistance to *Psuedomonas tabaci*.
2. **Resistant to herbicides:** In the recent past the fertile plants resistant to herbicides such as amitrol, 2,4-D, sodium chlorate, Picloram, Cholorosulfuron and sulfometuron methyl have been selected from cultivated cells of *Nicotiana*.

3. **Resistant to amino acids and their analogues:** A Potato cell line resistant to 5-methyl tryphtophan has been used to demonstrate the presence of two possible Isoenzymes of anthranilate synthase the first regulatory enzyme unique to tryptophan biosynthesis.
4. **Secondary metabolic production:** A few but well known established examples of secondary metabolite production in high amounts by selected cell lines are- Glutathione & Nicotine from *Nicotiana tobacum*, Anthraquinones from *Merinda citrifolia*, Rosmarinic acid from *Coleus blumei*, Ajmalicine and Serpentine from *Catharanthus roseus*, Diosgenin from *Dioscorea deltoidea*.
5. **Biotransformation**: Biotransformation means conversion of less useful compound into more useful compounds by the plant cells. The biotransformation of 'digitoxin' or 'β-methyldigitoxin' to 'digoxin' or 'B-Methyl digoxin' by specific hydroxylation of suspension cultures of *Digitalis lantana* can carry out biotransformation at 15 % conservation in 24 hours and 70 % in 7 days. Digoxin is used in cardiac treatment. The cell cultures of *Stevia rebaudiana* and *Digitalis purpurea* can biotransform the Steviol to steviolbiocide and stevioside.

3.2. Callus culture:

Callus is amorphous mass of unorganized thin wall-walled parenchyma cells. When a plant is wounded, callus formation occurs at the cut surfaces and is thought to be protective response by the plant to seal off damaged tissues. In culture callus is initiated by placing a fragment of plant tissue (an explant) on solid culture medium under aseptic conditions. Callus is induced and formed from proliferating cells at the cut surface of explant tissue. Depending upon species, callus can be initiated from a variety of tissue employing the appropriate growth medium. However, rapid cell division can be more easily induced in some tissues than the others. The *in vitro* formation and proliferation of callus is enhanced by the presence in the medium of hormones (auxins and cytokinins) that promotes cell division and elongation.

During the *in vitro* initiation of callus, the differentiation and specialization that occurred in the parent plant is reversed and cells of plants become de-differentiated. The process of de-differentiation is characterized by changes in metabolic activity, the disappearance of storage products and rapid cell division that gives rise to undifferentiated and unorganized parenchyma cells.

- Formation of callus is a fundamental step in the *in vitro* culture of many types of plant cells and tissues. For example, callus provides the most frequent used totipotent cells from which whole plants are regenerated via either organogenesis or somatic embryogenesis.
- Dispersal of friable callus into single cells or clumps of cells is used universally as the method of initiating cell suspension cultures.
- Callus cultures are important elements of almost all current transformation strategies. Callus cells are frequently used as targets for transformation both by micro projectile bombardment and by *Agrobacterium tumefaciens*.

3.3. Somatic Embryogenesis:

Somatic embryogenesis refers to the process in which somatic or non-sexual cells are induced to form bipolar embryos through a series of developmental steps similar to those occurring during in vivo embryogenesis. Somatic embryo formation has been achieved for a variety of plant species, including angiosperms and gymnosperms. Somatic embryogenesis is different from organogenesis in that regeneration and organization are bipolar; the shoots and roots meristems are formed simultaneously from group of cells (proembryonic masses). As with organogenesis, differentiation, morphogenesis and organization of somatic embryo takes place directly in the explant or from the callus. Somatic embryogenesis is often favored by cultured in an agitated liquid medium.

Pattern's of differentiation of somatic embryos are from the formation of globular structure to heart stage at which both shoot and root meristems can be clearly distinguished at two poles, to the "torpedo" stage at which elongation of shoots and roots takes place. This coordinated development results in the formation of well organized embryoids or plantlets.

With regard of place of origin, embryogenesis is of two types; direct embryogenesis and indirect embryogenesis. Juvenile explants like hypocotyls, cotyledons and young immature zygotic embryos are best material to initiate the embryogenic cultures. When the embryogenesis occurs directly on the explant without production of callus it is known as direct embryogenesis. When explant produces callus and the callus forms the embryos then it is cakes as indirect embryogenesis. An exogenously supplied auxin is needed in appropriate concentration for the induction of somatic embryogenesis from callus or explant. In case of direct embryogenesis cells of explant tissue are already determined for embryogenic development, and these are termed as pre-embryogenic determined cells (PEDC's). The starting material is completely rejuvenated eg. nucellus of citrus or epidermal cells of hypocotyls of *Brassica napus* and *Ranunculus* sp. In case of indirect embryogenesis cells require redetermination through a period in culture and this is termed as induced embryogenic determined cells (IEDC's). It has been suggested that this phenomenon is determined by epigenetic factors. Here, differentiated cells must firstly be differentiated and then redetermined as embryogenic cells after the cell division. Complete rejuvenation must take place. Eg. secondary

phloem of carrot, leaf explant of *Petunia, Vigna* and *Asparagus*.

Advantages of somatic embryogenesis:

- Rapid multiplication through production of somatic embryogenesis in cell cultures, and use of bioreactors for scale up industry, thus mass propagation of plants can be performed.
- Somatic embryos grown individually make the system easy to manipulate(to sub culture and to make transgenic plant).
- Provides an important resource for the analysis of the molecular and biochemical events that occur during induction and maturation of embryo.
- Presence of both roots and shoots avoid the rooting step required in organogenesis.
- It shortens the breeding cycle of deciduous trees and increases the germination of hybrid embryos.
- Somatic embryos provide potential plantlets in the form of synthetic seeds, which can be directly sowed in the soil. This gives impetus to increased agricultural crops.

3.4. Synthetic Seeds/Artificial Seeds/Somatic Seeds:

Synthetic seeds are defines as artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that can be used for sowing as a seed and which possess the capability to convert into plant under in vitro conditions.

- Hydrated synthetic seeds: consist of somatic embryos individually encapsulated in hydrogel such as calcium alginate.
- Desiccated synthetic seeds: are produced by coating somatic embryos in polyoxyethylene glycol.

Methods of Production: Following steps are followed for production of synthetic seeds:

1. Induction of somatic embryos from the cell suspension or callus culture.
2. Mixing the embryos with 2 % Na – alginate.
3. Immersing the embryos in the bath of calcium salt, eg. solution of calcium chloride or calcium nitrate for 30 min. This lead to very quick complex formation at surfaces due to exchange of ions i.e., Na^{+} and Ca^{++}. As a result, individual embryos is enclosed in clear and hardened beads of about 4 mm. Hardening of calcium alginate is modulated with the concentrations of sodium alginate along with the duration of complexing. Usually 30-100 mM Ca^{++} is used.
4. sieving the bead through a nylon mesh; $Ca(NO_3)_2$ solution is recycled, and
5. Testing the growth vigour of beads by plating in sand or soil amended with pesticides.

Thus synthetic seeds are living seed like structure derived from somatic embryoids after encapsulation by a hydrogel. Such preserved embryos are called as synthetics seeds. These seeds can resist unfavorable field conditions including microbial contamination, without desiccation. Such encapsulated seeds are used as substitute of natural seeds and can be grown directly in green house or fields.

Examples of plants produced from synthetic seeds sown in vitro and in soil include: *Apium graveolens, Brassica sps., Gossypium hirsutum, Medicago sativa, Oryza sativa, Zea mays, Daucos carota* and *Medicago sativa.*

Advantages of artificial Seeds:

- Reduced costs of transportation.
- Production of large number of identical embryos.
- Germplasm Conservation through cryopreservation.
- Study of seed coat formation is possible.
- Useful in tree genetic engineering.
- Production of synthetics seeds is possible in any time, in any season of year.
- Problems of dormancy can be avoided, thereby shortening the life cycle of plant.

3.5. Haploidy:

Haploid plants have the gametophytic number of chromosomes, a single set (n) of chromosomes in the sporophyte. Haploid cells or individual are those in which original chromosome number has been reduced by the half. These are the immense importance for plant improvement, useful in mutation and for production of homozygous diploid plants.

In theory, haploid plants could be produced by the in vitro culture of haploid gametophytic tissues, that is, unpollinated ovaries or ovules and anther or pollen. Viable haploid plants have been produced by ovary and ovule culture for some crops; the procedures are however technically laborious and the yields low. There are two in vitro procedures that are now routinely used in the production of haploids: (i) anther culture and (ii) culture of the isolated microspores. The development of haploid plants from the male gametophytic tissue is known as androgenesis.

a. Anther Culture: It is rapid and relatively simple procedure by which pollen producing anthers are collected from flowers at a certain stage of development; sometimes they are given a low or high temperature pre-treatment, before sterilization and culturing on a specific nutrient medium. Mature pollen (the male gametophyte) develops from haploid microspores that are in turn formed by the meiosis in pollen mother cells. Microspores

have a single nucleus and mature pollen three nuclei. When the anthers containing the microspores at the middle to late uninucleate stage are cultured on solid media, individual microspores form the callus. Plants with the half the chromosome number can be regenerated from the callus directly by embryogenesis or indirectly by organogenesis. Alternatively, microspores may give rise to embryoids (aggregates of cells with recognizable embryonic structures) that are capable of differentiation of embryo.

b. Microspore culture: It involves the physical isolation of microspores from the anthers at specific stage of development. After a suitable pre-treatment, the anthers are homogenized and microspores are isolated and purified from the homogenate by filtration and centrifugation steps. The purified microspores are then cultured in a liquid medium. Small embryos develop directly from the microspores (microspore embryogenesis) and are transferred to a regenerative medium. In microspore culture problems of anther culture such as mix ploidy types (mixoploidy) and albino plants in the regenerated plants are less frequent. Microspores cultures are the ideal material for genetic transformation.

Haploids have been very useful in agriculture and tree breeding.

They are useful for two reasons. (1) The presence of one set of chromosomes facilitates the isolation of mutants, and (2) isogenic diploids can be obtained by chromosome doubling. Isogenic lines are those individuals that have the same genotype irrespective of their homozygous or heterozygous nature. Through the anther culture technique, haploids can be obtained in a few weeks and by doubling their chromosome number homozygous diploids can be produced in one generation. Such fertile homozygous plants can be used for obtaining the inbreed lines required for hybridization programme.

Other **Advantages of haploid culture are-**

1. To produce virus free plants
2. In plant breeding to obtain pure line varieties.
3. Mutation Breeding
4.Tree Breeding.
5. Development of new varieties: ‘Mingo’-the first licensed cultivar of barley was produced using haploid.

3.6. Somaclonal Variations:

When the plants are grown in culture, much genomic variability is produced. This variability can be found in callus and suspension cultures, tissue cultures, protoplast cultures and other in vitro culture systems. Such variations observed among plants regenerated from tissue cultures are called somaclonal variations (Scrowcroft, 1985). Frequent genetic modifications are known to occur during the process of cell or tissue culture. These modifications tend to be inherited as mutations among the progeny of regenerated plants. The source of explant is critical factor or variable for somaclonal variations. Plant cell, tissues and somatic tissues developed from various explants are sources for generating somaclonal variation. Somaclonal variation has been reported in number of species such as sugarcane, potato, rice *Brassica*, tobacco, tomato and other various agronomic traits like disease resistance, plant height, maturity and for many physiological and biochemical traits.

Factors determining somaclonal variations:

- The methods of vegetative propagation used
- The genotype used to raise the primary culture
- Growth regulators such as 2,4-D, NAA leads to a high number of mutation
- The risk of mutation is high when starting material is highly differentiated.
- Repeated sub-culturing for many cycles enhances mutation.

Reasons for somaclonal variations:

- Pre-exisiting genetic variation in explant tissue
- Gene mutation during culturing
- Numerical and structural changes during *in vitro* growth
- Somatic crossing over involving symmetric and asymmetric recombination
- Due to transposoms activity.
- Intracellular mutagenic agents produced during in vitro growth

Applications:

- Increased genetic variability for agronomic traits
- In vitro selection has been used to select desirable somaclones, including tolerance to pathotoxins, herbicides and diseases. Eg. tobacco variant resistance to Psuedomonas syringae and Alternaria alternate have been obtained.
- Variants with improved traits have been obtained giving rise to new useful Germplasm, as well as cultivar. E.g., Tobacco germplasm having resistance to potato virus Y and rice dwarf cultivar with 10 % higher yield and tolerance to salt have been obtained.

3.7. Protoplast Fusion and Somatic Hybridisation:

The plant cell wall is a multilayered structure composed of polysaccharides and proteins. Careful removal of plant cell wall results in a viable, spherical cell called protoplast. Protoplasts contain original cell’s contents bounded by the plasma membrane. With the appropriate culture condition protoplast will resynthesize the cell wall and undergo cell division to form callus form which new plants can be regenerated.

Protoplasts can be isolated from variety of whole plant tissues and from plant tissue cultures like

callus and suspension cultures. Removal of the wall for protoplast is achieved either by mechanical means(Removal of cell wall by ultra knife of plasmolysed cell) or enzymatic digestion (which include a combination of cellulases, hemicellulases, and pectinases).

In plant breeding, the technique of somatic hybridization by protoplast fusion is a particularly useful way of introducing genetic variations from distant relatives into crop plants in circumstances where sexual hybridization is prevented by reproductive barriers. When two or more protoplast fuses to form a new cell, the parent nuclei may remain separate or fuse to form a somatic hybrid. If one of the nuclei is lost after fusion, the cytoplasm of two parent protoplast will still coalesce and form what is known as a cytoplasmic hybrids or cybrids.

Isolated protoplasts can fuse spontaneously, although this occurs infrequently because of mutual repulsion that results from the net negative surface charge or the protoplast's plasma membranes. Nevertheless, fusion can be induced either by the application of compounds known as fusogenic agents (eg. $CaNO_3$, PEG, High pH and Ca ions, Dextran) or by placing the protoplasts in an electric field (electrofusion / elecroporation).

Fusogenic agents tend to either neutralize or shield the surface charge so that adjacent protoplasts come in close proximity, adhere and coalesce. Fusion is induced by high concentration of Calcium ions at high pH (pH=10) and high temperature (37^0 C). However, the most widely used fusogenic agent is poly ethylene glycol (PEG).

Electrofusion takes place in two stages. First, the protoplast suspension is placed in a fusion cell between two metal electrodes and subjected to a non uniform alternating electric field. The Protoplast membrane becomes differentially charged and positive region are attracted to negative regions on adjacent protoplasts resulting ion the protoplast aligning in chains. In the second step, a high voltage DC pulse is applied to the aligned protoplasts that cause the breakdown of adjacent membranes and fusion. Flow cytometry can be used to identify the fusion products by cell sorting and estimation of nuclear DNA,

Advantages of Somatic Hybridization:

- This technique can be used as alternative to obtain tetraploids and amphidiploids, when mitotic doubling is impossible.
- It allows genetic recombination of sexually incompatible genotypes, which is impossible using conventional techniques of hybridization.
- It helps to produce hybrids between species that are too distant to be hybridized sexually.
- Both parental species contribute equally for hybrid in somatic hybridization.
- Protoplast can be pre-treated prior to fusion, which increases gene introgression.
- The wild relatives of cultivated variety having dominant and resistance genes can be exploited to improve crop yield. For instance, Wild relatives of cultivated sweet potato contain the dominant and resistant genes against root knot nematode.
- It can be employed in the improvement of plants wherein sexual reproduction is either difficult or absent such as banana, cassava, Potato, sugarcane and yam. Here, the fusion derived plants need not be fertile, circumventing the problems of infertility.

3.8. Cybrids:

These are obtained when nucleus is derived from one parent and cytoplasm is derived from both the parents. Cytoplasmic hybrids (cybrids) can be obtained by using any of following methods:

(a) Fusion of normal protoplast from one parent with enucleated protoplast from other parent.
(b) Fusion of normal protoplast from one parent and protoplast containing non-viable nuclei from the other parent.
(c) Selective elimination of one of the nuclei from the heterokaryon.
(d) Selective elimination of chromosomes of one parent at a later stage after fusion of nuclei.

Cybrids have been produced in many cases mainly to transfer cytoplasmic organelles. Cybridisation has been successfully employed to make intergeneric and interspecific transfer of cytoplasm in tobacco, petunia, rice and Brassica sps.

B2. Animal Cell culture: Requirements and Growth of animal cell culture

Animal cells are more difficult to culture than microorganisms because they require many more nutrients and typically grow only when attached to specially coated surfaces. Despite these difficulties, various types of animal cells, including both undifferentiated and differentiated ones, can be cultured successfully.

2.1 Nutritional Requirements:

Rich media are required for culture of animal cells. Nine amino acids, referred to as the *essential amino acids,* cannot be synthesized by adult vertebrate animals and thus must be obtained from their diet. Animal cells grown in culture also must be supplied with these nine amino acids, namely, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. In addition, most cultured cells require cysteine, glutamine, and tyrosine. In the intact animal, these three amino acids are synthesized by specialized cells; for example, liver cells make tyrosine from phenylalanine, and both liver and kidney cells can make glutamine. Animal cells both within the organism and in culture can synthesize the 8 remaining amino acids; thus these amino acids need not be present in the diet or culture medium. The other essential components of a medium for culturing animal cells are vitamins, which the cells cannot make at all or in adequate amounts; various salts; glucose; and *serum,* the noncellular part of the blood.

The nutrient media used for culture of animal cells and tissues must be able to support their survival as well as growth, i.e., must provide nutritional, hormonal and stromal factors. The various types of media used for tissue culture may be rouped into two broad categories:
(1) natural media and
(2) artificial media.

The choice of medium depends mainly on the type of cells to be cultured (normal, immortalized or transformed), and the objective of culture (growth, survival, differentiation, production of desired proteins).

Non-transformed or normal cells (finite life span) and primary cultures from healthy tissues require defined quantities of proteins, growth factors and hormones even in the best media developed so far. But immortalized cells (spontaneously or transfection with viral sequences) produce most of these factors, but may still need some of the growth factors present in the serum. In contrast, transformed cells (autonomous growth control and malignant properties) synthesize their own growth factors; in fact, addition of growth factors may even be detrimental in such cases. But even these cultures may require factors like insulin, transferrin, silenite, lipids etc.

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

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

b. Biological Fluids: Of the various biological fluids used as culture medium (e.g., amniotic fluid, ascitic and pleural fluid, aqueous humour from eye,insect haemolymph, serum etc.), serum is the most widely used. Serum may beobtained from adult human blood, placental cord blood, horse blood or calf blood(foetal calf serum, newborn calf serum, and calf serum); of these foetal calf serumis the most commonly used. Serum is the liquid exuded from coagulating blood. Different preparations of serum differ in their properties; they have to be tested for sterility and toxicity before use.

c. Tissue Extracts: Chick embryo extract is the most commonly used tissue extract, but bovine embryo extract is also used. Other tissue extracts that have been used are spleen, liver, bone marrow, leucocytes etc. extracts. Tissue extracts can often be substituted by a mixture of amino acids and certain other organic compounds.

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

2.1.2 Artificial Media

Different artificial media have been devised to serve one of the following purposes:
(1) immediate survival (a balanced salt solution, with specified pH and osmotic pressure is adequate),
(2) prolonged survival (a balanced salt solution supplemented with serum, or with suitable formulation of organic compounds),
(3) indefinite growth, and
(4) specialized functions.

The various artificial media developed for cell cultures ‘may be grouped into 4 classes: (i) serum containing media (ii) serumfree media, (iii) chemically defined media, and (iv) protein-free media.

(i) Serum Containing Media. The various defined media, e.g., Eagle’s minimum essential medium etc. (see, serum-free media) when supplemented with 5-20% serum are good nutrient media for culture of most types of cells. The serum provides various plasma proteins, peptides, lipids, carbohydrates, minerals, and some enzymes. Serum serves the following major functions.

It provides the basic nutrients for cells; the nutrients are Present both in the solution as well as are bound to the proteins.

It provides several hormones, e.g., insulin, which is Essential for growth of nearly all cells in culture,cortisone, testosterone, prostaglandin etc.

1. It contains several growth factors, e.g., platelet derived growth factor (PDGF), transforming growth factor P (TGFp), epidermal growth factor, fibroblast growth factor, endothelial growth factor etc.; these are presenting concentrations of µg/l. Both hormones and growth factors are involved in growth promotion and specialized cell function. A given hormone or growth factor may stimulate growth of one cell type, may have no effect on another and may even be inhibitory to some others. For example, PDGF induces proliferation in fibroblasts but induces differentiation of some types of epithelia. Further, proliferation of a single cell type may be induced by more than one growth factor, e.g., fibroblasts respond to PDGF, epidermal growth factor, fibroblast growth factor and somatomidins.
2. A major role of serum is to supply proteins, e.g., fibronectin, which promote attachment of cells to the substrate. It also provides spreading factors that help the cells to spread out before they can begin to divide. Although cells do produce these factors, but typsinized cells are usually unable to attach to the substrate.
3. It provides several binding proteins, e.g., albumin, transferrin, which carry other molecules into the cell. For example, albumin carries into cells lipids, vitamins, hormones etc. Transferrin usually carries Fe in a non basic form, but binding of transferrin to its receptor in cell membrane is believed to be mitogenic damages, e.g., shear forces during agitation of suspension cultures.
4. Protease inhibitors present in the serum protect cells, especially trypsinised cells, from proteolysis.
5. The serum also the viscosity of medium and, thereby, protects cells from mechanical provides several minerals, e.g., Na+, K+, Fe2+, Zn2+, Cu2+, etc.
6. It also acts as a buffer.

1. EBSS and HBSS are autociaved below pH 6.5 at 15 psi (pounds per square inch) for 20min. pH may be adjusted to 7.4 with sterile NaOH prior to use.
2. For use as a general handling, washing and dissection solution, $NaHCO_3$ and glucose are omitted from EBSS
and HBSS. For dissection, add to BSS : (i) penicillin(250 U/ ml), (ii) streptomycin (250 µg/ml), (iii) kanamycin (100 µg/ml) and (iv) fungizone(2.5 g/1)
3. PBSA is used as a washing solution before disaggregation, and as a diluent for trypsin(trypsin is used at 2.5 g, crude preparation, or 0.1 g, purified by repeated crystallization, per litre). PBSA is also used as the base solution for preparing EDTA solution (Na_2 EDTA.$2H_2O$ at 372 mg/l, 1 mM) which is autoclaved before using.

TABLE Composition of Earle's balanced salt solution (EBSS) and Hank's balanced salt solution (HBSS). Composition of Dulbecco's phosphate-buffered saline, solution A (PBSA, Ca2* and Mg^{2+}-free) is also given.

Constituent	Amount (mg/l)		
Inorganic salts	EBSS	HBSS	PBSA3
$CaCl_2$ (anhyd.)	20 (0.18 mM)	140(1.26mM)	
KC1	40 (0.536 mM)	400 (5.36 mM)	200 (2.68 mM)
KH_2PO_4	60 (0.44 mM)	200 (1.47 mM)	
$MgCl_2.6H_2O$	100 (0.49 mM)		
$MgSO_4.7H_2O$	200 (0.82 mM)	100 (0.41 mM)	
NaCl	6680(114.4 mM)	8000 (137 mM)	8000 (137 mM)
$NaHCO_3$	2220 (2.22 mM)	350 (0.35 mM)	
$Na_2HP0_4.7H_2O$	90 (0.34 mM)	2160 (8.06 mM)	
$NaH_2PO_4.H_2O$	140 (0.53 mM)		
Others			
D-glucose	1000 (5.55 mM)	1000 (5.55 mM)	
Phenol red		10	10

However, there are several disadvantages of using serum in the culture medium; these are summarised below.
1. Serum may inhibit growth of some cell types, e.g., epidermal keratinocytes.
2. Serum may contain some cytotoxic or potentially cytotoxic constituents. For example, foetal calf serum contains the enzyme polyamine oxidase which converts polyamines like spermidine and sperrnine (secreted by fast growing cells) into cytotoxic polyamino aldehydes.
3. There is a large variation in serum quality from one batch to another; this requires costly and time consuming testing
every time a new batch has to be used.
4. Some growth factors may be inadequate for specific cell types and may need supplementation.
5. It interferes with downstream processing when cell cultures are used for production of biochemicals.
6. The supply of serum is always lower than its demand.

(ii) Serum-Free Media. In view of the disadvantages due to serum, extensive investigations have been made to develop
serum-free formulations of culture media. These efforts were mainly based on the following 3 approaches:
(1) analytical approach based on the analysis of serum constituents,
(2) synthetic approach to supplement basal media by various combinations of growth factors, and
(3) limiting factor approach consisting of lowering the serum level in the medium till growth stops and then supplementing the medium with vitamins, amino acids, hormones etc. till growth resumes.

These approaches have resulted in several elaborate media formulations in which serum in sought to be replaced by a mixture of amino acids, vitamins, several other organic compounds etc.; hormones, growth factors and other proteins are supplemented when required. However, addition of

5-20% of serum even in these media is essential for optimum growth.

The various advantages of serum-free media may be summarised as under.
1. Improved reproducibility of results from different laboratories and over time since variation due to batch
change of serum is avoided.
2. Easier downstream processing of products from cultured cells.
3. Toxic effects of serum are avoided.
4. Biassays are free from interference due to serum proteins.
5. There is no danger of degradation of sensitive proteins by serum proteases.
6. They permit selective culture of differentiated and producing cell typesfrom the heterogenous cultures.

However, serum-free media suffer from the following disadvantages.
1. Most serum-free media are specific to one cell type. Therefore, different media may be required for different cell lines.
2. Reliable serum-free preparations, for most of the media formulations are not available commercially. This necessitates time consuming task of preparing the desired formulations in the laboratory.
3. A greater control of pH, temperature etc. is necessary as compared to that with serum containing media.
4. Growth rate and the maximum cell density attained are lower than those with serum containing media.
5. Cells tend to become fragile during prolonged agitated cultures unless biopolymers or synthetic polymers are added.

Several defined media have been evolved from the Eagle's minimal essential medium (MEM), e.g., Dulbecco's enriched modification (DME), Ham's F12, CMRL1066, RPMI 1640, McCoy's 5A and Iscove's modified Dulbecco's medium (IMDM); all are commercially available.

Often a 1 mixture of DME and F12 is used as a serum-free formulation. The most frequently used media are listed in. If needed, purified proteins and/or hormones may be added to the medium.

(iii) Chemically Defined Media. These media contain contamination- free ultra-pure inorganic and organic constituents, and may contain pure protein additives, like insulin, epidermal growth factor etc. that have been produced in bacteria or yeast by genetic engineering.

(iv) Protein-free Media. In contrast, protein-free media do not contain any protein; they only contain non-protein Constituents necessary for culture of the cells.

Several assays have developed for the determination of the best medium for a given cell type, i.e.,

(1) long-term (over several days) cell multiplication assay in form of clonal growth assay, cell growth curve analysis etc., and
(2) short-term (for several hours) [3H]-thymidine assay. It is desirable to use the long-term assays. Butoften the [3H]- thymidine methods are used for screening; these results should be compared with atleast growth curve analysis or, preferably, clonal growth assay.

2.2. Most Cultured Animal Cells Grow Only on Special Solid Surfaces: Within the tissues of intact animals, most cells tightly contact and interact specifically with other cells via various cellular junctions. The cells also contact the extracellular matrix, a complex network of secreted proteins and carbohydrates that fills the spaces between cells. The matrix, whose constituents are secreted by cells themselves, helps bind the cells in tissues together; it also provides a lattice through which cells can move, particularly during the early stages of animal differentiation.
The tendency of animal cells in vivo to interact with one another and with the surrounding extracellular matrix is mimicked in their growth in culture. Unlike bacterial and yeast cells, which can be grown in suspension, most cultured animal cells require a surface to grow on. Many types of cells can adhere to and grow on glass, or on specially treated plastics with negatively charged groups on the surface (e.g., SO_3^{2-}). The cultured cells secrete collagens and other matrix components; these bind to the culture surface and function as a bridge between it and the cells. Cells cultured from single cells on a glass or a plastic dish form visible colonies in 10 –14 days. Some tumor cells can be grown in suspension, a considerable experimental advantage because equivalent samples are easier to obtain from suspension cultures than from colonies grown in a dish.
a. Substrate material: Glass and disposal plastic: This was the original substrate because of its optical properties and surface charge. It is used in form of slides, cover slips and test tubes, which may not be variously modified. It is cheap, easily washed without loosing its growth supporting properties, but it has been replaced in most laboratories by synthetic plastic usually polystene, which has greater consistency and superior optical properties.
b. Palladium and metallic surface as substrate: For 90% of animal cells and tissue cultures, glass or plastic is employed as a substrate for growth of fibroblasts. Stainless steel discs and other metallic surfaces were also used.
c. Feeder layers: While matrix coating may also help attachment, growth and differentiation, some cultures particularly at low cell densities, require support from living cells in form of monolayers. This action is due partly to supplementation of the medium either metabolic leakage or secretion of growth factors from the fibroblasts, but may also be due to conditioning of substrate by cell products. These monolayers are called feeder layers, since they also feed the growing cultures. These feeder layer may consist of mouse embryo fibroblasts, normal fetal intestine, glial cells etc.

and has been effectively used for selective growth of breast and coelomic epithelium, central and peripheral neurons, and also for growing cells used for the production of transgenic transfection.

2.3. The gas phase for tissue culture: The animal cell and tissue cultures also require for their growth a gas phase consisting of O_2 and CO_2 even though cultures may vary in their oxygen requirement. These gases also influence the pH and HCO_3^- ion concentration on the culture. Most cell lines grow well at pH 7.4. The CO_2 in the gas phase appears in the medium as dissolved CO_2 in equilibrium with HCO_3^- and lowers the pH.

2.4. Temperature: The optimal temperature for cell growth is dependent on the body temperature of the animal from which cell s are obtained Any anatomical variation in temperature (eg. temperature of skin and testis may be lower then that of rest of body) and Incorporation of safety factors to allow for minor errors in regulating the incubator. Thus the temperature recommended for most human and warm-blooded animal cell lines is 37 ^{0}C, close to body heat.

B3. Primary Cell Culture

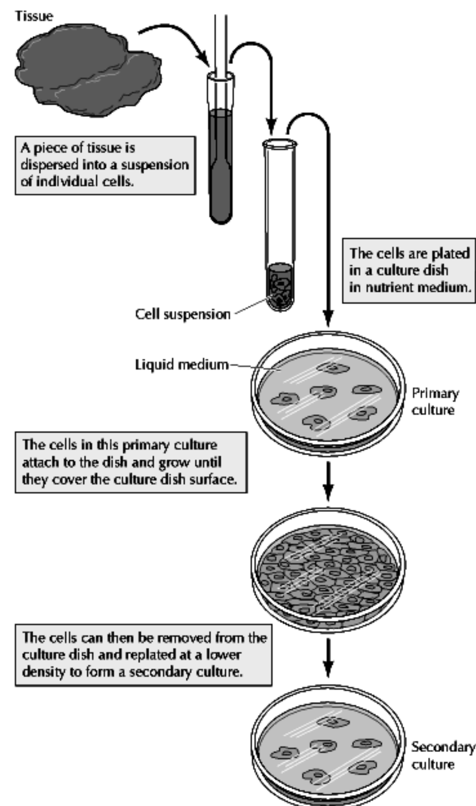
A primary culture is that stage of the culture following isolation of the cells, but before the first subculture. There are three stages to consider:
(1) isolation of the tissue,
(2) dissection and/or disaggregation, and
(3) culture following seeding into the culture vessel.

Following isolation, a primary cell culture may be obtained either by allowing cells to migrate out from fragments of tissue adhering to a suitable substrate or by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells, some of which will ultimately attach to the substrate. It appears to be essential for most normal untransformed cells with the exception of hematopoietic cells - to attach to a flat surface in order to survive and proliferate with maximum efficiency. Transformed cells, on the other hand, particularly cells from transplantable animal tumors, are often able to proliferate in suspension.

The enzymes used most frequently are crude preparations of trypsin, collagenase, elastase, hyaluronidase, DNase, pronase, dispase, alone or in various combinations. Crude preparations are often more successful than purified enzyme preparations, as the former contain other pro teases as contaminants, although the latter are generally less toxic and more specific in their action. Trypsin and pronase give the most complete disaggregation, but may damage the cells. Collagenase and dispase, on the other hand give incomplete disaggregation, but are less harmful Hyaluronidase can be used in conjunction with collagenase to digest the intracellular matrix, and DNase is employed to disperse DNA released from lysed cell as it tends to impair proteolysis and promote reaggregation.

Although each tissue may require a different set of conditions, certain requirements are shared by most primary cultures:

1. Fat and necrotic tissue is best removed during dissection.
2. The tissue should be chopped finely with sharp instruments to cause minimum damage.
3. Enzymes used for disaggregation should be removed subsequently by gentle centrifugation.
4. The concentration of cells in the primary culture should be much higher than that normally used for subculture, since the proportion of cells from the tissue that survives in primary culture may be quite low.
5. A rich medium, such as Ham's F12, is preferable to a simple medium, such as Eagle's MEM, and, if serum is required, fetal bovine often gives better survival than does calf or horse. Isolation of specific cell types will probably require selective media.
6. Embryonic tissue is preferable, as it disaggregates more readily, yields more viable cells, and proliferates more rapidly in primary culture than does adult tissue.



3.1 Isolation of the Tissue:

Before attempting to work with human or animal tissue, make sure that your work fits within medical ethical rules or current legislation on experimentation with animals. An attempt should be made to sterlize the site of dissection with 70% alcohol if the site is likely to be contaminated (e.g. skin). Remove the tissue aspetically and transfer it to the tissue culture laboratory in BSS or medium as soon as possible. Do not dissect the animal in the tissue culture laboratory, as the animals may carry microbial contamination. If a delay in transferring a tissue is unavoidable, it can be held at 4^0 C for up to 72 hours, although a better yield will result from the quicker transfer.

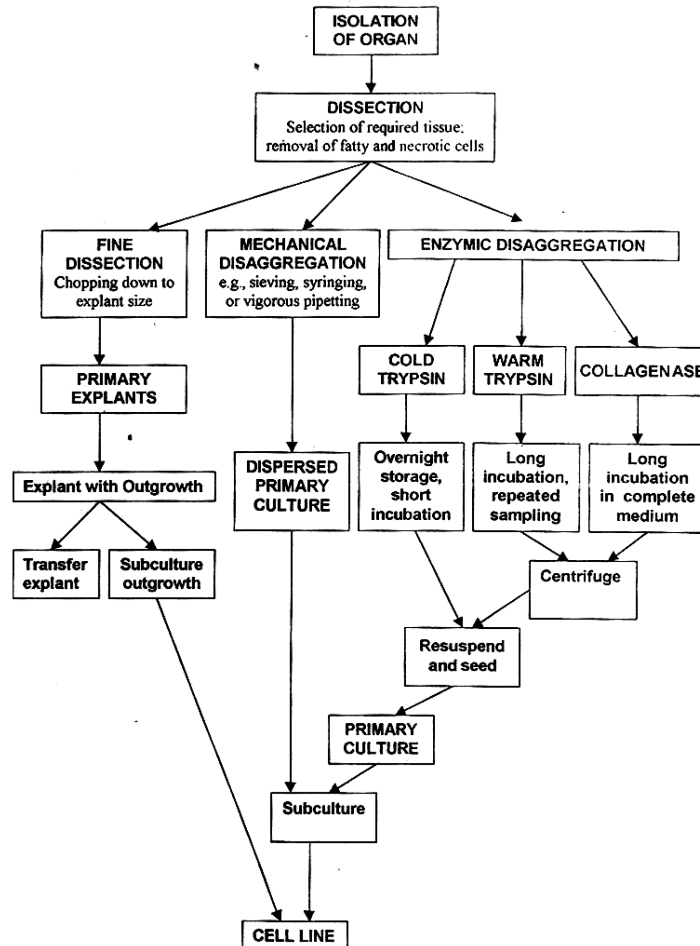
Mouse embryos are a convenient source of cells for undifferentiated fibroblastic cultures. They are often used as feeder layer. Chick embryos are easier to dissect, as they are larger then the equivalent stage of mouse embryo.

Like mouse embryos, chick embryos are used to provide predominantly mesenchymal cell primary cultures for cell proliferation analysis, to provide feeder layers, and as a substrate for viral propagation. Because of their larger size, it is also easier to dissect out individual organs to generate specific cell types, such as hepatocytes, cardiac muscle, and lung epithelium. As with the mouse embryo, the use of chick embryo is subject o animal legislation and working with embryos that are more than half-term requires a license.

For handling human biopsy material it is usually necessary to obtain consent from the hospital ethical committee, from the attending physician and from the patient or the patient's relatives. Furthermore, biopsy sampling is usually performed for diagnostic purposes, and hence the needs of the pathologist must be met first. There is also the difficult problem of ownership and subsequent patent right to deal with.

3.2 Primary Culture:

Several techniques have been devised for primary culture of isolated tissue. These techniques can be divided into purely mechanical techniques, involving dissection with or without some form of maceration, and techniques utilizing enzymatic disaggregation.(Fig 1) Briefly, dissection of primary explants is suitable when very small amounts of tissue are available, enzymatic disaggregation when more tissue is available but a high recovery is required, and mechanical dis-aggregation when large amounts of soft tissue are available and the size of the yield is not paramount.



3.3 Primary Explant

The primary-explant technique was the original method developed by Harrison [1907], Carrel [1912], and others for initiating a tissue culture. As originally performed, a fragment of tissue was embedded in blood plasma or lymph, mixed with heterologous serum and embryo extract, and placed on a coverslip which was inverted over a concavity slide. The clotted plasma held the tissue in place, and the explant could be examined with a conventional microscope. The embryo extract and serum, together with the plasma, supplied nutrients and stimulated migration out of the explant across the solid substrate. The heterologous serum was used to promote clotting of the plasma. This technique is still used, but has been largely replaced by the simplified method.

3.4 Enzymatic Disaggregation:

3.4.1. Trypsinization: Crude trypsin is by far the most common enzyme used in tissue disaggregation, as it is tolerated quite well by many cells, it is effective for many tissues, and any residual activity left after washing is neutralized by the serum of the culture medium, or by a trypsin inhibitor (e.g., soya bean trypsin inhibitor) when serum-free medium is used. It is important to minimize the exposure of cells to active trypsin in order to preserve maximum viability.

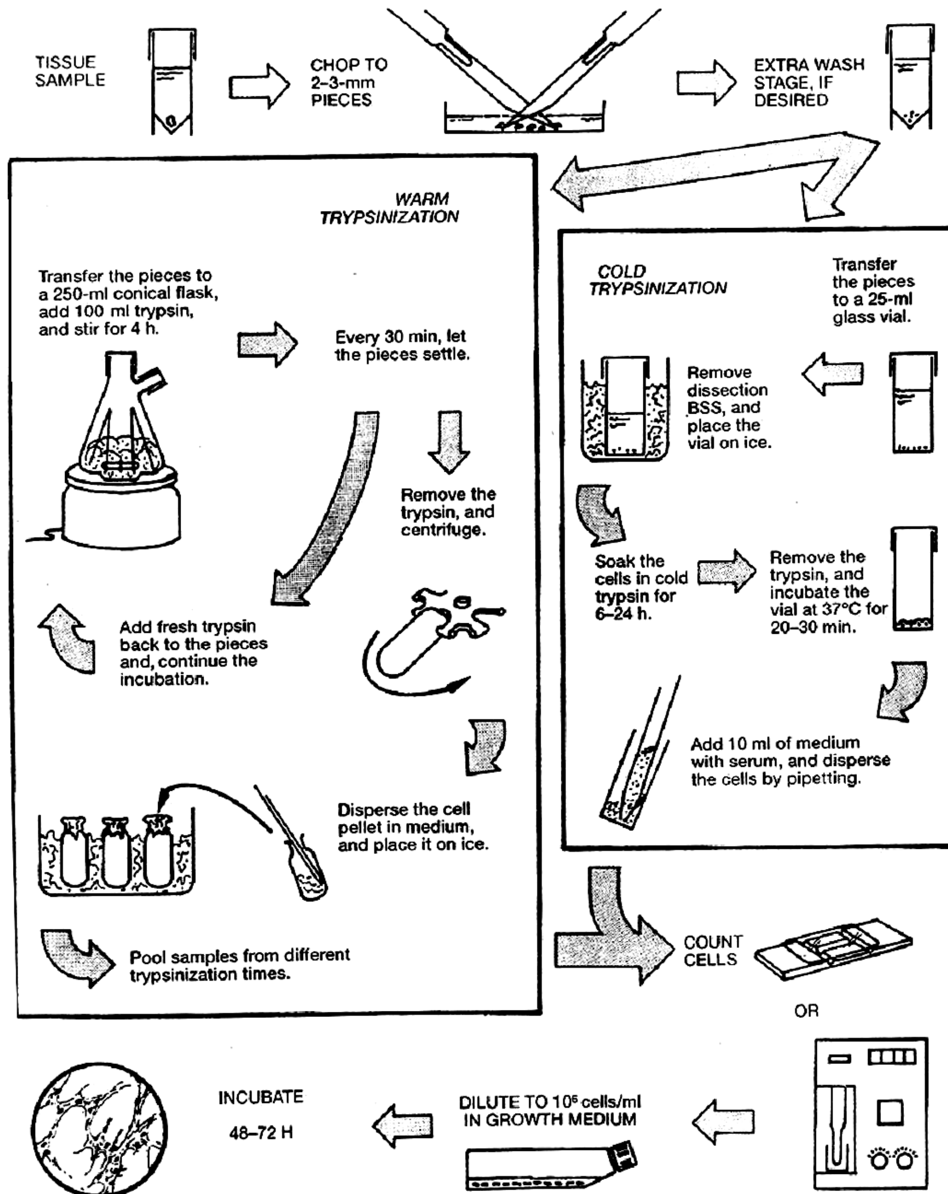


Fig. Trypsin Disaggregation. Preparation of primary culture by disaggregation in trypsin. The warm-trypsin method is shown on the left, and the cold-trypsin method is shown on the right.

Hence, when whole tissue is being trypsinized at 37 ^{0}C, dissociated cells should be collected every half hour, and trypsin should be removed by centrifugation and neutralized with serum in medium. Soaking the tissue for 6-18 hours in trypsin at 4° C allows the penetration with minimal tryptic activity and digestion may then proceed for more a shorter time (i.e. 20-30 min) at 37^0 C. Although the cold trypsin method gives a higher yield of viable cells and requires less effort, the warm trypsin method is still used extensively.

Warm Trypsinization: The tissue is chopped and stirred in trypsin for a few hours at 37^0 C. The dissociated cells are collected every half hour, centrifuged, and pooled in medium containing serum.

Trypsinization with Cold Pre-exposure: One of the disadvantages of using trypsin to disaggregate tissue is the damage that may result from prolonged exposure to the tissue to trypsin at 37° C - - hence the need to harvest cells after 30-min incubations in the warm-trypsin method rather than have them exposed for the full time (i.e., 3 to 4 h) required to disaggregate the whole tissue. A simple method of minimizing damage to the cells during disaggregation is to soak the tissue in trypsin at 4°C to allow penetration of the enzyme with little tryptic activity Following this procedure, the tissue will require much shorter incubation at 37° C for disaggregation.

The cold-trypsin method usually gives a higher yield of viable cells, with improved survival after 24 h culture and preserves more different cell types than the warm method. Cultures from mouse embryos contain more epithelial cells when prepared by the cold method, and erythroid cultures from 13-d fetal mouse liver respond to erythropoietin after this treatment, but not after the warm-trypsin method or mechanical disaggregation. The cold-trypsin method is also convenient, as no stirring or centrifugation is required, and the incubation at 4°C may be done overnight. This method does take longer than the warm-trypsin method however and is not as convenient when large amount of tissue (i.e., greater than 10 g) are being handled.

3.4.2 Other Enzymatic Procedures

Disaggregation in trypsin can be damaging (e.g., to some epithelial cells) or ineffective (e.g., for very fibrous tissue, such as fibrous connective tissue), so attempts have been made to utilize other enzymes. Since, the extracellular matrix often contains collagen, particularly in connective tissue and muscle, collagenase has been the obvious choice. Other bacterial proteases, such as pronase and dispase have also been used with varying degrees of success. The participation of carbohydrate in intracellular adhesion has led to the use of hyaluronidase and neuraminidase in conjunction with collagenase. Other proteases continue to appear on the market. With the selection now available, screening available samples is the only option if trypsin, collagenase, dispase, pronase, hyaluronidase, and DNase, alone and in combinations, do not prove to be successful. It is not possible to describe here all of the primary disaggregation techniques that have been used, but the method described in the next section has been found to be effective in several normal and malignant tissues.

Disaggregation in collagenase

This technique is very simple and effective for many tissues: embryonic, adult, normal, and malignant. It is of greatest benefit when the tissue is either too fibrous or too sensitive to allow the successful use of trypsin. Crude collagenase is often used and may depend, for some of its action, on contamination with other nonspecific pro teases. More highly purified grades are available if nonspecific proteolytic activity is undesirable, but they may not be as effective as crude collagenase. Finely chopped tissues are placed in complete medium containing collagenase and are incubated. When tissue is disaggregated, collagenase is removed by centrifugation, cells are seeded at a high concentration, and cultured.

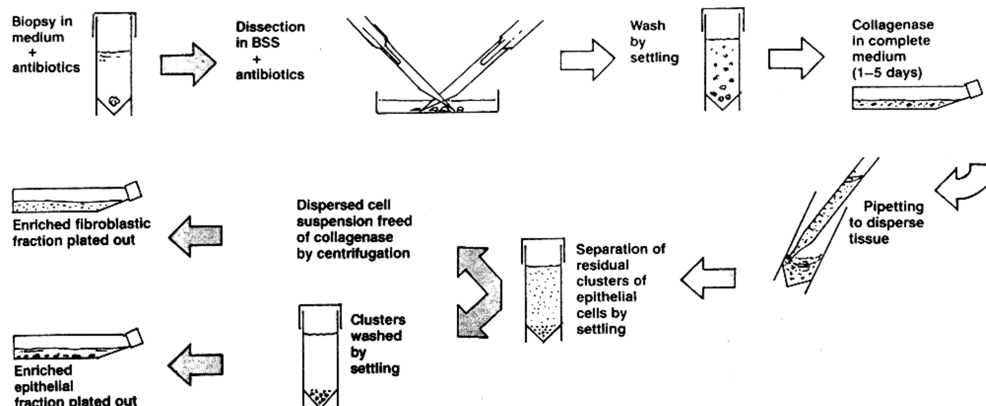


Fig. Stages in Disaggregation of Tissue for Primary Culture by Collagenase..

Some cells, particularly macrophages, may adhere to the first flask during the collagenase incubation. Transferring the cells to a fresh flask after collagenase treatment (and subsequent removal of the collegenase) removes many of the macrophages from the culture. The first flask may be cultured as well, if required. Light trypsinization will remove any adherent cells other than macro phages.

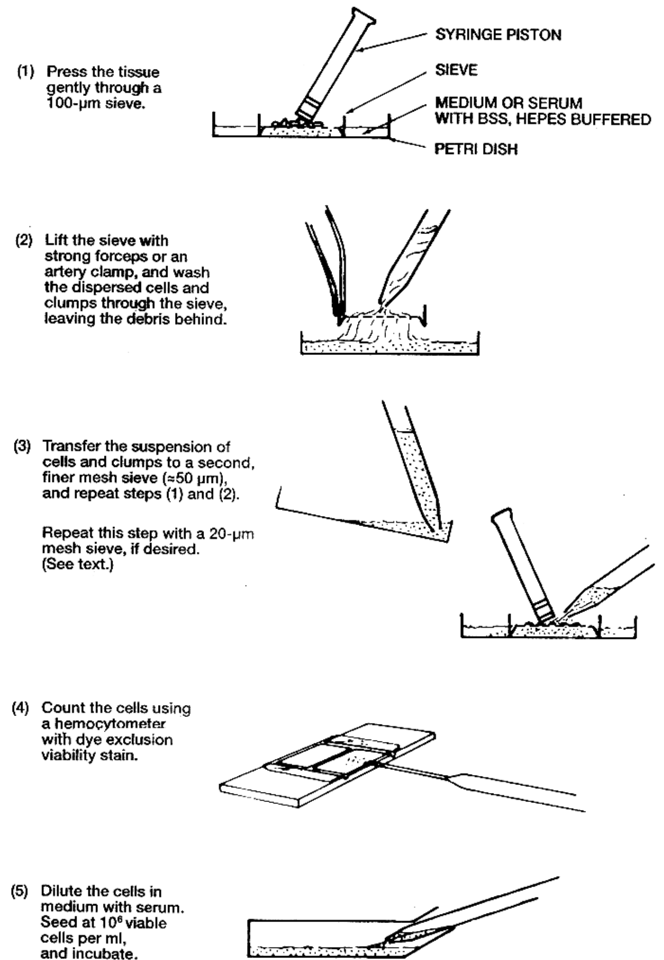
Disaggregation in collagenase has proved particularly suitable for the culture of human tumors, mouse kidney, human adult and feral brain, lung, and many other tissues, particularly epithelium. The process is gentle and requires no mechanical agitation or special equipment. With more than 1 g of tissue, however, it becomes tedious as the dissection stage and can be expensive, due to the amount of collagenase required. It will also release most of the connective tissue cells, accentuating the problem of fibroblastic outgrowth, so it may need to be followed by selective culture or cell separation.

The discrete clusters of epithelial cells produced by disaggregation in collagenase and by the cold-trypsin method can be selected under a dissection microscope and transferred to individual wells in a microtitration plate, alone or with irradiated or mitomycin C-treated feeder cells.

The addition of hyaluronidase aids disaggregation by attacking terminal carbohydrate residues on the surface of the cells. This combination has been found to be particularly effective for dissociating rat or rabbit liver, by perfusing the whole organ *in situ* and completing the disaggregation by stirring the partially digested tissue in the same enzyme solution for a further 1015 min, if necessary. This technique gives a good yield of viable hepatocytes and is a good starting point for further culture.

3.5 Mechanical Disaggregation

The outgrowth of cells from primary explants is a relatively slow process and can be highly selective. Enzymatic digestion, is rather more labor intensive, though, potentially, it gives a culture that is more representative of the tissue. As there is a risk of damaging cells during enzymatic digestion, many people have chosen to use the alternative of mechanical disaggregation-for example, collecting the cells that spill out when the tissue is carefully sliced, pressing the dissected tissue through a series of sieves for which the mesh is gradually reduced in size or, alternatively forcing the tissue fragments through a syringe and needle or simply pipetting it repeatedly. This procedure gives a cell suspension more quickly than does enzymatic digestion, but may cause mechanical damage. The following protocol is one method of mechanical disaggregation that has been found to be moderately successful with soft tissues, such as brain.



Protocol

1. After washing and preliminary dissection of the tissue, chop the tissue into pieces about 3-5 mm across, and place a few pieces at a rime into a stainless steel or polypropylene sieve of 1-mm mesh in a 9-cm Petri dish or centrifuge tube
2. Force the tissue through the mesh into medium *by* applying gentle pressure with the piston of a disposable plastic syringe. Pipette more medium through the sieve to wash the cells through it.
3. Pipette the partially disaggregated tissue from the Petri dish into a sieve of finer porosity, perhaps *100-µm* mesh, and repeat step 2.
4. The suspension may be diluted and cultured at this stage, or it may be sieved further through 20µ*m* mesh if it is important to produce a single cell suspension. In general, the more highly dispersed the cell suspension, the higher the sheer stress required and the lower the resulting viability.
5. Seed the culture flasks at 2×10^5, 1×10^6, and 2×10^6 cells/ml by diluting the cell suspension in medium.

Only soft tissues, such as spleen, embryonic liver, embryonic and adult brain, and some human and animal soft tumors, respond well to this technique.

Even with brain, for which fairly complete disaggregation can be obtained easily, the viability of the resulting suspension is lower than that achieved with enzymatic digestion, although the rime taken may be very much less. When the availability of tissue is nor a limitation and the efficiency of the yield is unimportant, it may be possible to produce, in a shorter amount of time, as many viable cells with mechanical disaggregation as with enzymatic digestion, but as the expense of very much more tissue.

Separation of Viable and Nonviable Cells

When an adherent primary culture is prepared from dissociated cells, nonviable cells are removed at the first change of medium. With primary cultures maintained in suspension, nonviable cells are gradually diluted our when cell proliferation starts. If necessary, however, nonviable cells may be removed from the primary disaggregate by centrifuging the cells on a mixture of Ficoll and sodium metrizoate (e.g., Hypaque or Triosil) . This technique is similar to the preparation of lymphocytes from peripheral blood. The dead cells will form a pellet at the bottom of the tube.

B4. Organ Culture:

Organ culture seeks to retain the original structural relationship of cells of the same or different types, and hence their interactive function, in order to study the effect of exogenous stimuli on further development. This relationship may be preserved by explanting the tissue intact or recreated by separating the constituents and recombining them.

4.1. Gas and Nutrient Exchange: A major deficiency in tissue architecture in organ culture is the absence of a vascular system, limiting the size (by diffusion) and potentially the polarity of the cells within the organ culture. When the cells are cultured as a solid mass of tissue, gaseous diffusion and the exchange of nutrients and metabolites becomes the limitating. The dimensions of individual cell cultured in suspension or as a monolayer are such that diffusion is rapid, but aggregates of cells beyond about 250µl in diameter (5,000 cells) start to become limited by diffusion, and at or above 1.0 mm in diameter (~2.5 X 10^5 cells) central necrosis is often apparent. To alleviate this problem, organ culture is usually placed at the interface between the liquid and gaseous phases, to facilitate gas exchange while remaining access to nutrients. Most systems achieve this by positioning the explant on a raft or gel exposed to the air (see Fig.), but explants anchored to a solid substrate can also be aerated by rocking the culture exposing it alternately to a liquid medium and a gas phase, or by using a roller bottle or tube

Anchorage to a solid substrate can lead to the development of an outgrowth from the explant and resultant alterations in geometry although this effect can be minimized by using a non- wettable surface. One of the advantages of culture at the gas -liquid interface is that the explant retains a spherical geometry if the liquid is maintained at the correct level. If the liquid is too deep, gas exchange is impaired; if it is too shallow, surface tension will tend to flatten the explant and promote outgrowth.

Increased permeation of oxygen can also be achieved by using increasing O_2, concentrations up to pure oxygen or by using hyperbaric oxygen. Certain tissues-e. g., thyroid and prostate, trachea, and skin, particularly from a newborn or an adult-may benefit from elevated O_2, tension, but often, this benefit is at the risk of O_2-induced toxicity. As increasing the O_2, tension will not facilitate CO_2 release or nutrient metabolite exchange, the benefits of increased oxygen may be overridden by other limiting factors.

4.2. Structural Integrity: Structural integrity, above other considerations, was and is the main reason for adopting organ culture as an *in vitro* technique in preference to cell culture. While cell culture utilizes cells dissociated by mechanical or enzymic techniques or spontaneous migration, organ culture deliberately maintains the cellular associations found in the tissue. Initially, organ culture was selected to facilitate histological characterization, but ultimately it was discovered that certain elements of phenotypic expression were found only if cells were maintained in close association.

It is now recognized that associated cells do exchange signals via junctional communications (gap junctions), via paracrine factors, and via cell adhesion molecules. Signaling between cells is most striking during organogenesis, but is probably also required for the maintenance of fully mature tissues.

Therefore, maintenance of the structural integrity of the original tissue may preserve the correct homologous and heterologous cellular interactions present in the original tissue and maintain the correct configuration of the extra cellular matrix.

4.3. Growth and Differentiation: There is a relationship between growth and differentiation such that differentiated cells no longer proliferate. It is also possible that cessation of growth may in itself contribute to the induction of differentiation, if only by providing a permissive phenotypic state that is receptive to exogenous inducers of differentiation. Because of density limitation of cell proliferation and the physical restrictions imposed by organ culture geometry, most organ cultures do not grow, or, if they do, proliferation is limited to the outer cell layers. Hence, the status of the culture is permissive to differentiation and,

given the appropriate cellular interactions and soluble inducers, should provide an ideal environment for differentiation to occur.

4.4. Types of Organ Culture: As techniques for organ culture have been dictated largely by the requirement to place the tissue at a location that allows optimal gas and nutrient exchange, most of these techniques put the tissue at the gas liquid interface on semisolid gel substrates of agar or clotted plasma or on a raft of microporous filter, lens paper, or rayon supported on a stainless steel grid or adherent to a strip of Perspex or Plexiglas. This type of geometry is now most easily attained with filter-well inserts. The following, protocol uses organ primordia from chick embryo but is applicable to many other types of tissue.

4.4.1 Culture of Embryonic organs: Embryonic organ culture is easier than to normal organ from adult animals. Following are the three techniques used for embryo culture.

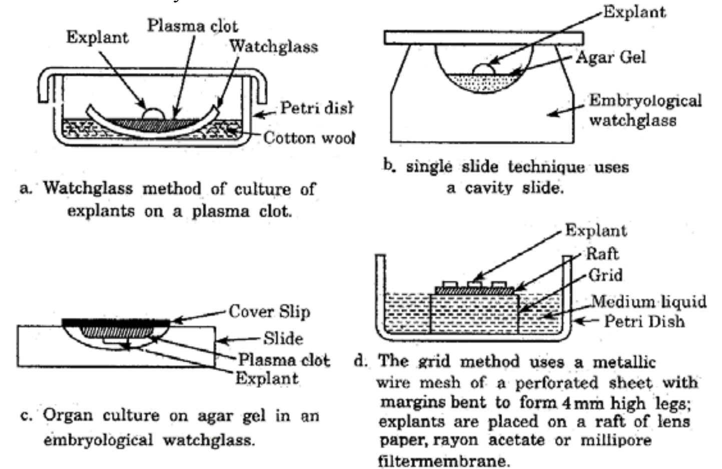
a. Organ culture on Plasma clots: It involves the following steps:

1. Prepare a plasma clot by mixing 15 drops of plasma with five drops of embryo extract in a watch glass.
2. Place a watch glass on a pad of cotton wool in Petridish; cotton wool is kept moist to prevent excessive evaporation from the dish.
3. Place a small, carefully dissected piece of tissue on top of the plasma clots in watch glass.

The technique has now been modified, and a raft of lens paper or rayon net is used on which the tissue is placed. Transfer of the tissue can then be achieved by raft easily. Excessive fluid is removed and the net with the tissue placed again on the fresh pool of medium.

b. Organ Culture on Agar Gel: In this approach, the medium (consisting of a suitable salt solution, se'rum, chick embryo extract or a mixture of certain amino acids and vitamins) is gelled with 1% agar. This method avoids immersion of explants into the medium and permits the use of defined media. Generally, explants need to be subcultured on fresh agar gels every 5-7 days. The agar gels are generally kept in embryological watchglasses (Fig. c) and sealed with paraffin wax. The explants can be examined using a stereoscopic microscope. This method has been used to study many developmental aspects of normal organs as wall as of tumours.

c. Grid Method: Initially devised by Trowell in 1954, the grid method utilizes 25 mm x 25 mm pieces of a suitable wire mesh or perforated satinless steel sheet whose edges are bent to form 4 legs of about 4 mm height. Skeletal tissues are generally placed directly on the grid but softer tissues like glands or skin are first placed on rafts which are then kept on the grids. The grids themselves are placed in a culture chamber filled with fluid medium upto the grid; the chamber is supplied with a mixture of O_2 and CO_2 to meet the high O_2 requirement of adult mammalian organs.A modification (Fig[d]) of the original grid method is widely used to study the growth and differentiation of adult and embryonic tissues.



a. Watchglass method of culture of explants on a plasma clot.

b. single slide technique uses a cavity slide.

c. Organ culture on agar gel in an embryological watchglass.

d. The grid method uses a metallic wire mesh of a perforated sheet with margins bent to form 4 mm high legs; explants are placed on a raft of lens paper, rayon acetate or millipore filtermembrane.

d. Cyclic Exposure to Medium and Gas Phase: This technique has been successful in long-term (upto 4-5 months) culture of human adult tissues like oesophagus, mammary epithelium, uterine endocervix etc. The explants are intermittently exposed to the fluid medium and the gas phase. The number of explants are intermittently exposed to the fluid medium and the gas phase. The number of explants per dish varies from 2-18 depending on the organ cultured. The explants are attached to the bottom of a plastic culture dish and are covered with fluid medium. The dishes are enclosed in a chamber containing a suitable gas mixture and mounted on a rocker platform. The chamber is rocked at several cycles/min to ensure cyclic exposure of the organ explants to the medium and the gas phases.

4.4.2 Whole Embryo Culture:

a. Culture of ChicK Embryo (Spratt,1956)

In this techniquw, 40 hours old embryos were used and the embryo development could be followed for another 24-48 hours, in vitro before the embryo dies.

Steps of Chick Embryo Culture:

- Prepare a suitable defined media and added to sterile watch glasses, palced on moist absorbent cotton wool pads in petri dish as grown in embryo culture.
- Incubate the hens egg at 38^0C for 40-42 hours to provide about a dozen embryos.
- The shell is wiped with alcohol and broken into sterile evoparating dish containing 50 ml chick saline or BSS.
- A circular cut is made using scissor into the vitelline membrane around the blastoderm and the latter is transferred to a petri dish containing BSS.
- The adherent vitelline membrane is removed with the aid of forceps and the embryo is

examined under the microscope to determine the stage of development.

- The blastoderm is transferred to the top of the medium in the watch glass prepared as above.
- The blstoderm is spread on agar gel (ventral side down) and the excess BSS is removed.
- Culture is incubated at 37.5^0C.

4. 5. Limitations of Organ Culture: Analysis of organ cultures depends largely on histological techniques and they do not lend themselves readily to biochemical and molecular analyses. Bio-chemical monitoring requires reproducibility between samples, which is less easily achieved in organ culture than in propagated cell lines, due to sampling variation in preparing an organ culture, minor differences in handling and geometry, and variations in the ratios of cell types among cultures. Organ cultures are also more difficult to prepare than replicate cultures from a passaged cell line and do not have the advantage of a characterized reference Stock to which they may be related. Organ cultures cannot be propagated, and hence each experiment requires recourse to the original donor tissue. Preparation is labor intensive, and as a result, the yield of usable tissue is often too low to be of value in biochemical or molecular assays. Furthermore, as the population of reacting cells may be a minor component of the culture, it is difficult to analyze the biochemical name of the response and attribute it to the correct cell type, other than by auto radiographic, histo-chemical, or immunochemical techniques, which tends to be more qualitative than quantitative.

4.6. Application of Organ culture: Organ culture is essentially a technique for studying the behavior of integrated tissues rather than isolated cells. It is precisely in this area that a future understanding of the control of gene expression (and ultimately of cell behavior) in multicellular organisms may lie, bur the limitations imposed by the organ culture system are such that recombinant systems between purified cell types may contribute the information at this particular stage. However, there is no doubt that organ culture has contributed a great deal to our understanding of developmental biology and tissue interactions and that it will continue to do so in the absence of adequate synthetic systems.

B5. Techniques for the mass culture of animal cell lines:

The cultivation of animal cells differs significantly from the techniques used with bacteria, yeasts, and fungi. Tissues excised from specific organs of animals, such as lung and kidney, under aseptic conditions are transferred into a growth medium containing serum and small amounts of antibiotics in small T -flasks. These cells form a **primary culture**. Unlike plant cells, *primary* mammalian cells do not normally form aggregates, but grow in the form of monolayers on support surfaces such as glass surfaces of flasks. Using the proteolytic enzyme trypsin, individual cells in a tissue can be separated to form single-cell cultures. The cells directly derived from excised tissues are known as the primary culture. A cell line obtained from the primary culture is known as the *secondary culture.* Cells are removed from the surfaces of flasks using a solution of EDT A, trypsin, collagenase, or pronase. The exposure time for cell removal is 5 to 30 min at 37°C. After cells are removed from surfaces, serum is added to the culture bottle. The serum-containing suspension is centrifuged, washed w1th buffered isotonic saline solution, and used to inoculate secondary cultures.

5.1. Secondary Culture: Cultures prepared directly from the tissues of an organism, that is, without cell proliferation *in vitro,* are called *primary cultures*. These can be made with or without an initial fractionation step to separate different cell types. In most cases, cells in primary cultures can be removed from the culture dish and made to proliferate to form a large number of so-called ***secondary cultures***; in this way, they may be repeatedly sub-cultured for weeks or months. Such cells often display many of the differentiated properties appropriate to their origin: fibroblasts continue to secrete collagen; cells derived from embryonic skeletal muscle fuse to form muscle fibers that contract spontaneously in the culture dish; nerve cells extend axons that are electrically excitable and make synapses with other nerve cells; and epithelial cells form extensive sheets with many of the properties of an intact epithelium. Because these phenomena occur in culture, they are accessible to study in ways that are often not possible in intact tissues

5.2. Cell Lines: To be able to clone individual cells, modify cell behavior, or select mutants, biologists often want to maintain cell cultures for many more than 100 doublings. This is possible with cells derived from some tumors and with rare cells that arise spontaneously because they have undergone genetic changes that endow them with the ability to grow indefinitely. The genetic changes that allow these cells to grow indefinitely are collectively called *oncogenic* transformation, and the cells are said to be *oncogenically transformed,* or simply *transformed.* A culture of cells with an indefinite life span is considered immortal; such a culture is called a cell line to distinguish it from an impermanent cell *strain.*

The ability of cultured cells to grow indefinitely or their tendency to be transformed varies depending on the animal species from which the cells originate. Normal chicken cells rarely are transformed and die out after only a few doublings; even tumor cells from chickens almost never exhibit immortality. Among human cells, only tumor cells grow indefinitely. The HeLa cell, the first human cell type to be grown in culture, was originally obtained in 1952 from a malignant tumor

(carcinoma) of the uterine cervix. This cell line has been invaluable for research on human cells.

In contrast to human and chicken cells, cultures of embryonic adherent cells from rodents routinely give rise to cell lines. When adherent rodent cells are first explanted, they grow well, but after a number of serial replatings they lose growth potential and the culture goes into crisis. During this period most of the cells die, but often a rapidly dividing variant cell arises spontaneously and takes over the culture. A cell line derived from such a variant will grow forever if it is provided with the necessary nutrients. Cells in spontaneously established rodent cell lines and in cell lines derived from tumors often have abnormal chromosomes. In addition, their chromosome number usually is greater than that of the normal cell from which they arose, and it continually expands and contracts in culture. Such cells are said to be *aneuploid* (i.e., have an inappropriate number of chromosomes) and are obviously mutants.

Although most cell lines are undifferentiated, some can carry out many of the functions characteristic of the normal differentiated cells from which they are derived. One example is certain hepatoma cell lines (e.g., HepG2) that synthesize most of the serum proteins made by normal hepatocytes (the major cell type in the liver) from which they are derived. These highly differentiated hepatoma cells are often studied as models of normal hepatocytes. Cultured *myoblasts* (muscle precursor cells) are another example of transformed cells that continue to perform many functions of a specialized, differentiated cell. When grown in culture, transformed myoblasts can be induced to fuse to form myotubes. These resemble differentiated multinucleated muscle cells and synthesize many of, if not all, the specialized proteins associated with contraction. Certain lines of epithelial cells also have been cultured successfully. One such line, Madin-Darby canine kidney (MDCK) cells, forms a continuous sheet of polarized epithelial cells one cell thick that exhibits many of the properties of the normal canine kidney epithelium from which it was derived. This type of preparation has proved valuable as a model for studying the functions of epithelial cells.

These highly differentiated cells are said to be *polarized* because the plasma membrane is organized into at least two discrete regions. For example, the epithelial cells that line the intestine form a simple columnar epithelium. That portion of the plasma membrane facing the lumen of the intestine, the *apical* surface, is specialized for absorption; the rest of the plasma membrane, the *basolateral* surface, mediates transport of nutrients from the cell to the blood and forms junctions with adjacent cells and the underlying extracellular matrix called the basal lamina.

Certain cells cultured from blood, spleen, or bone marrow adhere poorly, if at all, to a culture dish but nonetheless grow well. In the body, such nonadherent cells are held in suspension (in the blood), or they are loosely adherent (in the bone marrow and spleen). Because these cells often come from immature stages in the development of differentiated blood cells, they are very useful for studying normal blood cell differentiation and the abnormal development of leukemias.

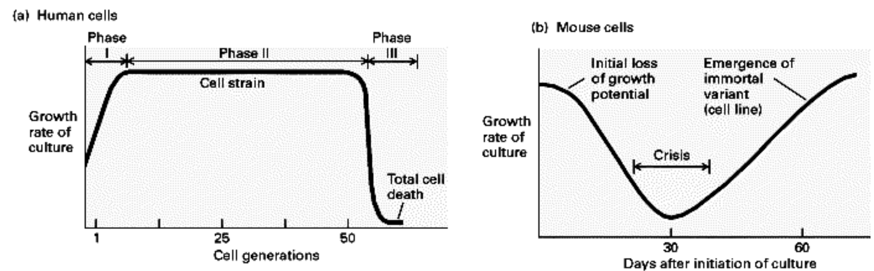


Figure : Stages in the establishment of a cell culture. *(a) When an initial explant is made of human cells, some cells die and others (mainly fibroblasts) start to grow; overall the growth rate increases (phase I). If the remaining cells are continually diluted, the cell strain grows at a constant rate for about 50 cell generations (phase II), after which the growth rate falls rapidly. During the ensuing period of increasing cell death (phase III), all the cells in the culture eventually die. (b) In a culture prepared from mouse or other rodent embryo cells, there is initial cell death coupled with the emergence of healthy growing cells. As these are diluted and allowed to continue growth, they soon begin to lose growth potential and most cells die (the culture goes into crisis). Very rare cells do not die but continue growing until their progeny overgrow the culture. These cells constitute a cell line, which will grow forever if it is appropriately diluted and fed with nutrients: the cells are immortal.*

When cells are removed from an embryo or an adult animal, most of the adherent ones grow continuously in culture for only a limited time before they spontaneously cease growing. Such a culture eventually dies out after many cell doublings, even if it is provided with fresh supplies of all the known nutrients that cells need to grow, including serum. For instance, when human fetal cells are explanted into cell culture, the majority of cells die within a relatively short time; "fibroblasts," although also destined to die, proliferate for a while and soon become the predominant cell type. They divide about 50 times before they cease growth. Starting with 10^6 cells, 50 doublings can produce $10^6 \times 2^{50}$, or more than 10^{20} cells, which is equivalent to the weight of about 10^5 people. Thus, even though its lifetime is limited, a single culture, if carefully maintained, can be studied through many generations. Such a lineage of cells originating from one initial primary culture is called a cell strain.

Pieces of an organ or whole organ can be cultured in vitro. For organ culture whole organs, or representative parts, are maintained as small fragments in culture. During this process organ retain their intrinsic distribution, numerical and spatial, of participating cells. The main objective is

to maintain architecture of the tissue and direct it towards normal development. In this technique, it is essential that the tissue should never be disrupted or damaged. It requires careful handling. Media used for growing organ culture are generally the same as those used for tissue culture. The techniques or organ culture can be divided into

(i) those employing a solid medium and

(ii) those employing liquid medium.

To start cultures of animal cells, excised tissues are cut into small pieces (-2 mm^3) and are placed in an agitated flask containing a dilute solution of trypsin (-0.25% w/v) for 120 min at 37°C in buffered saline. The cell suspension is passed through a presterilized filter to clear the solution, and cells are washed in the centrifuge. The cells are then resuspended in growth medium and placed in T-flasks or roller bottles. Cells usually attach onto the glass surface of the bottle and grow to form a monolayer. The cells growing on support surfaces are known as *anchorage-dependent* cells. Surface attachment is necessary for these cells to assume the three-dimensional shape necessary for the alignment of internal structures in a manner allowing growth. However, some cells grow in suspension culture and are known to be *nonanchorage-dependent* cells.

Many secondary lines can be adapted to grow in suspension and are nonanchorage dependent. Most differentiated mammalian cell lines (for example, human fibroblasts such as WI-38 and MRC-5 that are licensed for human vaccine production) are *mortal.* These cell lines undergo a process called *senescence.* Essentially, the cells, for reasons that are not completely understood, will only divide for a limited number of generations (for example, about 30 generations for the MRC-5 cells). Cells that can be propagated indefinitely are called *continuous, immortal,* or *transformed* cell lines. Cancer cells are naturally immortal. All cancerous cell lines are transformed, although it is not clear whether all transformed cell lines are cancerous.

Although mammalian cell lines have been the primary focus of work in animal cell culture, the two are not synonymous. Insect, fish, and crustacean cell cultures are evolving technologies. In particular, insect cell culture is unusually promising for biotechnological purposes. The baculovirus that infects insect cells is an ideal vector for genetic engineering because it is nonpathogenic to humans and has a very strong promoter that encodes for a protein that is not essential for virus production in cell culture. The insertion of a gene under the control of this promoter can lead to high expression levels (40% of the total protein as the target protein). Most cell lines are derived from ovaries or embryonic tissue, although at least one differentiated cell line (a BTI-EAA blood cell line) has been maintained in indefinite culture for 15 years. Insect cell lines are not transformed, but are naturally continuous. In contrast, senescence is observed with many fish cell lines.

Another important category is the culture of hybridomas. Hybridoma cells are obtained by fusing lymphocytes (normal blood cells that make antibodies) with myeloma (cancer) cells. Lymphocytes producing antibodies grow slowly and are mortal. After fusion with myeloma cells, hybridomas become immortal, can reproduce indefinitely, and produce antibodies. Using hybridoma cells, highly specific., monoclonal (originating from one cell) antibodies can be produced against specific antigens.

To produce hybridoma cells, animals (mice) are injected (immunized) with certain antigens. As a reaction to antigens, the animals produce antibodies. The antibody-producing cells (for example, spleen B lymphocytes) are separated from blood sera and fused with certain tumor cells (for example, myeloma cells with infinite capacity to proliferate derived from lymphocytes). The resulting cells are hybrid cells (hybridomas), which secrete highly specific antibodies (monoclonal Ab's) against the immunizing antigen.

Mammalian cell culture to produce proteins other than antibodies usually involves genetically engineered cells. Although many host cells can be used, Chinese hamster ovary (CHO) cells are particularly popular. Most of the vectors

5.3 Bioreactor Considerations for Animal Cell culture:

Mammalian cells are large (10 to 20 μ*m* diameter), slow growing (t_d = 10 to 50 h), and very shear sensitive. Moreover, some animal cells are anchorage dependent and must grow on surfaces of glass, especially treated plastics, natural polymers such as collagen, or other support materials; some are not anchorage dependent and can grow in suspension culture. Product concentration (titer) is usually very low (μg/ml), and toxic metabolites such as ammonium and lactate are produced during growth. These properties of animal cells set certain constraints on the design of animal cell bioreactors. Certain common features of these reactors are the following:

1. The reactor should be gently aerated and agitated. Some mechanically agitated reactors operating at agitation speeds over 20 rpm and bubble column and airlift reactors operating at high aeration rates may cause shear damage to cells. Shear sensitivity is strain dependent.
2. Well-controlled homogeneous environmental conditions (T, pH, DO, redox potential) and a supply of CO_2 enriched air need to be provided.
3. A large support material surface-volume ratio needs to be provided for anchorage-dependent cells.
4. The removal of toxic products of metabolism, such as lactic acid and ammo nium, and the concentration of high-value products, such as MAb's, vaccines, and Iymphokines, should be accomplished during cell cultivation.

5.4 Monolayer Culture

Monolayer cultures are essential for anchorage dependent cells. Scaling up of such cultures is based on increasing the available surface area by using plates, spirals, ceramics and microcarriers (most effective). The various culture vessels used are briefly described below.

(i) Roux Bottle: It is commonly used in laboratory, and is kept stationary so that only a portion of its internal surface is available for cell anchorage Each bottle provides ca.175-200 cm^2 surface area for cell attachment and occupies 750-1000 cm3 space.

(ii) Roller Bottle: This vessel permits a limited scale up as it is rocked or preferably, rolled so that all of its internal surface is available for anchorage. Several modifications of roller bottle further enhance the available surface, *e.g.*,

- Spira-Cel (spiral polystyrene cartridge),
- Glass tube (roller bottle packed with a parallel cluster of small glass tubes separated by silicone spacer rings), and
- Extended surface area roller bottle (the bottle surface is corrugated enhancing the surface by a factor of two) etc.

(iii) Multitray Unit: A standard unit has 10 chambers stacked on each other which have interconnecting channels; this enables the various operations to be carried out in one go for all the chambers. Each chamber has a surface area of 600 cm^2 and the total volume of the unit is 12.5 lit. This polystyrene unit is disposable and gives good results similar to plastic flasks.

(iv)Synthetic Hollow Fibre Cartridge: The fibres enclosed in a sealed cartridge provide a large surface area for cell attachment on the outside surface of fibres. The capillary fibres are made up of acrylic polymer, are 350 µm in diameter with 75 µm thick walls. The medium is pumped in through the fiber; it perfuses through the fiber walls and becomes available to the cells. The surface area available is very high (upto 30 cm^2/ml of medium volume). The system is mainly used for suspension cells, but is also suitable for cell anchorage if polysulphone type fiber is used.

(v) Opticell Culture System: It consists of a cylindrical ceramic cartridge in which 1 mm^2 channels run through the length of the unit, and perfusion loop to a reservoir is provided for environmental (medium, gas etc.) control. It gives about 40 cm^2 surface area/ml of medium. It is suitable for virus, cell surface antigen and monoclonal antibody production, and for both suspension and monolayer cell cultures.

(vi) Plastic Film: Teflon (fluoro ethylene poropylene copolymer) is biologically inert and highly permeable to gas. Teflon bags (5 x 30 cm) filled with cells and medium (2-10 mm deep) serve as good culture vessels; cells attach to the inside surface of bags. Alternatively, teflon tubes are wrapped round a reel with a spacer and the medium is pumped through the tube; cells grow on the inside surface of tube (a culture vessel called stericell is available).

(vii) Heli-Cell vessels: These vessels are packed with polystyrene ribbons (3 mm x 5-10 mm x 100 µm) that are twisted in helical shape. The medium is pumped through the vessel, the helical shape of ribbons ensuring good circulation; the cells adhere to the ribbon surfaces. All the culture vessels, in addition to the increased surface area due to the vessel design, allow further scaling up by the use of multiple units of the vessels. In contrast, the following three culture systems allow scaling up in a single unit by increasing the vessel volume. In addition, they make the monolayer culture system considerably similar to suspension cultures.

(viii)Bead Bed Reactors: These reactors are packed with 3-5 mm glass beads (which provide the surface for cell attachment) and the medium is pumped either up or down the bead column. Use of 5 mm beads gives better cell yields than that of 3 mm beads.

(ix) Heterogeneous Reactors: These reactors contain circular glass or stainless steel plates stacked 5-7 mm apart and fitted to a central shaft . Either an airlift pump is used for mixing or the shaft is rotated either vertically or horizontally. The chief disadvantage of the system is very low ratio of surface area to medium volume (1-2 cm^2/ml).

(x) Microcarrier cultures: These are use 90-300 mm dia particles as substrate for cell attachment. Initially, Dextron beads (Sephadex A-50) were used by Van Wezel in 1967; these were not entirely satisfactory due to the unsuitable charge of beads and possibly due to toxic effects. The microcarriers available for use at present range for Dextran, polystyrene, polyacrolein, glass, polyacrylamide, silica, DEAE sephadex, cellulose, gelatin to collagen; the specific gravity of microcarriers ranges from 1.02 to 1.05.

Microcarrier cultures are initiated by harvesting cells from 3 lit of logarithmic phase (log phase) culture and inoculating them in 1 lit of fresh medium to which 2-3 g/1 of microcarriers is then added. The culture is stirred at 15-25 rpm (revolutions per minute) for 3-8 hours. During this period, cells attach to microcarrier beads and later grow as a monolayer. The volume of culture is slowly increased to 3 lit and stirring is enhanced to the normal rates (20-100 rpm). As the cells grow, the beads become heavier and need to be agitated at higher speeds. The medium needs to be changed every 3 days. Samples of beads can be drawn for observations on cell morphology, growth and number.

Use of microcarriers permits the handling of monolayer systems as suspension cultures. However, cells do not grow to the same degree as they do in stationary cultures. Harvesting of cells from microcarrier beads in rather simple. Stirring is

stopped, the medium is drained off, the beads are washed in buffer, treated with trypsin or some other suitable enzyme, the culture is shaken at 75-125 rpm for 20-30 min, stirring is stopped for 2 min and the supernatant is poured and collected. Alternatively, the beads may be dissolved where possible, *e.g.,* gelatin beads are dissolved by trypsin, collagen-coated beads are treated with collagenase and dextranase is used for dextran beads; these treatments leave the cells free which are collected. Scaling up of microcarrier cultures can be done either by increasing the concentration of beads or by enlarging the culture vessel, when high microcarrier concentrations are used, medium perfusion becomes necessary, and efficient filters must be used to allow medium withdrawal without cells and microcarriers. The oxygen supply is problematic; it can be based on surface aeration, increased perfusion rate of fully aerated medium and sparging into the filter compartment.

B6. Application of Animal Cell Culture

Animal cell cultures are used to produce virus vaccines, as well as a veriety of useful biochemicals which are mainly high molecular weight proteins like enzymes, hormones, cellular biochemicals like interferons, and imunobiological compounds including monoclonal antibodies. Animal cells are also good hosts for the expression of recombinant DNA molecules and a number of commercial products have been/are being developed. Initially, virus vaccines were the dominant commercial products from cell cultures, but at present monoclonal antibody production is the chief commercial activity. It is expected that recombinant proteins would become the prime product from cell cultures in the near future.

TABLE 1 A list of products generated/likely to be generated from cultured animal cells.

Product	Cell line	Remarks
Virus Vaccines		
Canine distemper	Dog kidney	
Foot and mouth disease (FMD)	Cow kidney	
Hepatitis-B	Human plasma-derived	From blood of infecte individuals
Influenza	Chick embryo	
Measles	Chick embryo	
Mumps	Chick embryo	
* Polio	Primate kidney	First vaccine from cel culture
* Rabies	1. Chick embryo 2. Human diploid cells	
* Rubela (German measles, MMR)	Human diploid cells	
Cellular Biochemicals		
Angiogenic factor	Human tumour	
β-Interferon	Mouse fibroblast	
β- and γ-interferons	Chinese hamster ovary (CHO) cell lines	
α-Interferon	Human leucocytes	
Immunoregulatory Biochemicals		
Interleukin-2	Lymphoblastoid T-cell line	Continuous cell line
Lymphokines	—	
Enzymes		
Collagenase, pepsin, Renin, trypsin	—	
Urokinase (Plasminogen activator)	Human kidney	
Tissue-type plasminogen activator	CHO cell line	
Single chain urokinase type Plasminogen activator	Human kidney transformed cell line TCL 598	
Tyrosine hydroxylase		
Hormones		
Luteinizing hormone		
Follicle stimulating hormone		
Chorionic hormone		
Erythropoietin		
Growth hormone		
ACTH (Adenocorticotrophic hormone)		
Immunobiologicals		
Monoclonal antibodies	Hybridoma clone	Most dominant activity

* Being manufactured in India.

Transplantable tissues and organs are another very valuable product from cell cultures.Artificial skins are already in use for grafting in burn and other patients, and efforts are focussed on developing transplantable cartilage and other tissues.Animal cell products usually consist of high-molecular-weight proteins with or without glycosidic groups. A number of enzymes, hormones, vaccines, immunobiologicals (monoclonal antibodies, lymphokines) and anticancer agents can be produced using animal cell culture technology (Table 1).

The major types of immunobiologicals produced by animal cells are (1) monoclonal antibodies, (2) immunobiological regulators (interleukins, lymphokines), and (3) virus vaccines (prophylactics).

6.1. Monoclonal Antibodies: One of the most important products of animal cell culture has been monoclonal antibodies (MAb's). MAb's are produced by hybridoma cells and are used in diagnostic assay systems, for therapeutic purposes, and for biological separations (for example, affinity chromatography). MAb's have been used to determine well over 100 drugs, toxins, vitamins, and other biological compounds as diagnostic agents. MAb's are also used for chromatographic separations to purify protein molecules. Purification of interferon by affinity chromatography is an example of the use of MAb's for protein purification purposes. MAb's also may serve as "magic" bullets to target toxic agents to cancer tumors. However, the large size of MAb's has limited their ability to penetrate some tumors.

6.2. Immunobiological Regulators: Interferon (an anticancer glycoprotein secreted by animal cells upon exposure to cancer-causing agents) is an example of an immunoregulator produced by mammalian cells. Interferon can be produced by either animal cells or recombinant (genetically engineered) bacteria. Other immunoregulators are lymphokines (hormonal proteins regulating immune responses of human body), interleukines (anticancer agents), tissue plasminogen activator (a compound preventing blood clotting), and thymosin. The production of immunoregulators is a very promising area with a great potential for growth in the near future.

6.3. Virus Vaccines: Various virus vaccines (prophylactics) have been produced for animal and human use. Table 2 summarizes human vaccines produced by mammalian cells. The virus vaccines should provide lifelong immunity, be safe, and be given as a single dose.

Table 2: Animal Cell Virus Products for Human Use

Existing	Prospective	Future
Mumps	Hepatitis	A Common cold
Measles	Hepatitis B	Some cancers
Rubella	Herpes simplex type I	AIDS
Rabies	Herpes simplex type 2	
Yellow fever	Cytomegalovirus	
Polio	Varicella-zoster	
Influenza	Respiratory syncytial virus	
Adenovirus	Rift Valley fever	
Smallpox		

6.4. Hormones: Some animal hormones are large molecules (50 to 200 amino acids) and are glycosylated. These hormones can be produced by using cell cultures of the hormonesynthesizing organ. Some potential hormone products from animal cells are follicle-stimulating hormone, chorionic hormone, and erythropoetin. Some animal hormones are relatively small polypeptides (20 to 30 amino acids) and may be produced by chemical synthesis. Erythropoetin is a very successful commercial product useful in treating anemia in a wide range of disorders from patients on artificial kidneys to those with AIDS.

6.5. Enzymes: A number of enzymes can be produced by animal cell cultures. The synthesis and excretion of these enzymes are targets of genetic engineering. Glycosylation, posttranslational modification, and excretion from the host organism are potential problems. Some potential enzyme products from animal cell cultures are urodnase, rennin, asparaginase, collagenase, pepsin, trypsin, hyaluronidase, and blood-clotting compounds such as factor VII, factor VIII, and factor X. The large-scale production of tissue plasminogen activator is an important commercial process.

6.6. Insecticides: Animal cell cultures have been used to produce some insect viruses that are highly specific and safe to the environment. Several of the baculoviruses have federal approval for use as insecticides. Genetically engineered variants with greater virulence can now be produced. The commercial production of such viruses from issue culture has not yet been accomplished.

6.7. Whole Cells and Tissue Culture: The production of differentiated cells for medical use is under early development. Artificial skin for burn patients is one example. Artificial organs and semisynthetic bone and dental structures are potentially feasible.

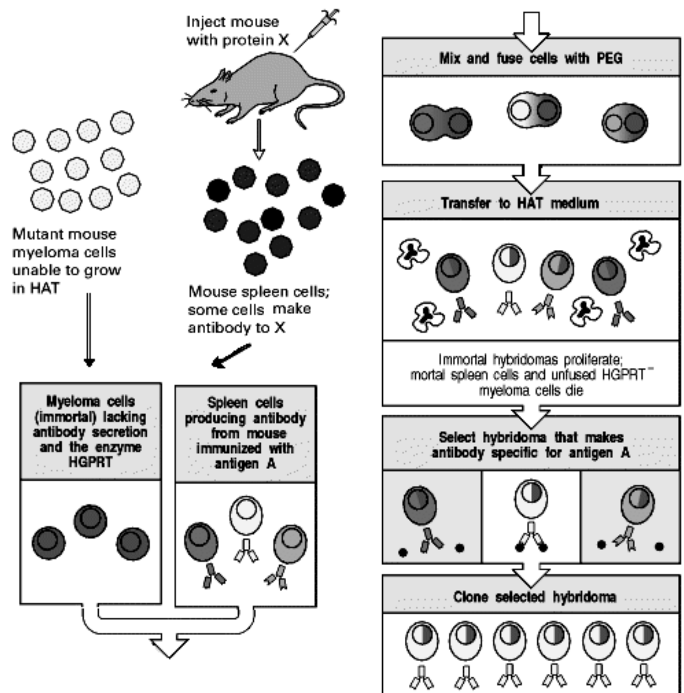
B7. Hybridoma Technology:

Georges Köhler and Cesar Milstein, devised a technique for producing a homogeneous population of antibodies of known antigenic specificity. They did this by fusing spleen cells from an immunized mouse to cells of a mouse myeloma to produce hybrid cells that both proliferated indefinitely and secreted antibody specific for the antigen used to immunize the spleen cell donor. The spleen cell provides the ability to make specific antibody, while the myeloma cell provides the ability to grow indefinitely in culture and secrete immunoglobulin continuously. By using a myeloma cell partner that produces no antibody proteins itself, the antibody produced by the hybrid cells comes only from the immune spleen cell partner. After fusion, the hybrid cells are selected using drugs that kill the myeloma parental cell, while the unfused parental spleen cells have a limited life-span and soon die, so that only hybrid myeloma cell lines or hybridomas survive. Those hybridomas producing antibody of the desired specificity are then identified and cloned by regrowing the cultures from single cells. Since each hybridoma is a clone derived from fusion with a single B cell, all the antibody molecules it produces are identical in structure, including their antigen-binding site and isotype. Such antibodies are called monoclonal antibodies. This technology has revolutionized the use of antibodies by providing a limitless supply of antibody of a single and known specificity. Monoclonal antibodies are now used in most serological assays, as diagnostic probes, and as therapeutic agents. So far, however, only mouse monoclonals are routinely produced and efforts to use this same approach to make human monoclonal antibodies have met with very limited success.

7.1. Procedure:

Mice are immunized with antigen A (protein X) and given an intravenous booster immunization three days before they are killed, in order to produce a large population of spleen cells secreting specific antibody. Spleen cells die after a few days in culture. In order to produce a continuous source of antibody they are fused with immortal myeloma cells by using polyethylene glycol (PEG) to produce a hybrid cell line called a hybridoma.

The myeloma cells are selected beforehand to ensure that they are not secreting antibody themselves and that they are sensitive to the hypoxanthine-aminopterin-thymidine (HAT) medium that is used to select hybrid cells because they lack the enzyme hypoxanthine:guanine phosphoribosyl transferase (HGPRT). The HGPRT gene contributed by the spleen cell allows hybrid cells to survive in the HAT medium, and only hybrid cells can grow continuously in culture because of the malignant potential contributed by the myeloma cells. Therefore, unfused myeloma cells and unfused spleen cells die in the HAT medium, as shown here by cells with dark, irregular nuclei. Individual hybridomas are then screened for antibody production, and cells that make antibody of the desired specificity are cloned by growing them up from a single antibody-producing cell. The cloned hybridoma cells are grown in bulk culture to produce large amounts of antibody. Each hybridoma produces a single antibody. Once a hybridoma that produces a desired antibody is identified, the clone can be cultured to yield large amounts of that antibody.As each hybridoma is descended from a single cell, all the cells of a hybridoma cell line make the same antibody molecule, which is thus called a monoclonal antibody



7.2 Fusion of Cultured Animal Cells Can Yield Interspecific Hybrids Useful in Somatic Cell Genetics:

Cultured animal cells infrequently undergo cell fusion spontaneously. The fusion rate, however, increases greatly in the presence of certain viruses that have a lipoprotein envelope similar to the plasma membrane of animal cells. A mutant viral glycoprotein in the envelope promotes cell fusion. Cell fusion also is promoted by polyethylene glycol, which causes the plasma membranes of adjacent cells to adhere to each other and to fuse. As most fused animal cells undergo cell division, the nuclei eventually fuse, producing viable cells with a single nucleus that contains chromosomes from both "parents." The fusion of two cells that are genetically different yields a hybrid cell called a heterokaryon.

Because some **somatic cells** from animals can be cultured from single cells in a well-defined medium, it is possible to select for genetically distinct cultured animal cells, just as is done with bacterial and yeast cells. Moreover, during mitosis

the chromosomes in an animal cell are large and highly visible after staining, making it easy to distinguish individual chromosomes. Genetic studies of cultured animal cells are called *somatic-cell genetics* to distinguish them from *classical genetics,* which deals with whole organisms derived from **germ cells** (sperm and eggs).

Cultured cells from different mammals can be fused to produce interspecific hybrids, which have been widely used in somatic-cell genetics. For instance, hybrids can be prepared from human cells and mutant mouse cells that lack an enzyme required for synthesis of a particular essential metabolite. As the human-mouse hybrid cells grow and divide, they gradually lose human chromosomes in random order, but retain the mouse chromosomes. In a medium that can support growth of both the human cells and mutant mouse cells, the hybrids eventually lose all human chromosomes. However, in a medium lacking the essential metabolite that the mouse cells cannot produce, the one human chromosome that contains the gene encoding the needed enzyme will be retained, because any hybrid cells that lose it following mitosis will die. All other human chromosomes eventually are lost.

By using different mutant mouse cells and media in which they cannot grow, researchers have prepared various panels of hybrid cell lines. Each cell line in a panel contains either a single human chromosome or a small number of human chromosomes, and a full set of mouse chromosomes. Because each chromosome can be identified visually under a light microscope, such hybrid cells provide a means for assigning, or "mapping," individual genes to specific chromosomes.

For example, suppose a hybrid cell line is shown microscopically to contain a particular human chromosome. That hybrid cell line can then be tested biochemically for the presence of various human enzymes, exposed to specific antibodies to detect human surface antigens, or subjected to DNA hybridization and cloning techniques to locate particular human DNA sequences. The genes encoding a human protein or containing a human DNA sequence detected in such tests must be located on the particular human chromosome carried by the cell line being tested. Panels of hybrids between normal mouse and mutant hamster cells also have been established; in these hybrid cells, the majority of mouse chromosomes are lost, allowing mouse genes to be mapped to specific mouse chromosomes.

7.3 Hybrid Cells Often Are Selected on HAT Medium:

One metabolic pathway has been particularly useful in cell-fusion experiments. Most animal cells can synthesize the purine and pyrimidine nucleotides de novo from simpler carbon and nitrogen compounds, rather than from already formed purines and pyrimidines. The folic acid antagonists amethopterin and aminopterin interfere with the donation of methyl and formyl groups by tetrahydrofolic acid in the early stages of de novo synthesis of glycine, purine nucleoside monophosphates, and thymidine monophosphate. These drugs are called *antifolates,* since they block reactions involving tetrahydrofolate, an active form of folic acid. Many cells, however, contain enzymes that can synthesize the necessary nucleotides from purine bases and thymidine if they are provided in the medium; these *salvage pathways* bypass the metabolic blocks imposed by antifolates.

A number of mutant cell lines lacking the enzyme needed to catalyze one of the steps in a salvage pathway have been isolated. For example, cell lines lacking thymidine kinase (TK) can be selected because such cells are resistant to the otherwise toxic thymidine analog 5-bromodeoxyuridine. Cells containing TK convert 5-bromodeoxyuridine into 5-bromodeoxyuridine monophosphate. This nucleoside mono- phosphate is then converted into a nucleoside triphosphate by other enzymes and is incorporated by DNA polymerase into DNA, where it exerts its toxic effects. This pathway is blocked in cells with a *TK* mutation that prevents production of functional TK enzyme.

Hence, TK^{-} mutants are resis- tant to the toxic effects of 5-bromodeoxyuridine. Similarly, cells lacking the HGPRT enzyme have been selected because they are resistant to the otherwise toxic guanine analog 6-thioguanine. As we will see next, $HGPRT^{-}$ cells and TK^{-} cells are useful partners in cell fusions with one another or with cells that have salvage-pathway enzymes but that are differentiated and cannot grow in culture by themselves.

The medium most often used to select hybrid cells is called *HAT medium,* because it contains hypoxanthine (a purine), aminopterin, and thymidine. Normal cells can grow in HAT medium because even though aminopterin blocks de novo synthesis of purines and TMP, the thymidine in the media is transported into the cell and converted to TMP by TK and the hypoxanthine is transported and converted into usable purines by HGPRT. On the other hand, neither TK^{-} nor $HGPRT^{-}$ cells can grow in HAT medium because each lacks an enzyme of the salvage pathway. However, hybrids formed by fusion of these two mutants will carry a normal *TK* gene from the $HGPRT^{-}$ parent and a normal *HGPRT* gene from the TK^{-} parent. The hybrids thus will produce both functional salvage-pathway enzymes and grow on HAT medium. Likewise, hybrids formed by fusion of mutant cells and normal cells can grow in HAT medium.

C1. Trangenic Animals-Introduction

Animals are made up of billions of cells. Producing a transgenic animal, which has the same new piece of DNA incorporated into each of its cells, seems at first glance to be a highly time-consuming process! Luckily, scientists can take advantage of a fundamental property of animal development: all the cells in a given animal can be traced back to one cell. In other words, every animal began as one cell, which divided over and over again to finally produce the billions of cells which make up that animal.

Therefore, to ensure that every cell in an animal contains the same new piece of DNA, scientists add the DNA to the one celled entity before it starts dividing. The new DNA becomes incorporated into the cell's genome, and will theoretically be present in all the cells that make up the animal.

Developing transgenic animals is more complex than generating transgenic bacteria or plants. Transgenic bacteria are fairly easy to produce, as they are unicellular. This means that the difficulty encountered with transgenic multicellular organisms of making sure that the new DNA is present in all of the organism's cells is avoided. For many plant types, plant biotechnologists can cause any transgenic plant cell to grow into a transgenic plant. However, transgenic animals must be generated by altering **germ cells**. Germ cells (like egg and sperm cells) are the only animal cells capable of giving rise to new offspring. Other cells in an animal (called somatic cells, like blood, skin, brain or heart cells) are not capable of giving rise to whole new animals.

One way to produce a transgenic animal is to use microinjection. The new DNA is injected directly into a fertilized egg cell (called a zygote) before it starts dividing. The new DNA gets incorporated into a chromosome in the nucleus, and will therefore be present in every cell of the resulting animal. The new DNA will also be present in the resulting animal's germ cells, meaning that the new DNA will be passed on to many of that animal's offspring.

C2. TRANSFECTION OF ANIMAL CELLS

The ability to introduce nucleic acids into cells has enabled the advancement of our knowledge of genetic regulation and protein function within eukaryotic cells, tissues and organisms. The process of introducing nucleic acids into cells by non-viral methods, such as the DEAE-dextran and calcium phosphate techniques, is defined as “transfection”. This process is distinct from “infection”, which is a viral method of nucleic acid introduction into cells. Progress in transfection technology was relatively slow until the advent of molecular biology techniques for cloning plasmid DNA. These techniques provided the means to prepare and manipulate DNA sequences and the ability to prepare virtually unlimited amounts of relatively pure DNA for transfection experiments. Cloned sequences could also be used to generate RNA in vitro with phage RNA polymerase using DNA templates with the corresponding polymerase promoter. As the ability to prepare DNA and RNA for transfection became easier, additional methods, such as electroporation and liposome-mediated transfer, were developed to enable more efficient transfer of the nucleic acids to a broad range of cultured mammalian cells. The development of reporter gene systems and selection methods for stable gene expression of transferred DNA greatly expanded the applications for gene transfer technology.

In 1982, Gorman et al. initiated the reporter gene concept with the bacterial chloramphenicol acetyltransferase (CAT) gene and associated CAT assay system. Using a reporter gene that is not endogenous to the cell, coupled with a sensitive assay system for that gene product, allows investigators to clone regulatory sequences of interest upstream of the reporter gene to study expression of the reporter gene under various conditions. This technology, together with the availability of transfection reagents, provides the foundation for studying promoter and enhancer sequences, trans-acting proteins such as transcription factors, mRNA processing, protein/ protein interactions, translation, and recombination events. Since the introduction of the CAT gene and assay system several other reporter systems have been developed for various in vitro and in vivo applications including luciferase, β-galactosidase, alkaline phosphatase and green fluorescent protein.

Integration of DNA into the chromosome, or stable episomal maintenance, of reporter genes and other genes occurs with a relatively low frequency. The ability to select for these cells is made possible using genes that encode resistance to a lethal drug. An example of such a combination is the marker gene for neomycin phosphotransferase with the drug Geneticin. Individual cells that survive the drug treatment expand into clonal groups that can be individually selected, propagated and analyzed.

Today the study of gene regulation, the analysis of the expression and function of proteins within mammalian cells, the generation of transgenic organisms and in vivo/ex vivo gene therapy strategies are all made possible by the availability of gene transfer technologies, nucleic acid molecular biology and reporter gene systems.

Many transfection techniques have been developed. Desirable features include high

efficiency transfer of nucleic acid to the appropriate cellular organelle (for example, DNA into the nucleus), minimal intrusion or interference with normal cell physiology, low toxicity, ease of use, reproducibility, successful generation of stable transfectants, and in vivo efficacy. The techniques developed for gene transfer can be broadly classified as either chemical reagents or physical methods.

2.1 Chemical Reagents

a. **DEAE-dextran** was one of the first chemical reagents used for transfer of nucleic acids into cultured mammalian cells. Mammalian Transfection System-DEAE-Dextran provides reagents for this transfection technique. DEAE-dextran is a cationic polymer that associates with negatively charged nucleic acids. An excess of positive charge, contributed by the polymer in the DNA/polymer complex allows the complex to come into closer association with the negatively charged cell membrane. Uptake of the complex is presumably by endocytosis. This method is successful for delivery of nucleic acids into cells for transient expression; that is, for short-term expression studies of a few days in duration. However, this technique is not generally useful for stable transfection studies that rely upon integration of the transferred DNA into the chromosome. Other synthetic cationic polymers have been used for the transfer of DNA into cells, including polybrene, polyethyleneimine and dendrimers.

b. **Calcium phosphate** co-precipitation became a popular transfection technique following the systematic examination of this method by Graham and van der Eb in the now-classic paper published in 1972. Their study examined the effect of different cations, cationic and phosphate concentrations, and pH on the parameters of transfection. Calcium phosphate co-precipitation is widely used because the components are easily available and reasonable in price, the protocol is easy to use and many different types of cultured cells can be transfected. This method is routinely used for both transient and stable transfection of a variety of cell types. The protocol involves mixing DNA with calcium chloride, adding this in a controlled manner to a buffered saline/phosphate solution and allowing the mixture to incubate at room temperature. This step generates a precipitate that is dispersed onto the cultured cells. The precipitate is taken-up by the cells via endocytosis or phagocytosis. The calcium phosphate also appears to provide protection against intracellular and serum nucleases.

By 1980, artificial liposomes were being used to deliver DNA into cells. The next advancement in liposomal vehicles was the development of synthetic cationic lipids by Felgner and colleagues. Liposome-mediated delivery offers advantages such as relatively high efficiency of gene transfer, ability to transfect certain cell types that are intransigent to calcium phosphate or DEAE-dextran, successful delivery of DNA of all sizes from oligonucleotides to yeast artificial chromosomes, delivery of RNA, and delivery of protein. Cells transfected by liposome techniques can be used for transient and for longer-term experiments that rely upon integration of the DNA into the chromosome or episomal maintenance.

Unlike the DEAE-dextran or calcium phosphate chemical methods, liposome-mediated nucleic acid delivery can be used for in vivo transfer of DNA and RNA to animals and humans. A lipid with overall net positive charge at physiological pH is the most common synthetic lipid component of liposomes developed for gene delivery. Often the cationic lipid is mixed with a neutral lipid such as L-dioleoyl phosphatidyl- ethanolamine (DOPE). The cationic portion of the lipid molecule associates with the negatively charged nucleic acids, resulting in compaction of the nucleic acid in a liposome/nucleic acid complex. For cultured cells, an overall net positive charge of the liposome/nucleic acid complex generally results in higher transfer efficiencies, presumably because this allows closer association of the complex with the negatively charged cell membrane. Following endocytosis, the complexes appear in the endosomes, and later in the nucleus. It is unclear how the nucleic acids are released from the endosomes and traverse the nuclear membrane. DOPE is considered a “fusogenic” lipid and it is thought that its role may be to release these complexes from the endosomes, as well as to facilitate fusion of the outer cell membrane with the liposome/nucleic acid complexes.

2.2 Physical Methods

a. Microinjection: Direct microinjection into cultured cells or nuclei is an effective, although laborious technique to deliver nucleic acids into cells. This method has been used to transfer DNA into embryonic stem cells that are used to produce transgenic organisms. However, this technique is not appropriate for studies that require a large number of transfected cells.

b. Electroporation: It was first reported for gene transfer studies in 1982. This technique is often used for cell types such as plant protoplasts that are particularly recalcitrant to milder methods of gene transfer. The mechanism for entry into the cell is based upon perturbation of the cell membrane by an electrical pulse, which forms pores that allow the passage of nucleic acids into the cell. The technique requires fine-tuning and optimization for duration and strength of the pulse for each type of cell used. A critical balance must be achieved between conditions that allow efficient delivery and conditions that kill cells.

c. Another physical method of gene delivery is biolistic particle delivery: This method relies upon high velocity delivery of nucleic acids on microprojectiles to recipient cells. This method has been successfully employed to deliver nucleic acid to cultured cells, as well as to cells in vivo.

Transfer of genetic material to whole cell can be exercised at the level of (i) individual chromosomes or fragments or (ii) isolated genes or gene fragments.

d. Transfection of fertilized eggs or embryos: The production of transgenic animals requires transfection of specialized cells or embryos, since only eggs or embryos can develop into whole animals. The eggs may be modified by transfer of whole nuclei, whole chromosomes or parts thereof DNA fragments.

e. Transfer of whole nuclei (or split embryos): Transfer of whole nucleus from a somatic cell of the superior donor to the enucleated egg can be achieved using the following steps: (i) enucleation of unfertilized eggs is achieved by centrifuging cytochalasin-B treated cells, such that nuclei detach from the eggs and pellet at the bottom of the tube, leaving enucleated eggs in the supernatant. (ii) Karyoplasts (nuclei with only some residual plasma membrane) are similarly obtained from the blastula stage of the developing embryo of the donor; (iii) karyoplast derived from the donor are incubated with enucleated eggs in the presence of PEG (poly ethylene glycol), and fusion is achieved; (iv) the manipulated egg is transferred to the uterus of surrogate mother for development.

f. Transfer of whole individual chromosomes or fragments: Chromosomes may be isolated from metaphase cells by hypotonic lysis. Incubation of these isolated chromosomes with whole cells after coprecipitation with the calcium phosphate results in their incorporation into the nuclei. Chromosomes once isolated may also may be subjected to fractionation using density centrifugation or flow cytometry and individual specific chromosomes pairs may be inserted into recipient cells.

g. DNA microinjection into the egg: Transfer of individual cloned genes is generally achieved by inserting into fertilized egg, a large number of copies of interest, using the technique of DNA microinjection. The DNA is microinjected into the fertilized into a fertilized egg before the fusion of male and female nuclei. The egg is first immobilized by applying mild suction to the large, blunt holding pipette and DNA is then injected through the sharp end of narrow glass microneedle. Through recombination at the DNA level, the inserted gene may get integrated into the host genome and inherited in a mendellian manner. Transgenic mice have been produced in many laboratories by this technique.

C3. Vectors for Gene Transfer and Expression in Animal Cells

In recent years progress in the design, sophistication, and availability of vectors for gene expression in mammalian cells has been phenomenal. Vectors have many applications, including the study of gene regulation, DNA sequencing, molecular cloning, protein production, antigen expression for vaccination, and gene therapy. There is a large number of vectors available; for example, Vector Database on the Web lists more than 2600 vectors. In spite of the plethora of available vectors, however, robust protein production in mammalian cells is not necessarily a routine matter.

Efficient expression of genes in mammalian cells depends on many factors, including both transcriptional and translational control elements, RNA processing, gene copy number, mRNA stability, the chromosomal site of gene integration, potential toxicity of recombinant proteins to the host cell, as well as the genetic properties of the host. Gene transfer into mammalian cells may be effected either by infection with virus that carries the recombinant gene of interest or by direct transfer of plasmid DNA. Due to space limitations, the emphasis here is on nonviral vectors for high-level protein production. There is extensive literature on mammalian vectors of viral origin with applications in protein production, gene therapy, and vaccine development. Table 1 represents selected references on viral-based vectors.

Viral-based vector
DNA viruses
Cytomegalovirus
Herpes simplex virus
Epstein-Barr virus
Simian virus 40
Bovine papillomavirus
Adeno-associated virus
Adenovirus
Vaccinia virus
Baculovirus
RNA viruses
Semliki Forest virus[a]
Sindbis virus
Poliovirus
Rabies virus
Influenza virus
SV5
Respiratory syncytial virus
Venezuelan equine encephalitis virus
Kunjin virus
Sendai virus
Vesicular stomatitis virus
Retroviruses
Chimeric viral vectors
Adenovirus-Sindbis virus
Adenovirus-adeno-associated virus

The choice of an expression system for production of recombinant proteins depends on many factors, including cell growth characteristics, expression levels, intracellular and extracellular expression, posttranslational modifications and biological activity of the protein of interest, as well as

regulatory issues and economic considerations in the production of therapeutic proteins. Key advantages of mammalian cells over other expression systems are the ability to carry out proper protein folding, complex *N*-linked glycosylation and authentic *O*-linked glycosylation, as well as a broad spectrum of posttranslational modifications.

The essential elements of mammalian expression vectors (Fig. 1) include

1. A constitutive or inducible promoter capable of robust transcriptional activity.
2. Optimized mRNA processing and transla tional signals that include a Kozak sequence, translation termination codon, mRNA cleavage and polyadenylation signals, as well as mRNA splicing signals.
3. A transcription terminator.
4. Selection markers for the preparation of stable cell lines and for gene amplification and
5. Prokaryotic origin of replication and selection markers for vector propagation in bacteria.

The inclusion of the SV40 origin of replication facilitates transient gene expression in COS cells. Other genetic elements for specific applications include fusion moieties, protease cleavage sites, sequences for gene or protein targeting, and IRES elements for the construction of polycistronic vectors (Fig. 1D).

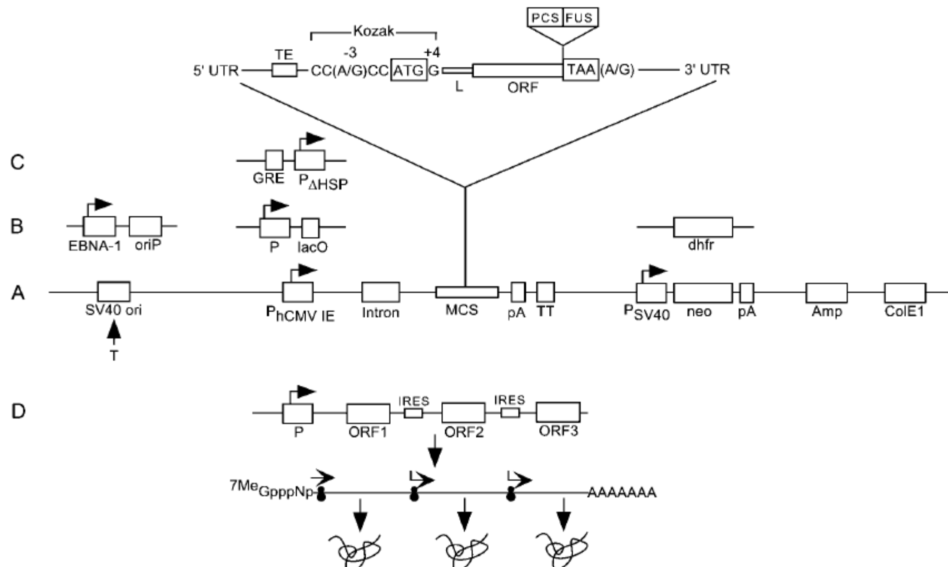


FIG. 1. Configuration of genetic elements in monocistronic (A) and polycistronic (D) expression vectors. Specific elements are shown for illustrative purposes and are not drawn to scale. The source, position, and combination of different components in the vector may vary in order to meet specific experimental requirements. SV40 ori is required for transient gene expression in COS cells. EBNA-1 and oriP facilitate high-copy episomal replication in primate and canine cell lines. The various promoter (P) elements allow constitutive (A) or inducible (B, C) expression. The optimal translational initiation sequence (Kozak) and termination codon followed by purines are shown. The ColE1 origin of replication and the ampicillin-resistance gene allow vector propagation in bacteria. The neomycin-resistance gene facilitates selection in mammalian cells, and the *dhfr* gene allows both selection and gene amplification. In a polycistronic vector (D) IRES elements allow multiple ORFs to be efficiently translated from a single transcript. See text for details. Amp, ampicillin resistance gene; ColE1, prokaryotic origin of replication; dhfr, dihydrofolate reductase; EBNA, Epstein-Barr virus nuclear antigen; FUS, fusion moiety; GRE, glucocorticoid response element; hCMV IE, human cytomegalovirus immediate early enhancer/promoter; HSP, heat shock protein; IRES, internal ribosome entry site; lacO, *lac* operator; L, leader (targeting sequence); MCS, multiple cloning site; neo, neomycin resistance gene; ORF, open reading frame; ori, origin of replication; oriP, Epstein-Barr virus origin of replication; P, promoter; pA, polyadenylation signal; PCS, protease cleavage site; T, SV40 large tumor (T) antigen; TE, translational enhancer; TT, transcription terminator; UTR, untranslated region.

Some Common animal vectors which are being used:

The various animal vectors are based on one or the other virus, *e.g.,* SV40 vectors, bovine papillamovirus vectors, retrovirus vectors etc., or on a transposable element, *e.g., Drosophila P* element vectors (Table 3). The essential features of some of these vectors are briefly described and are summarized in Table 3. It may be pointed out that some of the these vectors, *e.g.,* early or late region replacement SV 40 vectors, retrovirus vectors etc., behave like viruses in that they produce virions which are used to infect the host cells. Some other vectors are like bacterial plasmids, e.*g.,* SV 40 based plasmid vectors, bovine papillomavirus vectors and polyoma virus vectors; these vectors have to be introduced in the cells using a suitable transfection technique. Most of the vectors are replicating in that they have the ability to replicate in suitable animal host cells, but some of them are non-replicating, *e.g.,* the passive transforming SV 40 vectors; the latter are used to carry DNA sequences into the host cells for integration into the animal genomes.

Vectors for Gene Expression in Mammalian Cells

Vector	Promoter	Induction	Selection marker		f1/M13 origin	Reporter/ Purification tag/epitope	MCS	Vector size (kbp)	Comments
			Mammal	Bacteria					
pSVK3	SV40 Early			Amp	f1		8	3.9	Transient
pSVL	SV40 Late			Amp			5	4.9	Transient
pMSG	MMTV-LTR (mouse mammary tumor virus)	Dexamethasone	gpt	Amp			4	7.6	Transient/stable
pCH110	SV40 Early			Amp			1	7.1	Promoter screen
pTargeT	hCMV-IE[b] (cytomegalovirus immediate early)		Neo	Amp	f1		10	5.7	Transient/stable
pSI	SV40 Early			Amp	f1		10	3.6	Transient
pCI	hCMV-IE			Amp	f1		11	4.0	Transient
pCI-neo	hCMV-IE		Neo	Amp	f1		9	5.5	Transient/stable
pOPRSVI/MCS	RSV-LTR (Rous Sarcoma virus)	IPTG[c]	Neo	Amp	f1		8	5.6	Requires cotransfection with pCMVLacI
pBK-CMV	hCMV		Neo	Kana	f1		17		Phagemid
pBK-RSV	RSV-LTR		Neo	Kana	f1		17		Phagemid
pCMV-Script	hCMV		Neo	Kana	f1		17	4.3	
pDual	hCMV (mutated)		Neo	Kana	f1	CBP[d]		5.5	Mammalian and bacterial expression
pCMV-Tag series	hCMV		Neo	Kana	f1	FLAG[e], *c-myc*	1–13	~4.3	
pFLAG series	hCMV			Amp	f1	FLAG			Transient

TABLE 3 : The different types of vectors used for gene transfers in animals.

Vector	*Derived from*	*Features*
1. SV40 vectors (*a*) Early region replacement vectors	Replacement of large-T gene of SV40	1. Produce virions which infect host cells 2. Transient gene expression 3. Mammalian cells are hosts of SV40
(*b*) Late region replacement vectors	Replacement of VP1, VP2, and VP3 genes of SV40, *e.g.*, SVGT-5	
(*c*) Plasmid vectors	Origin of replication and large-T gene of SV40	—
(*d*) Shuttle plasmid vectors	Plasmid vector [item 1 (c)] plus *p*BR322 origin and *amp*ʳ gene, *e.g.*, *p*SV2, *p*SV3 etc.	—
	Rous sarcoma virus promoter in place of SV40 early promoter, *e.g.*, *p*RSV.	Strong expression of the marker gene
(*e*) Passive transfecting vectors	SV40 transcription regulator and polyadenylation sequences, plus *p*BR322 origin and *amp*ʳ gene	Shuttle vectors; used for gene integration in mammalian cells
2. BPV vectors	Bovine papilloma virus "Transforming region" + *p*BR322.	Shuttle vector; often *p*BR322 sequence deleted prior to transfection; plasmid-like vector
3. Retrovirus vectors	*p*BR322 + retrovirus sequences	Shuttle vector; integrates as provirus into mammalian genome; produces virions
4. Polyoma virus vectors	Polyoma virus origin and early region + *p*BR32 sequences	Similar to SV40 vectors; mouse cells used as host.
5. Vaccinia virus	DNA insert placed within the thymidine kinase gene of virus by a process of recombination.	Promising as live vaccines; DNA insert is a pathogen gene encoding an antigen.
6. P element vectors	*Drosophila* transposable element *P*; minimum of 31 bp inverted repeat borders and the neighbouring regions, plus an *E. coli* vector, *e.g.*, *p*UC8; DNA insert of upto 40 kb placed within the two boders.	Gene transfer in *Drosophila;* a helper *P* element is needed to provide the transposase necessary for transposition or insertion of the recombinant *P* vector into the *Drosophila* genome.
7. Bacculo-virus vectors	Nuclear polyhedroma virus (NPV) polyhedrin gene replaced by DNA insert; *e.g.*, AcNPV (*Autographia california* nuclear polyhedroma virus) and BmNPV (*Bombyx mori* nuclear polyhedroma virus) vectors.	Produce virions; expression vector for production of transgenic proteins in slik worm larvae (BmNPV vectors) and in *Spodoptera frugiperda* larvae or cultured cells (AcNPV vectors)

A typical fish vector is a plasmid, *e.g.*, pRSV. These vectors usually contain a selectable marker, *e.g.*, ampicillin resistance *(amp')*, and the origin of replication *(ori)* from *E. coli* plasmid pBR322, and an enhancer/promoter sequence (8V 40 or Roux Sarcoma virus promoter), a multiple cloning site for insertion of the DNA insert and a termination site including the polyadenylation site. In addition, it has the SV 40 origin of replication as well.

Drosophila P elements have been developed as valuable vectors for this invaluable genetic material. The 31 bp inverted repeat border and the neighbouring sequences of the transposable *P* elements are combined with a suitable *E, coli* plasmid, *e.g.*, pUC8, to produce a shuttle vector. DNA insert of upto 40 kb can be placed between the two border sequences. The recombinant *P* vector is injected into *Drosophila* larvae alongwith a helper P element which produces transposase. Transposase enables the transposition of recombinant *P* element from the recombinant vector into the *Drosophila* genome thereby bringing about the integration of DNA insert as well.

Bacculovicus vectors have been developed for transfection of insects. Two nuclear polyhedrosis viruses (NPV), *e.g.*, AcNPV *(Autographa california* NPV) and BmNPV *(Bombyx mori* NPV), have been exploited for this purpose. The NPV polyhedrin gene has a very strong promoter and the polyhedrin protein is not needed for NPV replication. Therefore, the general strategy is to replace the NPV polyhedrin coding sequence by the DNA insert so that the polyhedrin promoter drives the transgene. The recombinant NPV vectors form virions, infect silk worm larvae or cultured cells, and replicate to yield upto 50 Ilg vector DNA per larva. BmNPV vectors are used for infection of silkworm larvae, while AcNPV vectors are multiplied and expressed in the larvae or cultured cells of the insect *Spodoptera frugiperda.*

It may be seen from Table 3 that most of the animal vectors are disigned to replicate and express in animal cells; only the passive transducing SV 40 vectors are incapable of replication. Retrovirus and transposon vectors integrate into the genome of host cells in a manner similar to the natural retroviruses and transposons, respectively. Both circular and linearized vectors can integrate into the host genome, but the latter (linearized vectors) are far more readily integrated than the former. It has also been found that the presence of additional vector-DNA alongwith the integrated gene construct interferes with the expression of introduced gene or transgene. Therefore, it is often desirable to introduce the transgene with a minimum of vector-DNA associated with it.

C4. STEM CELLS

Research on stem cells is advancing knowledge about how an organism develops from a single cell and how healthy cells replace damaged cells in adult organisms. This promising area of science is also leading scientists to investigate the possibility of cell-based therapies to treat disease, which is often referred to as regenerative or reparative medicine.

Stem cells are one of the most fascinating areas of biology today. But like many expanding fields of scientific inquiry, research on stem cells raises scientific questions as rapidly as it generates new discoveries.

4.1 Introduction to Stem Cells:

Stem cells have two important characteristics that distinguish them from other types of cells. First, they are unspecialized cells that renew themselves for long periods through cell division. The second is that under certain physiologic or experimental conditions, they can be induced to become cells with special functions such as the beating cells of the heart muscle or the insulin-producing cells of the pancreas. Scientists primarily work with two kinds of stem cells from animals and humans: embryonic stem cells and adult stem cells, which have different functions and characteristics that will be explained in this document. Scientists discovered ways to obtain or derive stem cells from early *mouse* embryos more than 20 years ago. Many years of detailed study of the biology of mouse stem cells led to the discovery, in 1998, of how to isolate stem cells from *human* embryos and grow the cells in the laboratory. These are called human embryonic stem cells. The embryos used in these studies were created for infertility purposes through in vitro fertilization procedures and when they were no longer needed for that purpose, they were donated for research with the informed consent of the donor.

Stem cells are important for living organisms for many reasons. In the 3 to 5 day old embryo, called a blastocyst, a small group of about 30 cells called the inner cell mass gives rise to the hundreds of highly specialized cells needed to make up an adult organism. In the developing fetus, stem cells in developing tissues give rise to the multiple specialized cell types that make up the heart, lung, skin, and other tissues. In some adult tissues, such as bone marrow, muscle, and brain, discrete populations of adult stem cells generate replacements for cells that are lost through normal wear and tear, injury, or disease.

It has been hypothesized by scientists that stem cells may, at some point in the future, become the basis for treating diseases such as Parkinson's disease, diabetes, and heart disease. Scientists want to study stem cells in the laboratory so they can learn about their essential properties and what makes them different from specialized cell types.

As scientists learn more about stem cells, it may become possible to use the cells not just in cell-based therapies, but also for screening new drugs and toxins and understanding birth defects. However, as mentioned above, human embryonic stem cells have only been studied since 1998. Therefore, in order to develop such treatments scientists are intensively studying the fundamental properties of stem cells, which include:

1) determining precisely how stem cells remain unspecialized and self renewing for many years; and
2) identifying the signals that cause stem cells to become specialized cells.

This primer on stem cells is intended for anyone who wishes to learn more about the biological properties of stem cells, the important questions about stem cells that are the focus of scientific research, and the potential use of stem cells in research and in treating disease. The primer includes information about stem cells derived from the embryo and adult.

4.2 Unique Properties of Stem Cells

Stem cells differ from other kinds of cells in the body. All stem cells — regardless of their source — have three general properties: they are capable of dividing and renewing themselves for long periods; they are unspecialized; and they can give rise to specialized cell types.

a. Stem cells are unspecialized: One of the fundamental properties of a stem cell is that it does not have any tissue specific structures that allow it to perform specialized functions. A stem cell cannot work with its neighbors to pump blood through the body (like a heart muscle cell); it cannot carry molecules of oxygen through the bloodstream (like a red blood cell); and it cannot fire electrochemical signals to other cells that allow the body to move or speak (like a nerve cell). However, unspecialized stem cells can give rise to specialized cells, including heart muscle cells, blood cells, or nerve cells.

b. Stem cells are capable of dividing and renewing themselves for long periods: Unlike muscle cells, blood cells, or nerve cells — which do not normally replicate themselves — stem cells may replicate many times. When cells replicate themselves many times over it is called proliferation. A starting population of stem cells that proliferates for many months in the laboratory can yield millions of cells. If the resulting cells continue to be unspecialized, like the parent stem cells, the cells are said to be capable of long-term self-renewal.

c. Stem cells can give rise to specialized cells: When unspecialized stem cells give rise to specialized cells, the process is called differentiation. Scientists are just beginning to understand the signals inside and outside cells that

trigger stem cell differentiation. The internal signals are controlled by a cell's genes, which are interspersed across long strands of DNA, and carry coded instructions for all the structures and functions of a cell. The external signals for cell differentiation include chemicals secreted by other cells, physical contact with neighboring cells, and certain molecules in the microenvironment.

4.3 Types of Stem Cells

4.3.1 Embryonic Stem Cells: Embryonic stem cells, as their name suggests, are derived from embryos. Specifically, embryonic stem cells are derived from embryos that develop from eggs that have been fertilized *in vitro* - in an *in vitro* fertilization clinic - and then donated for research purposes with informed consent of the donors. They are *not* derived from eggs fertilized in a woman's body. The embryos from which human embryonic stem cells are derived are typically four or five days old and are a hollow microscopic ball of cells called the blastocyst. The blastocyst includes three structures: the trophoblast, which is the layer of cells that surrounds the blastocyst; the blastocoel, which is the hollow cavity inside the blastocyst; and the inner cell mass, which is a group of approximately 30 cells at one end of the blastocoel.

Culture of stem cells: Growing cells in the laboratory is known as cell culture. Human embryonic stem cells are isolated by transferring the inner cell mass into a plastic laboratory culture dish that contains a nutrient broth known as culture medium. The cells divide and spread over the surface of the dish. The inner surface of the culture dish is typically coated with mouse embryonic skin cells that have been treated so they will not divide. This coating layer of cells is called a feeder layer. The reason for having the mouse cells in the bottom of the culture dish is to give the inner cell mass cells a sticky surface to which they can attach. Also, the feeder cells release nutrients into the culture medium. Recently, scientists have begun to devise ways of growing embryonic stem cells without the mouse feeder cells. This is a significant scientific advancement because of the risk that viruses or other macromolecules in the mouse cells may be transmitted to the human cells.

Over the course of several days, the cells of the inner cell mass proliferate and begin to crowd the culture dish. When this occurs, they are removed gently and plated into several fresh culture dishes. The process of replating the cells is repeated many times and for many months, and is called subculturing. Each cycle of subculturing the cells is referred to as a passage. After six months or more, the original 30 cells of the inner cell mass yield millions of embryonic stem cells. Embryonic stem cells that have proliferated in cell culture for six or more months without differentiating, are pluripotent, and appear genetically normal, are referred to as an **embryonic stem cell line.**

Once cell lines are established, or even before that stage, batches of them can be frozen and shipped to other laboratories for further culture and experimentation.

Laboratory Tests used to Identify Embryonic Stem Cells: At various points during the process of generating embryonic stem cell lines, scientists test the cells to see whether they exhibit the fundamental properties that make them embryonic stem cells. This process is called characterization. As yet, scientists who study human embryonic stem cells have not agreed on a standard battery of tests that measure the cells' fundamental properties. Also, scientists acknowledge that many of the tests they do use may not be good indicators of the cells' most important biological properties and functions. Nevertheless, laboratories that grow human embryonic stem cell lines use several kinds of tests. These tests include:

- growing and subculturing the stem cells for many months. This ensures that the cells are capable of long-term self renewal. Scientists inspect the cultures through a microscope to see that the cells look healthy and remain undifferentiated.
- using specific techniques to determine the presence of surface markers that are found only on undifferentiated cells. Another important test is for the presence of a protein called Oct-4, which undifferentiated cells typically make. Oct-4 is a transcription factor, meaning that it helps turn genes on and off at the right time, which is an important part of the processes of cell differentiation and embryonic development.
- examining the chromosomes under a microscope. This is a method to assess whether the chromosomes are damaged or if the number of chromosomes has changed. It does not detect genetic mutations in the cells.
- determining whether the cells can be subcultured after freezing, thawing, and replating.
- testing whether the human embryonic stem cells are pluripotent by 1) allowing the cells to differentiate spontaneously in cell culture; 2) manipulating the cells so they will differentiate to form specific cell types; or 3) injecting the cells into an immunosuppressed mouse to test for the formation of a benign tumor called a teratoma. Teratomas typically contain a mixture of many differentiated or partly differentiated cell types — an indication that the embryonic stem cells are capable of differentiating into multiple cell types.

Stimulation of Embryonic Stem Cells for Differentiation: As long as the embryonic stem cells in culture are grown under certain conditions, they can remain undifferentiated (unspecialized). But if cells are allowed to clump together to form embryoid bodies, they begin to differentiate spontaneously. They can form muscle cells, nerve

cells, and many other cell types. Although spontaneous differentiation is a good indication that a culture of embryonic stem cells is healthy, it is not an efficient way to produce cultures of specific cell types. So, to generate cultures of specific types of differentiated cells - heart muscle cells, blood cells, or nerve cells, for example - scientists try to control the differentiation of embryonic stem cells. They change the chemical composition of the culture medium, alter the surface of the culture dish, or modify the cells by inserting specific genes. Through years of experimentation scientists have established some basic protocols or “recipes” for the directed differentiation of embryonic stem cells into some specific cell types If scientists can reliably direct the differentiation of embryonic stem cells into specific cell types, they may be able to use the resulting, differentiated cells to treat certain diseases at some point in the future. Diseases that might be treated by transplanting cells generated from human embryonic stem cells include Parkinson’s disease, diabetes, traumatic spinal cord injury, Purkinje cell degeneration, Duchenne’s muscular dystrophy, heart disease, and vision and hearing loss.

4.3.2 Adult Stem Cells: An adult stem cell is an undifferentiated cell found among differentiated cells in a tissue or organ, can renew itself, and can differentiate to yield the major specialized cell types of the tissue or organ. The primary roles of adult stem cells in a living organism are to maintain and repair the tissue in which they are found. Some scientists now use the term somatic stem cell instead of adult stem cell. Unlike embryonic stem cells, which are defined by their origin (the inner cell mass of the blastocyst), the origin of adult stem cells in mature tissues is unknown.

Research on adult stem cells has recently generated a great deal of excitement. Scientists have found adult stem cells in many more tissues than they once thought possible. This finding has led scientists to ask whether adult stem cells could be used for transplants. In fact, adult blood forming stem cells from bone marrow have been used in transplants for 30 years. Certain kinds of adult stem cells seem to have the ability to differentiate into a number of different cell types, given the right conditions. If this differentiation of adult stem cells can be controlled in the laboratory, these cells may become the basis of therapies for many serious common diseases. The history of research on adult stem cells began about 40 years ago. In the 1960s, researchers discovered that the bone marrow contains at least two kinds of stem cells. One population, called hematopoietic stem cells, forms all the types of blood cells in the body. A second population, called bone marrow stromal cells was discovered a few years later. Stromal cells are a mixed cell population that generates bone, cartilage, fat, and fibrous connective tissue.

Also in the 1960s, scientists who were studying rats discovered two regions of the brain that contained dividing cells, which become nerve cells. Despite these reports, most scientists believed that new nerve cells could not be generated in the adult brain. It was not until the 1990s that scientists agreed that the adult brain does contain stem cells that are able to generate the brain’s three major cell types — astrocytes and oligodendrocytes, which are non-neuronal cells, and neurons or nerve cells.

Location and Function of adult stem cells: Adult stem cells have been identified in many organs and tissues. One important point to understand about adult stem cells is that there are a very small number of stem cells in each tissue. Stem cells are thought to reside in a specific area of each tissue where they may remain quiescent (non-dividing) for many years until they are activated by disease or tissue injury. The adult tissues reported to contain stem cells include brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, skin and liver.

Scientists in many laboratories are trying to find ways to grow adult stem cells in cell culture and manipulate them to generate specific cell types so they can be used to treat injury or disease. Some examples of potential treatments include replacing the dopamine-producing cells in the brains of Parkinson’s patients, developing insulin-producing cells for type I diabetes and repairing damaged heart muscle following a heart attack with cardiac muscle cells.

Tests used for identifying adult stem cells: Scientists do not agree on the criteria that should be used to identify and test adult stem cells. However, they often use one or more of the following three methods: (1) labeling the cells in a living tissue with molecular markers and then determining the specialized cell types they generate; (2) removing the cells from a living animal, labeling them in cell culture, and transplanting them back into another animal to determine whether the cells repopulate their tissue of origin; and (3) isolating the cells, growing them in cell culture, and manipulating them, often by adding growth factors or introducing new genes, to determine what differentiated cells types they can become.

Also, a single adult stem cell should be able to generate a line of genetically identical cells — known as a clone — which then gives rise to all the appropriate differentiated cell types of the tissue. Scientists tend to show either that a stem cell can give rise to a clone of cells in cell culture, or that a purified population of candidate stem cells can repopulate the tissue after transplant into an animal. Recently, by infecting adult stem cells with a virus that gives a unique identifier to each individual cell, scientists have been able to demonstrate that individual adult stem cell clones have the ability to repopulate injured tissues in a living animal.

4.4 Differentiation of stem Cells into particular organ:

As indicated above, adult stem cells occur in many tissues and that they enter normal differentiation pathways to form the specialized cell types of the tissue in which they reside. Adult stem cells may also exhibit the ability to form specialized cell types of other tissues, which is known as transdifferentiation or plasticity.

4.4.1 Normal differentiation pathways of adult stem cells: In a living animal, adult stem cells can divide for a long period and can give rise to mature cell types that have characteristic shapes and specialized structures and functions of a particular tissue. The following are examples of differentiation pathways of adult stem cells .

- Hematopoietic stem cells give rise to all the types of blood cells: red blood cells, B lymphocytes, T lymphocytes, natural killer cells, neutrophils, basophils, eosinophils,monocytes, macrophages, and platelets.
- Bone marrow stromal cells (mesenchymal stem cells) give rise to a variety of cell types: bone cells (osteocytes), cartilage cells (chondrocytes), fat cells (adipocytes), and other kinds of connective tissue cells such as those in tendons.
- Neural stem cells in the brain give rise to its three major cell types: nerve cells (neurons) and two categories of nonneuronal cells - astrocytes and oligodendrocytes.
- Epithelial stem cells in the lining of the digestive tract occur in deep crypts and give rise to several cell types: absorptive cells, goblet cells, Paneth cells, and enteroendocrine cells.
- Skin stem cells occur in the basal layer of the epidermis and at the base of hair follicles. The epidermal stem cells give rise to keratinocytes, which migrate to the surface of the skin and form a protective layer. The follicular stem cells can give rise to both the hair follicle and to the epidermis.

4.4.2 Adult stem cell plasticity and trans-differentiation: A number of experiments have suggested that certain adult stem cell types are pluripotent. This ability to differentiate into multiple cell types is called plasticity or transdifferentiation. The following list offers examples of adult stem cell plasticity that have been reported during the past few years.

- Hematopoietic stem cells may differentiate into: three major types of brain cells (neurons, oligodendrocytes, and astrocytes); skeletal muscle cells; cardiac muscle cells; and liver cells.
- Bone marrow stromal cells may differentiate into: cardiac muscle cells and skeletal muscle cells.
- Brain stem cells may differentiate into: blood cells and skeletal muscle cells.

Current research is aimed at determining the mechanisms that underlie adult stem cell plasticity. If such mechanisms can be identified and controlled, existing stem cells from a healthy tissue might be induced to repopulate and repair a diseased tissue.

4.4.3 Similarities and differences between embryonic and adult stem cells: Human embryonic and adult stem cells each have advantages and disadvantages regarding potential use for cell-based regenerative therapies. Of course, adult and embryonic stem cells differ in the number and type of differentiated cells types they can become. Embryonic stem cells can become all cell types of the body because they are pluripotent. Adult stem cells are generally limited to differentiating into different cell types of their tissue of origin. However, some evidence suggests that adult stem cell plasticity may exist, increasing the number of cell types a given adult stem cell can become.

Large numbers of embryonic stem cells can be relatively easily grown in culture, while adult stem cells are rare in mature tissues and methods for expanding their numbers in cell culture have not yet been worked out. This is an important distinction, as large numbers of cells are needed for stem cell replacement therapies.

A potential advantage of using stem cells from an adult is that the patient’s own cells could be expanded in culture and then reintroduced into the patient. The use of the patient’s own adult stem cells would mean that the cells would not be rejected by the immune system. This represents a significant advantage as immune rejection is a difficult problem that can only be circumvented with immunosuppressive drugs. Embryonic stem cells from a donor introduced into a patient could cause transplant rejection. However, whether the recipient would reject donor embryonic stem cells has not been determined in human experiments.

C5. Transgenic Plants

The plants, in which a functional foreign gene has been incorporated by any biological methods that geneally not present in plant, are called transgenic plants. However, a number of transgenic plants carrying genes for traits of economic importance have either been released for commercial cultivation or are under field trials, There are several methods discussed in previous chapter which are used in gene transfer,

These includes: (i) electroporation, (ii) particle bombardment, (iii) microinjection, *(iv) Agrobacterium-mediated* gene transfer, *(v)* co-cultivation (protoplast transformation) method, *(vi)* leaf disc transformation method, *(vii)* virus-mediated transformation, *(viii)* pollen-mediated transformation, *(ix)* liposome-mediated transformation, etc

During the last 20 years, considerable progress has been made on isolation, characterization and introduction of novel genes into plants. As per estimate made in 2002, transgenic crops are cultivated world-wide on about 48 million acres (587 million hectares) lands by about 5.5 million farmers. Transgenic crop plants have many beneficial traits like insect- resistance, herbicide tolerance, delayed fruit ripening, improved oil quality, weed control, etc.

Initially, some plants were produced by using reporter genes. Later on several genes for known traits of economic importance were incorporated into many crop plants. In others promoter sequences have been used that reduced/enhanced tissue specific expression of the adjacent genes according to requirement. In some cases antisense RNA genes have been introduced to inhibit expression of some existing genes in a desirable manner. All these approaches led to the development of transgenic crop plants of economic importance. More than 1000 field trial tests with transgenic crop plant have been conducted. Some of the commercially grown transgenic crop plants in developed countries are: 'Flavr Savr' and 'Endless Summer' tomatoes, 'Freedom II' squash, 'High-lauric' rapeseed (canola) and 'Roundup Ready' soyabean, etc. During 1995, in the USA full registration was granted to genetically engineered *Bf* gene containing insect resistant 'New Leaf' (potato), 'Maximizer' (com), 'BollGard' (cotton) (Gupta. 1996).

Currently, India is importing both grain legumes and edible oils to meet people's demand. by 2050, India's population is expected to reach about 1.5 billion. It is hoped that 30% India's population will be suffering from malnutrition. Therefore, nutritional security for everyone would require the extensive availability of grain legumes, edible oil fruits and vegetables, milk and poultry products. These challenges can be met by better resource management and producing more nutritious and more productive crops.

To strengthen further research in the area of crop biotechnology, a new institute, the National Centre for Plant Genome Research (NCPGR) has been established in New Delhi to strengthen plant biotechnology research in India. Department of Biotechnology (DBT) (Ministry of Science of Technology) has made enough fund for promotion of crop biotechnology. So far more than 60 transgenic dicot plants including herbs, shrubs and trees and several monocots *(e.g.* maize, oat, rice, wheat, etc.) have been produced. In future, the number of these crops certainly will go up. These transgenic plants contain certain selected traits such as herbicide resistance, insect resistance, virus resistance, seed storage protein, modified ripening, modified seed oil, agglutinin, etc. Moreover, in the light of future need, the transgenic plants are being looked up as bioreactor for molecular farming *i.e.* for the production of novel biomedical drugs such as growth hormones, vaccines, antibodies, interferon, etc.

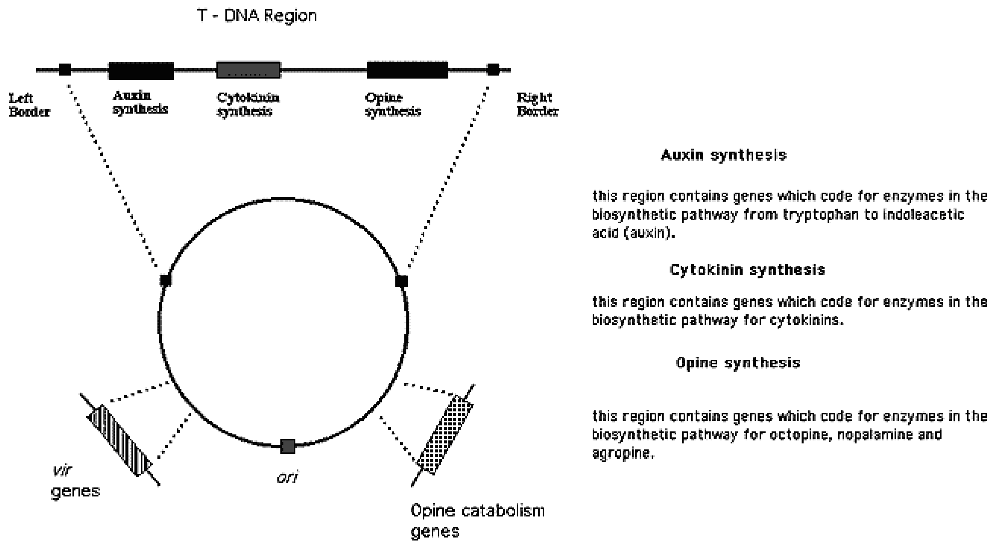
C6. Methods of gene transfer into Plant cells

Gene transfer methods have broadened the available gene pool as the gene(s) may come to plants from unrelated plants, viruses, bacteria, fungi, insects, animals, human beings and even from chemical synthesis in the laboratory. The essential requirements to produce transgenic plants include the availability of appropriate gene construct, its transfer to plant cells, integration and expression of transgenes and finally the characterization of transgenic plants. Depending upon the mode of transfer of foreign gene to plants two types of methods i.e. vector mediated and direct gene transfer are usually app~ied. In vector mediated approach the trans gene is combined with a vector which takes it to the target cells for integration. In direct gene transfer, on the other hand, the gene is physically delivered to the target tissue. The systems available under each of these two categories are:

6.1 Vector mediated gene transfer:

The vector mediated transformation is strongly linked to regeneration capabilities of the host plant. The target explants for vector mediated gene transfer usually include: protoplasts, suspension cells, callus cell clumps, cell layers, tissue slices or even whole organ sections. The cells to be used for transformation must be replicating DNA which is available in wounded or dedifferentiated cells or protoplasts.

6.1.1 *Agrobacterium-mediated Transformation:* *Agrobacterium* is naturally occurring gram negative, soil bacterium with two common species viz. *A.tumefaciens* and *A.rhizogenes.* These are known as **natural genetic engineers** for their ability to transform plants. In its natural environment, wild type *Agrobacterium tumefaciens* causes a crown gall disease. Genes for recognition of susceptible cells by the bacterium and its binding to these cells are located on bacterial chromosomes. However, the capability of bacterium to transfer DNA that is incorporated into plant's chromosome (transformation), resistance to antibiotics and pathogenicity are encoded on a plasmid. A plasmid is a segment of DNA which is separate from bacterial chromosome and can replicate independently of the chromosome. Wild type plasmid of *A. tumefaciens* is responsible for inducing tumour and hence termed as tumor inducing (Ti) plasmid.



The Ti plasmid of *Agrobacterium tumafasciens*

The Ti plasmid has two major regions of interest in transformation i.e. T-DNA and the *vir* region. The T-DNA region of the Ti plasmid of *Agrobacterium* is the part which is transferred to plant cell and incorporated into the nuclear genome of cells in an infected wound. The T - DNA part of plasmid carries genes which produce galls through regulation of phytohormones. Overproduction of phytohormones at the site of infection is responsible for the proliferation of wound cells into a gall (tumour) that can harbour a population of the bacteria. Transfer of the T-DNA is mediated by genes in another region of Ti-plasmid called *vir* (virulence) genes. So after infection of plants by bacteria, the single stranded T-DNA gets transferred to plant cells which actually express the plasmid genes to result in galls i.e. disease symptoms. If disease-inducing (phytohormone) genes are removed from T-DNA region of plasmid i.e. plasmid is **disarmed**, the bacterium does not cause disease. On the other hand, if some other gene is placed in the T-DNA region, it is transferred to plant. So modified Ti plasmids are constructed that lack the undesirable tumour inducing genes but contain a foreign gene e.g. for resistance to disease, and a closely linked

selectable marker gene e.g. for antibiotic resistance within the T-DNA region. In this way any desired DNA-sequence i.e. gene can be transferred to plants throughp1asmids that becomes the basis of *Agrobacterium* mediated gene transfer. These properties of *Agrobacterium* DNA transfer system are invaluable for developing a powerful vector system for plant transformation. Any gene put in the T-DNA region of plasmid gets transferred to the plant genome and is inherently stable once in the plant genome because neither the border nor the virulence genes are transferred. Wild type Ti plasmid carrying phytohormone genes in the T - DNA region interferes with the plant regeneration process. Therefore, disarmed Ti plasmid (Ti plasmid withotlt phytohormone genes) is generally used in the transformation process. Vector is maintained in the *Agrobacterium* either as cointegrating vector (where vector gets integrated into the Ti-plasmid) or as binary vector (where vector possesses autonomous replication). Transfer of genes *through Agrobacterium* is achieved either through co-culture of bacterium with plant cells or through application *of Agrobacterium* to wounded plant organs.

Method of transformation: Intact plants and seedling explants such as cotyledons, hypocotyls, roots, calli and protoplasts can be used for co-cultivation with *Agrobacterium* cells containing recombinant plasmids. However, leaf disc method has been widely used where surface sterilized leaf discs are infected with the appropriate strain of *Agrobacterium* carrying the vector of choice and co-cultured on regeneration medium for two or three days. During this time, the virulence genes in the bacteria are induced, the bacteria bind to the plant cells around the wounded site and the gene transfer occurs. The leaf discs are then transferred to regeneration/ selection medium which contains 500 μg/ml carbenicillin to kill the Agrobacteria and the appropriate antibiotic, usually kanamycin, to inhibit the growth of untransformed plant cells. During next 4-5 weeks the transformed shoots are obtained which are rooted and transferred to soil. Transgenic plants regenerated from various tissues are called T_0 plants whereas their subsequent generations are called as T_1 T_2 T_3 etc. When *Agrobacterium rhizogenes* infects plants, adventitious roots rather than tumour are formed at the site of infection. This is mediated by Ri (root inducing) plasmid. Vectors such as pRiAu which are based on Ri plasmid have been developed. The Ri. vectors are particularly useful for studying nodulation and manipulating root cultures for secondary metabolite and Vasicular Arbuscular Mycorrhiza (VAM) production. *Agrobacterium and host range Agrobacterium* mediated transformation has been a method of choice in dicotyledonous plant species where plant regeneration systems are well established. The host range of this pathogen includes about 60% of gymnosperms and dicotyledonous angiosperms.

6.1.2 Plant Viruses: On account of their ability to cause systemic infections in the plants, the viruses are being investigated as vectors for gene transfer in plants. Genetic engineering of the genomes of DNA and RNA viruses has been accomplished with the introduction of foreign DNA sequences. The foreign genes replace a part of the viral genome and create a defective viral particle that can infect the target plant only in the presence of a helper virus. The most promising viruses belong to two groups having DNA genome viz. Cauliflower Mosaic Virus (CaMV) and gemini virus. But viral vectors have not been developed to a stage where these can be routinely used for plant transformation.

6.2. Direct gene transfer methods: The problems associated with the species specificity and the inability of *Agrobacterium* to transfer multiple genes has been circumvented through direct gene transfer methods without the involvement of biological agents like bacteria and viruses. Here the DNA to be inserted in the target cells is delivered either through direct uptake *by* cell or through certain physical and chemical processes. A number of methods have been developed which include chemical methods, electroporation, particle gun delivery, lipofection, microinjection, macroinjection, pollen transformation, delivery via growing pollen tubes, laser induced or fibre-mediated gene transfers. Some of these like lipofection and electroporation involve delivery of DNA to protoplasts and thus require regeneration of plants from transformed protoplasts. The techniques like micro- or macroinjection on the other hand can be applied to a number of explants.

6.2.1 ***Physico-chemical Uptake of DNA:*** It is based on the ability of the protoplast to uptake the foreign DNA from the surrounding solution. An isolated plasmid DNA (vector) is mixed with the protoplasts in the presence of polyethylene glycol (PEG), polyvinyl alcohol and calcium phosphate which enhance the uptake of DNA by protoplasts. After 15-20 minutes of incubation, the protoplasts are cultured in the presence of appropriate selective agent. Protoplasts are regenerated and putative transgenic plants are further characterized for confirmation. This method, however, depends on the plant regeneration ability of the protoplasts and has been successfully used to produce transgenic plants in brassica, strawberry, lettuce, rice, wheat and maize.

6.2.2 ***Liposome Encapsulation:*** Liposomes are small lipid bags enclosing large number of plasmids. The procedure of liposome encapsulation was developed to protect the foreign DNA during the transfer process. The DNA enclosed in the lipid vesicles when mixed with protoplasts under appropriate conditions, penetrates into the protoplasts where lipase activity of the protoplast dissolves the lipid vesicles and DNA gets released for integration into the host genome. This method has not been commonly used as it is difficult to construct the lipid vesicles and the success depends upon the protoplast regeneration.

6.2.3 *Electroporation of Protoplasts:* Electroporation involves the creation of pores in the protoplast membrane using electrical impulses of a high field strength. Reversible breakdown of the membrane allows the entrance of foreign DNA into cytoplasm. Generally, protoplasts are used since they have exposed plasma membrane. Protoplasts are suspended in buffered saline solution containing plasmid DNA in a cuvette and an electrical pulse is applied across two platinum electrodes in the cuvette. The protoplasts are then cultured to regenerate the plants Electroporation has been successfully used for obtaining transgenics in tobacco, maize and rice.

6.2.4 *Microinjection*: Although it has been extensively used for animal cells but microinjection of DNA into plant cells has not been very successful. It is largely because of difficulties in getting the protoplasts immobilized and in injecting DNA into the protoplast without damaging the tonoplast which surrounds the plant cell vacuole. Tonoplast damage leads to the release of several kinds of toxic substances from the vacuoles into the cytoplasm. Crossway et al. (1986) developed the "holding pipette" technique to microinject tobacco protoplasts to overcome some of these problems. Protoplasts are held onto a 5µm pipette by gentle suction. Foreign DNA (about 2 picolitres) is injected into nucleus of the protoplast using 0.2 µm diameter injection pipette. After microinjection the protoplasts are cultured to obtain entire plants. The process of microinjection is time consuming and technically difficult but it raises the possibility of microinjecting a variety of materials such as chromosomes and even chloroplasts and mitochondria can also be transferred by microinjection. It has been successfully used in tobacco, alfalfa and *Brassica* spp.

3. 2.5 *DNA Injection into Intact Plants (Macroinjection):* Transgenic plants have been reported through direct DNA injection in the intact plants of rye. Macroinjection involves injection of a relatively large volume of exogenous DNA solution into inflorescence using a syringe. An aqueous solution of DNA was introduced into developing fl9ral tillers 14 days prior to meiosis. Transformed seeds were obtained from these injected tillers after cross pollination with other injected tillers. However, the mechanism by which DNA entered the zygotic tissue is yet unknown.

3.2.6 *Incubation of Seeds with DNA:* In some of the earlier experiments transformations were observed when germinating seeds were put into the DNA solution. Dry seeds without seed coats also take up DNA when imbibed in a DNA solution.

3.2.7 *Pollen Tube Pathway:* Transgenic plants have been reported in rice by the percolation of DNA solution through the pollen tube which eventually resulted in the formation of transformed seeds. The stigmas were cut after pollination exposing the pollen tubes and the DNA was introduced onto the cut surface that presumably diffused through the germinating pollen tube into the ovule. The mechanism of DNA transfer into zygote through this method is not yet established.

6.2.8 *Laser Microbeam:* This method of introducing DNA into plant cell has been described by Weber et al. (1988). Small pores in the membrane are created by laser microbeam. The DNA from the surrounding solution then enters the cell through these small pores to cause transformation.

6.2.9 *Electroporation into Tissues/embryos:* A procedure has been developed to deliver DNA by electroporation into intact cultured cells, immature and mature embryos. This procedure has been successfully used to obtain transgenic plants by delivering DNA into enzymatically or mechanically wounded immature embryos, embryogenic callus and bisected mature embryos of rice. Concerted efforts are required to improve the transformation frequency of this inexpensive procedure.

C7. Selectable Marker genes

When plant cells are transformed by any of the transformation methods as given earlier, it is necessary to isolate the transformed cells/tissue. However, it is possible to do now. There are certain selectable marker genes present in vectors that facilitate the selection process. In transformed cells, the selectable marker genes are introduced through vector. The transformed cells are cultured on medium containing high amount of toxic level of substrates such as antibiotic, herbicides, etc. For each marker gene there is one substrate (Table 1). For a model transgenic system, tobacco is the most common plant that is found everywhere. The young explants such as leaf discs are aseptically cut into pieces. These pieces are transferred onto tissue regeneration medium supplemented with an antibiotic, kanamycin. From the transformed discs shoots grow directly. The cells which do not undergo transformation will die due to kanamycin. Therefore, antibiotics and herbicides should be used carefully because even in low concentration many cells are damaged. When regeneration has accomplished, selection should be done thereafter. Besides, another difficulty associated with successful selection is the regeneration of shoots from transformed calli because the ex-plants may be heterogeneous and non-transformed cells could not be selected. Therefore, such methods should be used that can ensure escape of only few non-transformed shoots from selection. However, it is ensured by using leaf discs as only the cells which are in direct contact of medium containing antibioticlherbicide will undergo regeneration.

Table 1 Selectable marker genes of vectors and their applications.

Substrates used for selection	*Marker genes*
1. Antibiotics	
Bleomycin	Gene *ble* (unknown enzyme)
G418, Kanamycin, Neomycin	Neomycin phosphotransferese (*npt*II)
Gentamycin	Gentamycin acetyl transferase (*gat*)
Hygromycin B	Hygromycin phosphotransferase (*hpt*)
Methotrexate trimethoprim	Dihydrofoate reductase (*dfr*)
Streptomycin	Streptomycin phosphotransferase (*spt*)
2. Herbicides	
Chlorosulfuron imidazolinones	Mutant form of acetolactase synthase (*als*)
Bromoxynil	Bromoxynil nitrilase (*bnl*)
Glyphosate	5-enolpyruvate shikimate-3-phosphate (EPSP)-synthase (*aro*A)
PPT (L-phosphinothricin, also called bialaphos)	Phosphinothricin acetyltransferase (*bar*)

In addition, there is an alternative procedure where there will be no selection pressure imposed on cells/shoot that develop from ex-plants. In this method, samples of tissue from regenerated shoot are taken, the samples are tested for expression of a marker gene. There is a number of marker genes which are commonly described as *reporter genes* or *scoreable genes* or *screenable genes* (Table 2).

Table 2. Examples of some of the reporter genes used as screenable markers.

Reporter genes	*Enzymes expressed*	*Substrate/ assay*	*Identification*
cat	Chloramphenicol acetyl transferase	^{14}C-chloramphenicol +Acetyl CoA (TLC) separation)	Detection of acteyl chloramphenicol by autoradiography
gus	β-glucuronidase	Glucuronides (PNPG, X-GLUC, REG, NAG)	Detection of fluorescence, colorimetric, photometric
lacZ	β-galactosidase	β-galactosidase + X-gal	Colony colour
lux	Luciferase	Decan and $FMNH_2$, ATP+O_2+luciferin	Bioluminescence on exposure of X ray film
*npt*II	Neomycin phosphotransferase	Kan+^{32}P-ATP (*in situ* assay)	Detection of radioactivity
nos	Nopaline synthase	Arginine+ketoglutaric acid + NADH	Electrophoresis
ocs	Octopine synthase	Arginine+pyruvate+NADH	Electrophoresis

Some of the reporter genes which are most commonly used in plant transformation are: *cat, gus, lux, nptII.,* etc. They are briefly discussed as below:

***(a)* Chloramphenicol acetyl transferase (CAT) gene:** The *cat* gene is not used as a selectable but as reporter gene. It was first isolated from the bacterium *E.coli* but it is absent in higher plants and mammals. In transformed cells, its presence can be detected by assaying the enzyme CAT on 32P-chloramphenicol mixed growth medium. Therefore, the enzyme uses acetyl Co-A-chloramphenicol-p32 as substrate and transfer acetyl CoA to chloramphenicol converting the latter into acetyl chloramphenicol which is detected autoradiographically.

(b) Neomycin Phosphotransrerase (NPTII) Gene (*nptII* Gene): The *nptII* gene confer resistance against kanamycin and detoxifies it by phosphorylation. It encodes enzyme NPTII. Presence of *nptII* gene in transformed tissue can be detected by selecting them on kanamycin supplemented medium. Similarly, the presence of this enzyme is also detected in transgenic plants or transformed tissue. Commonly *nos* promoter is linked with *nptII* gene so that synthesis of enzyme NPTII may be started well. However, if *nptII* gene has adverse effect on expression of desirable gene, its expression can be improved by using an alternative approach.

Reiss *et al.* (1984) have discussed in detail the assay of enzyme NPTII. Firstly, NPTII is fractionated by using non-denaturing polyacrylamide gel electrophoresis (PAGE). In agar layer, radiolabelled 32p_ATP is used with kanamycin. The gel (that contains the enzyme NPTII) is covered with agar containing both 32p_ ATP and kanamycin. The entire preparation is incubated at 35°C. As a result of phosphorylation

of kanamycin 32p is incorporated into it, the presence of which is detected autoradiographically.

(c) Luciferase Gene *(lux* Gene): The *lux* gene is found in glow-worm, firefly and bacteria that secretes the enzyme luciferase. Due to secretion of this enzymes the glow-worm becomes luminescent in dark. The *lux* gene has been transferred into tobacco through Ti-plasmid of *Agrobacterium.* Consequently *lux* gene containing bioluminescent tobacco plants were produced.

Similarly a green fluorescent protein (GFP) isolated from the jellyfish, *Aequorea victoria* are used as reporter gene or tag in a wide variety of organisms. These act as visible marker for gene expression.

(d) The β-galactosidase Gene *(lacZ* Gene): The *lacZ* gene that encodes β-galactosidase is a polylinker as it contains several restriction sites but maintains the proper reading frame. Most DNA fragments cloned into polylinker disrupt *lacZ* gene and abolish f3-galactosidase activity. When a foreign gene fused with *lacZ* gene is inserted into a microbial cell, its presence and function can be detected. When the genetically engineered microbiaUplant/animal cells contained a reporter gene is allowed to grow on medium containing a chemical Xgal *(i.e.* 5-bromo -4-chloro-3-indolyl-β-D-galacto-pyranoside), β-galactosidase hydrolyses X-gal, and releases an insoluble blue dye. The release of dye shows the presence of foreign gene. If there appears no colour, it means the gene is disrupted.

D1. Transgenic Plants for Crop Improvement

For crop improvement several genetic traits have been introduced in plants and thereby plant's efficiency has been increased several times as compared to the normal plants. In this regard some of the plants have been described:

1.1. Engineered Resistance against Herbicide

The development of crop plants that are tolerant to herbicide is an important approach to control weeds. Most of the modem herbicides interfere with amino acid biosynthesis in plants. Once the mode of action of a herbicide is established, the transfer of resistance to the herbicide can be done by two different approaches: (1) the first approach is modification of the target side and, (2) detoxification of herbicide. Transgenic plants carrying the genes for herbicide tolerance has been produced in tobacco, petunia, tomato, potato, Populus etc. This approach has been made for developing resistance against at least three herbicides viz. glyphosate, sulphonyl urea and imidazolinones.

a. Resistance against glyphosate: Glyphosate, an active components of round up herbicides, is used as nonselective post-emergence herbicides. It is suggested that glyphosate inhibits aromatic amino acid biosynthesis by competitive inhibitor of enzyme 5-enol-pyruvyl shikimate-3 phosphate synthase [EPSPS). EPSPS catalyzes the synthesis of phenylalanine, tyrosine and tryptophan. The **aro A** gene which codes EPSPS has been isolated and sequenced from *E. coli, Salmonella typhimurium* and *Arabidopsis thaliana*. There are two possible methods for producing glyphosate resistant plants by genetic engineering: (a) transfer of a glyphosate sensitive EPSPS under the control of a powerful promoter causing over expression of the protein' and/or (b) transfer of gene which codes a mutated, glyphosate resistant EPSPS. cDNA of the EPSPS gene with 35 S cauliflower mosaic virus

An alternative method is insertion and expression of mutated bacterial aro A gene coding for glyphosate resistant EPSPS. The source of the aro A gene was a mutagenized strain of S. typhimuriuum or E.coli and was transfen-ed to tomato and/or tobacco. Transgenic tobacco plants, showing tolerance to commercial levels of glyphosate are field tested in USA.

b. Resistance against sulfonylurea and imidazolinone herbicides : The herbicidal action of sulfonylureas and imidazolinones is based on their ability to inhibit non-competitively an enzyme of branched chain amino acid biosynthesis, acetolactate synthase (ALS). ALS is the first enzyme in the biosynthetic chain resulting in the synthesis of the branched chain amino acids valine, leucine and isoleucine. Mutated strains resistant to sulfonylureas have been obtained from bacteria, fungi and plant cell cultures. The resistance of all these mutants can be attributed to possession of mutated forms of the ALS. Transgenic tobacco plants expressing a mutant ALS gene from tobacco or Arabidopsis, were produced that were tolerant to sulfonylurea herbicides.

c. Resistance against Phosphinothricin: L-Phosphinothricin (PPT) is a naturally occurring amino acid with herbicidal activity and is active ingredient of herbicide 'Basta'. PPT is a potent inhibitor of glutamine synthase and thus causes rapid increase in ammonia concentration h1 plants. This leads to death of plant cell. 'bar' gene from *Streptomyces hygroscopicus* ,encodes phosphinothricin acetyl transferase enzyme that acetylates the free-NH2 groups of phophinothricin and renders it inactivates the herbicide Basta. This 'bar' gene with CaMV 35S promoter has been transferred to tobacco, tomato and potato plants using Agrobacterium Ti plasmid vector system. The transgenic plants have been tested for 'Basta' resistance.

1.2. **Engineered Resistance against Pest:** For transgenic plant production with pest resistance properties, two major approaches have been developed: a) the introduction of protein delta endotoxins from Bt and b) introduction of protease inhibitors in plants.

a. Bacillus thuringiensis endotoxins: The entomological bacterium *Bacillus thuringiensis* upon sporulation normally produces a parasporal crystalline toxin. When ingested by a susceptible insect, a combination of the high pH and proteinases of the insects mid-gut solubilizes the crystal and yields active toxin. The effects of the toxin occur within minutes of indigestion, leading to disruption of insect's midgut cells. Bt toxin activity has been found against many species of insects within the orders of Lepidoptera, Diptera and Coleoptera. These **delta endotoxins** are very specific in their action and are safe insecticides, but their use is limited due to high production cost and due to instability of crystal proteins when exposed in the field. The gene for above toxin (ht2) from *B. thuringiensis* has been isolated and was used for Agrobacterium plasmid mediated transformation of tobacco, cotton, potato and tomato plants. These transgenic plants were resistant to the feeding damage of Manducta sexta larvae (tobacco horn worm), a pest of tobacco.

b. Protease inhibitors: Protease inhibitors are proteins with anti-metabolic activity against wide range of insects. Such inhibitors are widely distributed within the plant kingdom and can accumulate to high levels in seeds and storage organs. Other major advantages of protease inhibitors are their, inactivation with cooking.In cowpea (*Vigna unguiculata*), trypsin inhibitor (CpTi) level was shown to be responsible for its resistance to attack by the major storage pest of its seeds (i.e. bruchid beetle = *Callosobruchus maculatus)* A variety of insects have shown to be toxic to CpTi. This CpTi gene was joined with a CaMV 35S promoter, and one marker gene with the transgene and was used to infect tobacco leaf discs. Agrobacterium was used for transformation and the transgenic tobacco plants express a high level of CpTi. The CpTi gene in transgenic plants is stably inherited and there is no serious 'yield penalty'. Thus like Bt toxin, CpTi can also be used as a protectant against insect attack in transgenic plants. However, extensive field trials are necessary before releasing these transgenic plants to the farmers.

1.3. Engineered Resistance against Viral Infection: Over seven hundred plant viruses are recognised causing various diseases and significant crop losses. Due to the lack of 'viricides', the practical control depends on methods that prevent or restrict virus infection. Various strategies have been applied for protecting viral infection; few are described here like:

Cross protection: If susceptible strain of a crop is inoculated with a mild strain of a virus, the susceptible strain, as a result, develops resistance against more virulent strains. The phenomenon is known as cross protection. This method has been used to reduce yield losses in crops like tomatoes against tomato moisac virus and in potato against potato spindle tuber viroid.

Coat protein - mediated resistance: Coat protein - mediated resistance to viruses has been one of the successes of plant genetic engineering. Coat protein gene from tobacco mosaic virus (TMV), a positive strand RNA virus, has been transferred to genome of tobacco plants. In the transgenic plant, expression of coat protein (CP), as well as, low and delayed infection was observed when inoculated with TMV

1.4. Engineered Resistance against fungal pathogen: Genetic engineering has opened vistas with isolation of resistance genes and gene transformation methods to develop transgenic plants. Resistance to fungal pathogen is achieved by isolating and transferring fungal resistance genes to the desired susceptible crop. Compounds toxic to fungus can also be used to produce transgenic plants as done in case of tobacco. Antifungal proteins like endochitinase and 6-1,3-endoglucanases are thought to inhibit fungal growth. Transgenic tobacco plants with endochitinase CH5B gene with Ca MV 35S promoter exhibits reduced seedling mortality when grown in presence of Rhizoctonia so/ani.

1.5. Genetic Engineering and Storage proteins: Human nutrition requires a balanced source of amino acids and the amino acid balance of many plant products is unsatisfactory. Genetic engineering may be carried out in crops making following three approaches:

1. expressing a desirable and heterologous storage protein
2. increasing the level of a desirable, but little expressed, endogenous protein and
3. suppressing the expression of anti-nutritional proteins.

A heterologous species of sulphur-rich storage protein in Brazil nut containing 18% methionine and 8% cysteine has been expressed in tobacco and rapeseed. In transgenic tobacco, phaseolin gene has been expressed, leading to accumulation of methionine rich phaseolin.

1.6. Genetic engineering for nitrogen fixation

a) Transfer of nif-genes from Klebsiella pneumoniae to E. coli: *Klebsiella pneumoniae* is a non symbiotic nitrogen fixing bacterium, nif - genes of *K. pneumoniae* were transferred into *E. coli* cells by conjugation. The trans gene in the new host produced functional nitrogenase system.

b) Transfer of nif-genes of eukaryotic organisms: nif-genes from Rhizobium have been transferred to *Agrobacterium tumefaciens*. The expression of transferred gene has been successful in cereal crops like wheat, paddy etc. through the Ti-plasmids of *A. tumefaciens*.

c) Transfer of nod-genes to increase host range: Cloned nod-genes of *Rhizobium leguminosarum* that nodulated pea have been transferred to *R. phaseoli* (this normally nodulates bean). The transformed R. phaseoli can now nodulate pea plants also.

D2. Transgenic animal and Molecular Pharming

A transgenic organism carries in all its cells a foreign gene that was inserted by laboratory techniques. Each transgenic organism is produced by introducing cloned genes, composed of deoxyribonucleic acid (DNA) from microbes, animals, or plants, into plant and animal cells. Transgenic technology affords methods that allow the transfer of genes between different species.

2.1. Transgenic Mice in Immunology:

Introduction of rearranged immunoglobin (Ig) genes into transgenic mice has helped enormously to clarify mechanisms of rearrangements of Ig genes and the importance of this phenomenon to the regulation of their expression. The study indicated that the expression of rearranged heavy chain genes in T cells indicates that the inability of the T cell to express heavy-chain genes, is related to the absence of gene rearrangement in these lineages.

*2.2. **Transgenic Mice in Oncology:***

Insertion of oncogenes and proto-oncogenes into embryos of mice followed by tracing their effects in the normally differentiating cells of an intact organism has contributed in understanding the neoplastic disease and its relationship to aberrant gene expression.

*2.3. **Transgenic Mice as Animal Models of Human Disease:***

Animal models for human illnesses are useful for studying the pathogenesis of diseases as well as for developing and testing new therapies. Human diseases can be induced in transgenic mice by expression of transferred genes, or by insertional disruption of endogenous sequences. Some examples of models created by transgene expression are listed below.

a) **Hepatitis B** is a human disease that lacks a readily workable animal model. Introduction of the HBsAg gene into mice results in development of transgenic mice that mimic the carrier state with production of HBsAg in the liver but with an absence of disease.
b) **Progressive multifocal leukencephalopathy** can be recreated in mice by injection of the IC viral genome. Another disease of the nervous system, neurofibromatosis, can be induced in mice by introduction of the gene for tyrosine amino transferase (TAT) of HTLV-I.
c) **Osteogenesis imperfect** is a dominant disorder that results from a mutation of the gene for A I (I) collagen. The simple, resultant amino acid substitution results in a change in the helical structure of mature collagen. This condition has been induced in mice by microinjection of a mutant gene for collagen A I (I), created in the laboratory by site directed mutagenesis

*2.4. **Transgenic animals for xeno-transplantation:*** Use of animal organs for human transplantation is called xeno-transplantation. In 1992, a Cambridge biotechnology company mutran produced a genetically engineered pig called Astrid that carries genes from the human immune system. Organs from the genetically engineered pigs contain genes that produce a human protein. Such organs would not be recognized as foreign when transplanted into primates or man.

2.5 Molecular Pharming

Pharming relies on two related technologies, transgenesis and cloning. Transgenesis allows scientists to create animals with an extra foreign gene (which makes the protein of interest) incorporated into their own genetic material. This gene is attached to other 'regulatory' genes so it only produces the foreign protein in specific tissues, usually the mammary glands or the liver.

Transgenesis is very imprecise and is successful in around only 4-6% of experiments. The remaining 94 to 96% of animals are unwanted and killed, as they do not carry the transgene in the correct position or, in many cases, do not have the transgene at all. Since transgenesis is so unreliable and expensive, cloning is used to make 'copies' of those animals that have been successfully genetically modified.

Proponents of this technology envisage these techniques being used together to create entire flocks or herds of cloned transgenic animals all producing commercially valuable proteins. However, the imprecise nature of these technologies and the tremendous loss of life they entail are proving considerable stumbling blocks.

2.5.1. Biopharming: This is an experimental application of biotechnology in which organisms are genetically modified to produce pharmaceutical proteins and chemicals they do not produce naturally. A few examples include a contraceptive, a potent growth hormone, a blood clotting agent, blood thinners, industrial enzymes, and vaccines.

Pharming with animals: Some animals have been genetically modified to produce human proteins in their milk. The animals are like living pharmaceutical ‘factories’ and the technique is often referred to as ‘pharming’. Animals which may be used include cows, sheep, pigs, goats, rabbits and mice.

The section of human **DNA** containing the genes for the specific **protein** required is injected into the

host animal embryo. The embryo is then placed into the uterus of a **surrogate** mother where it develops to full term. As an adult, the animal produces the human protein in its milk. The animal is milked and the protein is purified from the milk for therapeutic use for humans.

A GM animal produced for pharmaceutical production should be able to produce the desired drug at high levels without endangering its own health, and pass this ability to its offspring.

The first use of this technology was in 1987 with mice that produced a human drug, tissue plasminogen activator (tPA), which is used to treat blood clots. Other research has included:

- cows that produce human serum albumin, used to maintain fluid balance in the blood, and **lactoferrin** which is a protein from breast milk that promotes infant growth.
- sheep that produced a number of proteins including **factor VIII and factor IX** which are essential for blood clotting, natural anticoagulants used during heart surgery, and proteins to treat lung and liver disease.
- rabbits that produce human interleukin-2, a protein essential in the **immune response** to infection and may be useful in fighting some types of cancer.

2.5.2 Animal Transformation:

Through transgenic animal transformation, new genetic information is introduced into an animal in one generation without compromising or limiting the overall pool of genetic information. Transgenic animals are produced by inserting genes into embryos prior to birth. Each transferred gene is assimilated by the genetic material or chromosomes of the embryo and subsequently can be expressed in all tissues of the resulting animal. The objective is to produce animals which possess the transferred gene in their germ cells (sperm or ova). Such animals are able to act as "founder" stock to produce many offspring that carry a desirable gene or genes.

Transgenic animals have been produced by three methods: microinjection of cloned gene(s) into the pronucleus of a fertilized ovum, injection of embryonic stem cells into embryos, and exposure to retroviruses. The third method is not discussed in this article.

The first method is the one that is most widely and successfully used for producing transgenic mice. After microinjection, the recently fertilized single cell embryos are removed from the animal. Micromanipulators on a specially equipped microscope are used to grasp each embryo. A glass pipette drawn or pulled to a fine point immobilizes the embryo on one side, as shown in the photos to the right. On the opposite side, the foreign DNA is injected into the embryo's pronucleus--either of two nuclei (male or female) containing half the chromosomes of a fertilized ovum--with a second finely drawn injection needle. After the injection, the embryos are transferred back into the hormonally prepared or pseudopregnant recipient females or foster mothers. The recipients follow normal pregnancy and deliver full-term young. This method is presently the most efficient for generating transgenic animal lines: about 1 to 4 percent of the injected embryos result in a transgenic offspring.

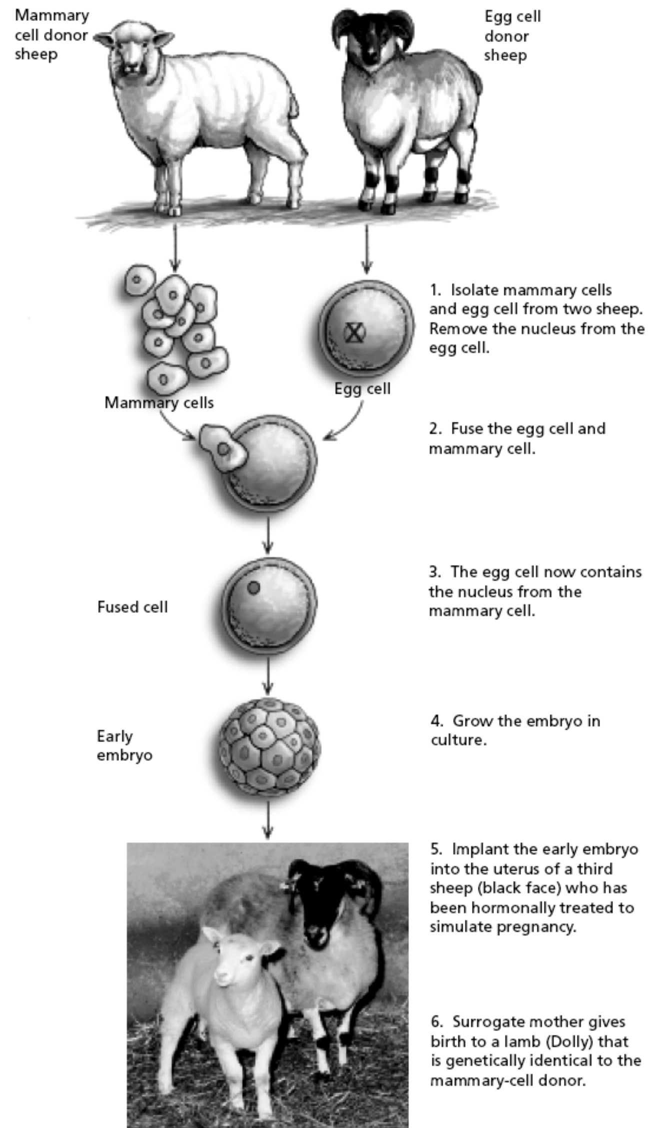


Figure 1. Dolly. This lamb is the result of a successful attempt to clone a mammal. She was genetically identical to her cell-donor mother.

The second method involves microinjection of embryonic stem (ES) cells derived from the inner cell mass of blastocyst-stage embryos (about 7 days postfertilization) into embryos to produce "hybrid" embryos of two or more distinct cell types. The ES cells are able to produce all tissues of an individual. Once isolated, ES cells may be grown in the lab for many generations to produce

an unlimited number of identical cells capable of developing into fully formed adults. These cells may then be altered genetically before being used to produce embryos. When these transformed cells participate in the formation of sperm and eggs, the offspring that are produced will be transgenic. Results have shown this method to be promising for producing transgenic mice. Studies are presently under way at the University of Illinois Department of Animal Sciences to develop ES cell lines for livestock species such as swine, cattle, and sheep.

Cloning offspring from adults with desirable traits has been successfully performed on cattle, goats, mice, cats, pigs, rabbits, and sheep. In fact, the animal that brought cloning to the attention of the public was a ewe named Dolly. Dolly was cloned when Scottish scientists took cells from the mammary gland of an adult female sheep and fused it with an egg cell that had previously had its nucleus removed. Treated egg cells were then placed in the uterus of an adult ewe that had been hormonally treated to support pregnancy. Scientists had to try many, many times before this **nuclear transfer** technique worked. In all, 277 embryos were constructed before one was able to develop into a live lamb (Figure 1). Dolly was born in 1997.

The research that led to Dolly's birth was designed to provide a method of ensuring that cloned livestock would have the genetic traits that made them most beneficial to farmers. Sheep that produced the most high-quality wool and cattle that produced the best beef would be cloned. This is more efficient than allowing two prize animals to breed because each animal would only give half its genes to the offspring. Therefore, there is no guarantee that the offspring of two prize animals will have the desired traits. There is no equivalent reason for cloning humans,

D3. Gene Knockout

Another goal of transgenic technology is the creation of engineered animals that lack specific genes (knockout), or have these genes replaced by one that has been engineered in a specific way.

For example, transplantation of organs or tissues from non-primates (such as pigs) to humans (xenotransplantation) is currently impossible, due in part to a dramatic ("hyperacute") immune response by human recipients to a carbohydrate on the surface of pig cells (galactose-1,3-galactose); this carbohydrate is not found in old-world primates (Galili, 2001). Inactivation of the enzyme (galactosyl transferase, GT) in donor pigs could alleviate this problem, and pigs with one allele of the gene encoding this enzyme recently have been produced (Lai et al., 2002), giving rise to expectation that completely GT-deficient animals soon will be available. Another important goal is to eliminate from cattle the gene encoding prion-related protein (PrP), the protein associated with scrapie in sheep and bovine spongiform encephalopathy (BSE, or mad cow disease). Removal of this gene from mice has, at most, subtle phenotypic consequences, yet renders them completely resistant to these diseases (Bueler et al., 1992). If the mouse model holds true in cattle, homozygous knockout of bovine PrP could lead to the elimination of BSE.

In order to study the relationship between proteins and gene function, scientists now can prevent the manufacturing of a protein by a specific gene. By disabling a gene from a test organism, and then producing descendants that contain two copies of the disabled gene, it is possible to observe the descendants' development in the absence of a particular protein. This practice, referred to as knockout technology, is an attempt to shut down or turn off a particular gene. Thus far, the mouse has been the mammal in which knockout technology has been most generally applied. In essence, a "knockout" organism (e.g., the mouse) is created when an embryo cell (an embryonic stem cell—or ESC—which is a cell that has yet to divide into different tissue cells) is genetically engineered, and then inserted into a developing embryo. The embryo then is inserted surgically into the womb of a host (e.g., a female mouse). Once the embryo has matured, a portion of its stem cells will produce egg and sperm with the knocked-out gene. A gene also can be altered in function, in contrast to being deleted. When a gene is altered but not shut down, a "targeted mutation" effect is created. This practice is referred to as knockin technology, whereby a life form has an altered gene "knocked" into it

Gene knockout/knockin technology is well established as an experimental tool in mice due to the availability of ES cell lines. The principleis to take advantage of a rather rare event that occurs after introduction of DNA into cells—homologous recombination between identical sequences in the genome and the transfecting DNA. In the most common protocol, a selectable marker (such as the neomycin resistance gene) is inserted within a piece of DNA corresponding to a portion of a gene of interest. After transfection of cells by this construct and selection for the marker (by growth in a medium containing the neomycin-related antibiotic G418, in this example), the selected cells are screened to identify the small fraction that has one copy of the gene of interest disrupted by the marker. Progeny animals derived from the cells will be heterozygous for the "knockedout" or "knocked-in" gene; breeding to obtain homozygous animals is straightforward. Because the process is so inefficient, very large numbers of transfected cells must be screened, making the use of cultured cells essential, since it would be impracticable to screen large numbers of progeny from microinjected eggs. The galactosyl transferase-knockout pigs discussed above were generated from cultured fetal fibroblasts manipulated in this way.

D4. Gene Therapy

The general approach of gene therapy is nothing more than an extension of the technique for clone selection by functional complementation. The functions absent in the recipient as a result of a defective gene are introduced on a vector that inserts into one of the recipient's chromosomes and thereby generates a transgenic animal that has been genetically "cured." The technique is of great potential in humans because it offers the hope of correcting hereditary diseases. However, gene therapy is also being applied to mammals other than humans.

The first example of gene therapy in a mammal was the correction of a growth hormone deficiency in mice. The recessive mutation little (lit) results in dwarf mice. Even though a mouse's growth hormone gene is present and apparently normal, no mRNA for this gene is produced. The initial step in correcting this deficiency was to inject homozygous lit / lit eggs with about 5000 copies of a 5-kb linear DNA fragment that contained the rat growth hormone structural gene (RGH) fused to a regulator–promoter sequence from a mouse metallothionein gene (MP). The normal job of metallothionein is to detoxify heavy metals, so the regulatory sequence is responsive to the presence of heavy metals in the animal. The eggs were then implanted into pseudopregnant mice, and the baby mice were raised. About 1 percent of these babies turned out to be transgenic, showing increased size when heavy metals were administered in the course of development. A representative transgenic mouse was then crossed with a homozygous lit / lit female.

The site of insertion of the introduced DNA in mammals is highly variable, and the DNA is generally not found at the homologous locus. Hence, gene therapy most often provides not a genuine correction of the original problem but a masking of it.

Similar technology has been used to develop transgenic fast-growing strains of Pacific salmon, with spectacular results. A plasmid containing a growth hormone gene placed next to a metallothionein promoter (all derived from salmon) was microinjected into salmon eggs. A small proportion of the resulting fish proved to be transgenic, testing positive when their DNA was probed with the plasmid construct. These fish were on average 11-fold heavier than the nontransgenic controls. Progeny inherited the transgene in the same manner as the mice in the earlier example.

4.1. Human Gene Therapy

Perhaps the most exciting and controversial application of transgenic technology is in human gene therapy, the treatment and alleviation of human genetic disease by adding exogenous wild-type genes to correct the defective function of mutations. We have seen that the first case of gene therapy in mammals was to "cure" a genotypically dwarf fertilized mouse egg by injecting the appropriate wild-type allele for normal growth. This technique has little application in humans, because it is currently impossible to diagnose whether a fertilized egg cell carries a defective genotype without destroying the cell. (However, in an early embryo containing only a few cells, one cell can be removed and analyzed with no ill effects on the remainder.)

Two basic types of gene therapy can be applied to humans, germ line and somatic. The goal of germ line gene therapy is the more ambitious: to introduce transgenic cells into the germ line as well as into the somatic cell population. Not only should this therapy achieve a cure of the person treated, but some gametes could also carry the corrected genotype. We have seen that such germinal therapy has been achieved by injecting mice eggs. However, the protocol that is relevant for application to humans is the removal of an early embryo (blastocyst) with a defective genotype from a pregnant mouse and injection with transgenic cells containing the wild-type allele. These cells become part of many tissues of the body, often including the germ line, which will give rise to the gonads. Then the gene can be passed on to some or all progeny, depending on the size of the clone of transgenic cells that lodges in the germinal area. However, no human germ line gene therapy has been performed to date.

We have seen that most transforming fragments will insert ectopically throughout the genome. This is a disadvantage in human gene therapy not only because of the possibility of the ectopic insert causing gene disruption, but also because, even if the disease phenotype is reversed, the defective allele is still present and can segregate away from the transgene in future generations. Therefore, for effective germinal gene therapy, an efficient targeted gene replacement will be necessary, in which case the wild-type transgene replaces the resident defective copy by a double crossover.

Somatic gene therapy focuses only on the body (soma). The approach is to attempt to correct a disease phenotype by treating some somatic cells in the affected person. At present, it is not possible to render an entire body transgenic, so the method addresses diseases whose phenotype is caused by genes that are expressed predominantly in one tissue. In such cases, it is likely that not all the cells of that tissue need to become transgenic; a portion of cells being transgenic can ameliorate the overall disease symptoms. The method proceeds by removing some cells from a patient with the defective genotype and making these cells transgenic through the introduction of copies of the cloned wild-type gene. The transgenic cells are then reintroduced into the patient's body, where they provide normal gene function.

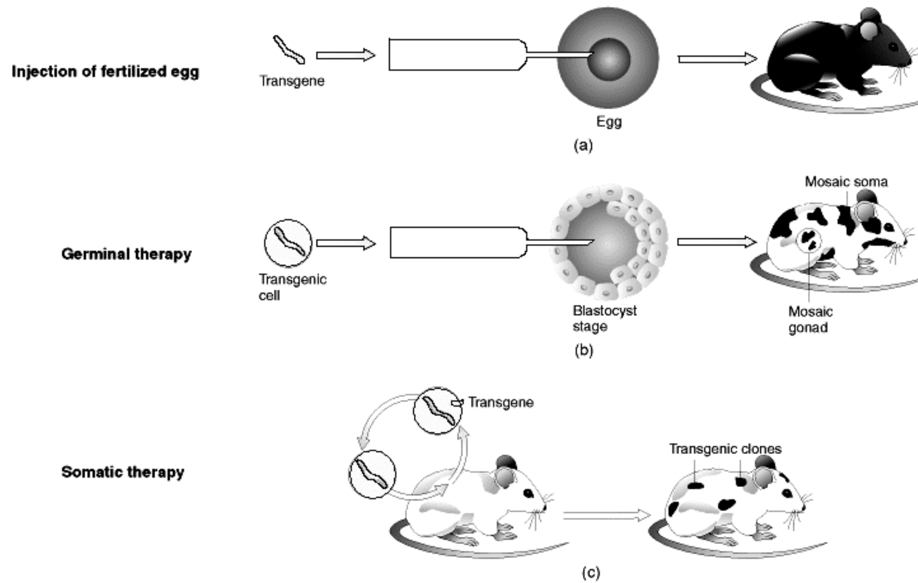


Figure 1. Types of gene therapy in mammals.

Currently, there are two ways of getting the transgene into the defective somatic cells. Both methods use viruses. The older method uses a disarmed retrovirus with the transgene spliced into its genome, replacing most of the viral genes. The natural cycle of retroviruses includes the integration of the viral genome at some location in one of the host cell's chromosomes. The recombinant retrovirus will carry the transgene along with it into the chromosome. This type of vector poses a potential problem, because the integrating virus can act as an insertional mutagen and inactivate some unknown resident gene, causing a mutation. Another problem with this type of vector is that a retrovirus attacks only proliferating cells such as blood cells. This procedure has been used for somatic gene therapy of severe combined immunodeficiency disease (SCID), otherwise known as bubble-boy disease. This disease is caused by a mutation in the gene coding for the blood enzyme adenosine deaminase (ADA). In an attempt at gene therapy, blood stem cells are removed from the bone marrow, the transgene is added, and the transgenic cells are reintroduced into the blood system. Prognosis on such patients is currently good.

Even solid tissues seem to be accessible to somatic gene therapy. In a dramatic case, gene therapy was administered to a patient homozygous for a recessive mutant allele of the LDLR gene for low-densitylipoprotein receptor (genotype $LDLR^{-}$ / $LDLR^{-}$). This mutant allele increases the risk of atherosclerosis and coronary disease. The receptor protein is made in liver cells, so 15 percent of the patient's liver was removed, and the liver cells were dissociated and treated with retrovirus carrying the LDLR+ allele. Transgenic cells were reintroduced back into the body by injection into the portal venous system, which takes blood from the intestine to the liver. The transgenic cells took up residence in the liver. The latest reports are that the procedure seems to be working and the patient's lipid profile has improved.

The other vector used in human gene therapy is the adenovirus. This virus normally attacks respiratory epithelia, injecting its genome into the epithelial cells. The viral genome does not integrate into a chromosome but persists extrachromosomally in the cells, which eliminates the problem of insertional mutagenesis by the vector. Another advantage of the adenovirus as a vector is that it attacks nondividing cells, making most tissues susceptible in principle. Inasmuch as cystic fibrosis is a disease of the respiratory epithelium, adenovirus is an appropriate choice of vector for treating this disease, and gene therapy for cystic fibrosis is currently being attempted using this vector. Viruses bearing the wild-type cystic fibrosis allele are introduced through the nose as a spray. It is also possible to use the adenovirus to attack cells of the nervous system, muscle, and liver.

A promising type of construct that should find use in gene therapy is the human artificial chromosome (HAC). HACs contain essentially the same components as YACs. They have been made by mixing human telomeric DNA, genomic DNA, and arrays of repetitive a-satellite DNA (thought to have centromeric activity). To this unjoined mixture was added lipofectin, a substance needed for passage through the membrane, and the complete mixture was added to cultured cells. Some cells were observed to contain small new chromosomes that seemed to have assembled de novo inside the cell from the added components. When the technology has been perfected, these HACs should be potent vectors capable of transferring large amounts of human DNA into cells in a stable replicating form.

E. Bioresource and uses of biodiversity.

Bioresources are the product which we harness from biodiversity. Biodiversity is the variation of life forms within a given ecosystem, biome or for the entire Earth. Biodiversity is often used as a measure of the health of biological systems. The biodiversity found on Earth today consists of many millions of distinct biological species, which is the product of nearly 3.5 billion years of evolution.

Definitions

The most straightforward definition is "variation of life at all levels of biological organization".[3] A second definition holds that biodiversity is a measure of the relative diversity among organisms present in different ecosystems. "Diversity" in this definition includes diversity within a species and among species, and comparative diversity among ecosystems.

A third definition that is often used by ecologists is the "totality of genes, species, and ecosystems of a region". An advantage of this definition is that it seems to describe most circumstances and present a unified view of the traditional three levels at which biodiversity has been identified:

- **genetic diversity** - diversity of genes within a species. There is a genetic variability among the populations and the individuals of the same species. (See also population genetics.)
- **species diversity** - diversity among species in an ecosystem. "Biodiversity hotspots" are excellent examples of species diversity.
- **ecosystem diversity** - diversity at a higher level of organization, the ecosystem. Diversity of habitat in a given unit area. To do with the variety of ecosystems on Earth.

The 1992 United Nations Earth Summit in Rio de Janeiro defined "biodiversity" as "the variability among living organisms from all sources, including, 'inter alia', terrestrial, marine, and other aquatic ecosystems, and the ecological complexes of which they are part: this includes diversity within species, between species and of ecosystems". This is, in fact, the closest thing to a single legally accepted definition of biodiversity, since it is the definition adopted by the United Nations Convention on Biological Diversity.

If the gene is the fundamental unit of natural selection, according to E. O. Wilson, the real biodiversity is genetic diversity. For geneticists, biodiversity is the diversity of genes and organisms. They study processes such as mutations, gene exchanges, and genome dynamics that occur at the DNA level and generate evolution.

For ecologists, biodiversity is also the diversity of durable interactions among species. It not only applies to species, but also to their immediate environment (biotope) and their larger ecoregion. In each ecosystem, living organisms are part of a whole, interacting with not only other organisms, but also with the air, water, and soil that surround them..

Measurement

It has been suggested that some content from this article be split into a separate article entitled Measurement of biodiversity.

Polar bears on the sea ice of the Arctic Ocean, near the north pole.Biodiversity is a broad concept, so a variety of objective measures have been created in order to empirically measure biodiversity. Each measure of biodiversity relates to a particular use of the data.

For practical conservationists, this measure should quantify a value that is broadly shared among locally affected people. For others, a more economically defensible definition should allow the ensuring of continued possibilities for both adaptation and future use by people, assuring environmental sustainability.

As a consequence, biologists argue that this measure is likely to be associated with the variety of genes. Since it cannot always be said which genes are more likely to prove beneficial, the best choice for conservation is to assure the persistence of as many genes as possible. For ecologists, this latter approach is sometimes considered too restrictive, as it prohibits ecological succession.

Biodiversity is usually plotted as taxonomic richness of a geographic area, with some reference to a temporal scale. Whittaker described three common metrics used to measure species-level biodiversity, encompassing attention to species richness or species evenness:

- Species richness - the least sophisticated of the indices available.
- Simpson index
- Shannon-Weaver index

There are three other indices which are used by ecologists:

- Alpha diversity refers to diversity within a particular area, community or ecosystem, and is measured by counting the number of taxa within the ecosystem (usually species)
- Beta diversity is species diversity between ecosystems; this involves comparing the number of taxa that are unique to each of the ecosystems.
- Gamma diversity is a measure of the overall diversity for different ecosystems within a region.

Distribution

A conifer forest in the Swiss Alps (National Park).Selection bias continues to dedevil modern estimates of biodiversity. In 1768 Rev. Gilbert White succinctly observed of his Selborne, Hampshire "all nature is so full, that that district produces the most variety which is the most examined."

Nevertheless, biodiversity is not distributed evenly on Earth. It is consistently richer in the tropics and in other localized regions such as the California Floristic Province. As one approaches polar regions one generally finds fewer species. Flora and fauna diversity depends on climate, altitude, soils and the presence of other species. In the year 2006 large numbers of the Earth's species were formally classified as rare or endangered or threatened species; moreover, many scientists have estimated that there are millions more species actually endangered which have not yet been formally recognized. About 40 percent of the 40,177 species assessed using the IUCN Red List criteria, are now listed as threatened species with extinction - a total of 16,119 species.

A biodiversity hotspot is a region with a high level of endemic species. These biodiversity hotspots were first identified by Dr. Norman Myers in two articles in the scientific journal The Environmentalist. Dense human habitation tends to occur near hotspots. Most hotspots are located in the tropics and most of them are forests.

Brazil's Atlantic Forest is considered a hotspot of biodiversity and contains roughly 20,000 plant species, 1350 vertebrates, and millions of insects, about half of which occur nowhere else in the world. The island of Madagascar including the unique Madagascar dry deciduous forests and lowland rainforests possess a very high ratio of species endemism and biodiversity, since the island separated from mainland Africa 65 million years ago, most of the species and ecosystems have evolved independently producing unique species different from those in other parts of Africa.

Many regions of high biodiversity (as well as high endemism) arise from very specialized habitats which require unusual adaptation mechanisms. For example the peat bogs of Northern Europe.

Evolution

Apparent marine fossil diversity during the Phanerozoic Eon.Biodiversity found on Earth today is the result of 4 billion years of evolution. The origin of life has not been definitely established by science, however some evidence suggests that life may already have been well-established a few hundred million years after the formation of the Earth. Until approximately 600 million years ago, all life consisted of archaea, bacteria, protozoans and similar single-celled organisms.

The history of biodiversity during the Phanerozoic (the last 540 million years), starts with rapid growth during the Cambrian explosion—a period during which nearly every phylum of multicellular organisms first appeared. Over the next 400 million years or so, global diversity showed little overall trend, but was marked by periodic, massive losses of diversity classified as mass extinction events.

The apparent biodiversity shown in the fossil record suggests that the last few million years include the period of greatest biodiversity in the Earth's history. However, not all scientists support this view, since there is considerable uncertainty as to how strongly the fossil record is biased by the greater availability and preservation of recent geologic sections. Some argue that corrected for sampling artifacts, modern biodiversity is not much different from biodiversity 300 million years ago. Estimates of the present global macroscopic species diversity vary from 2 million to 100 million species, with a best estimate of somewhere near 13–14 million, the vast majority of them arthropods.

Most biologists agree however that the period since the emergence of humans is part of a new mass extinction, the Holocene extinction event, caused primarily by the impact humans are having on the environment. It has been argued that the present rate of extinction is sufficient to eliminate most species on the planet Earth within 100 years.

New species are regularly discovered (on average between 5–10,000 new species each year, most of them insects) and many, though discovered, are not yet classified (estimates are that nearly 90% of all arthropods are not yet classified). ost of the terrestrial diversity is found in tropical forests.

Human Benefits

Summer field in Belgium (Hamois).There are a multitude of anthropocentric benefits of biodiversity in the areas of agriculture, science and medicine, industrial materials, ecological services, in leisure, and in cultural, aesthetic and intellectual value. Biodiversity is also central to an ecocentric philosophy. It is important for contemporary audiences to understand the reasons for believing in conservation of biodiversity. One way to identify the reasons why we believe in it is to look at what we get from biological diversity and the things that we lose as a result of species extinction, which has taken place over the last 600 years. Mass extinction is the direct result of human activity and not of natural phenomena which is the perception of many modern day thinkers. There are many benefits that are obtained from natural ecosystem processes. Some ecosystem services that benefit society are air quality, climate (both global CO_2 sequestration and regional and local), water purification, disease control, biological pest control, pollination and prevention of erosion. Along with those come non- material benefits that are obtained from ecosystems which are spiritual

and aesthetic values, knowledge systems and the value of education that we obtain today. However, the public remains unaware of the crisis in sustaining biodiversity. Biodiversity takes a look into the importance to life and provides modern audiences with a clear understanding of the current threat to life on Earth.

Agriculture

For some foodcrops and other economic crops, wild varieties of the domesticated species can be reintroduced to form a better variety than the previous (domesticated) species. The economic impact is gigantic, for even crops as common as the potato (which was bred through only one variety, brought back from the Inca), a lot more can come from these species. Wild varieties of the potato will all suffer enormously through the effects of climate change. A report by the Consultative Group on International Agricultural Research (CGIAR) describes the huge economic loss. Rice, which has been improved for thousands of years by humans, can through the same process regain some of its nutritional value that has been lost since (a project is already being carried out to do just this).

Crop diversity is also necessary to help the system recover when the dominant crop type is attacked by a disease:

- The Irish potato blight of 1846, which was a major factor in the deaths of a million people and migration of another million, was the result of planting only two potato varieties, both of which were vulnerable.
- When the rice grassy stunt virus struck rice fields from Indonesia to India in the 1970s, 6273 varieties were tested. Only one was luckily found to be resistant, a relatively feeble Indian variety, known to science only since 1966, with the desired trait. It was hybridised with other varieties and now widely grown.
- In 1970, coffee rust attacked coffee plantations in Sri Lanka, Brazil, and Central America. A resistant variety was found in Ethiopia, coffee's presumed homeland, which mitigated the rust epidemic.
- Monoculture, the lack of biodiversity, was a contributing factor to several agricultural disasters in history, including the Irish Potato Famine, the European wine industry collapse in the late 1800s, and the US Southern Corn Leaf Blight epidemic of 1970.

Higher biodiversity also controls the spread of certain diseases as pathogens will need to adapt to infect different species.

Amazon Rainforest in Brazil.Biodiversity provides food for humans. Although about 80 percent of our food supply comes from just 20 kinds of plants, humans use at least 40,000 species of plants and animals a day. Many people around the world depend on these species for their food, shelter, and clothing. There is untapped potential for increasing the range of food products suitable for human consumption, provided that the high present extinction rate can be stopped.

Science and medicine

A significant proportion of drugs are derived, directly or indirectly, from biological sources; in most cases these medicines can not presently be synthesized in a laboratory setting. About 40% of the pharmaceuticals used in the US are manufactured using natural compounds found in plants, animals, and microorganisms. Moreover, only a small proportion of the total diversity of plants has been thoroughly investigated for potential sources of new drugs. Many drugs are also derived from microorganisms.

Through the field of bionics, considerable technological advancement has occurred which would not have without a rich biodiversity.

Industrial materials

A wide range of industrial materials are derived directly from biological resources. These include building materials, fibers, dyes, resirubber and oil. There is enormous potential for further research into sustainably utilizing materials from a wider diversity of organisms.

Other ecological services

Biodiversity provides many ecosystem services that are often not readily visible. It plays a part in regulating the chemistry of our atmosphere and water supply. Biodiversity is directly involved in water purification (eg sewage), recycling nutrients and providing fertile soils. Experiments with controlled environments have shown that humans cannot easily build ecosystems to support human needs; for example insect pollination cannot be mimicked by human-made construction, and that activity alone represents tens of billions of dollars in ecosystem services per annum to humankind.

Leisure, cultural and aesthetic value

Many people derive value from biodiversity through leisure activities such as hiking in the countryside, birdwatching or natural history study.

Biodiversity has inspired musicians, painters, sculptors, writers and other artists. Many cultural groups view themselves as an integral part of the natural world and show respect for other living organisms.

Popular activities such as gardening, caring for aquariums and collecting butterflies are all strongly dependent on biodiversity. The number of species involved in such pursuits is in the tens of thousands, though the great majority do not enter mainstream commercialism.

The relationships between the original natural areas of these often 'exotic' animals and plants and commercial collectors, suppliers, breeders, propagators and those who promote their understanding and enjoyment are complex and poorly understood. It seems clear, however, that the general public responds well to exposure to rare and unusual organisms—they recognize their inherent value at some level, even if they would not want the responsibility of caring for them. A family outing to the botanical garden or zoo is as much an aesthetic or cultural experience as it is an educational one.

Philosophically it could be argued that biodiversity has intrinsic aesthetic and/or spiritual value to mankind in and of itself. This idea can be used as a counterweight to the rather notion that tropical forests and other ecological realms are only worthy of conservation because they may contain medicines or useful products.

Human Hindrances

Funds: Humans have generally expanded and developed their territory throughout history. An active approach is the only way to halt the expansion but this often requires funds or wise stewardship. Currently the United States Environmental Protection Agency has an annual budget of $7.3 billion (2007).

Preservation of invertebrate and plant species: Biodiversity is most well known to the public as a loss of animals with a backbone, when in fact there exist 20 times that number of insects and five times as many flowering plants. While many of these species may be highly valuable to the human race for the above reasons, the vast majority are often completely unknown to anyone but specialists and useless to mankind but still ellicit a strong response when endangered. In fact, while it is thought that about 90% of mammals have been identified, it is estimated that less than half and perhaps less than two-thirds of earth organisms have even been identified often due to the level of sublty and overlap many inverdibrate orders contain.

Numbers of species

As a soft guide, however, the numbers of identified modern species as of 2004 can be broken down as follows:

- 287,655 plants, including: 15,000 mosses, 13,025 ferns, 980 gymnosperms, 199,350 dicotyledons, 59,300 monocotyledons;
- 74,000–120,000 fungi; 10,000 lichens;
- 1,250,000 animals, including: 1,190,200 invertebrates: 950,000 insects, 70,000 mollusks, 40,000 crustaceans,
- 130,200 others; 58,808 vertebrates: 29,300 fish, 5,743 amphibians, 8,240 reptiles, 10,234 birds, (9799 extant as of 2006) 5,416 mammals.

However the total number of species for some phyla may be much higher:

- 10–30 million insects;
- 5–10 million bacteria;
- 1.5 million fungi;
- ~1 million mites

Threats to Biodiversity

Destruction of habitats: Most of the species extinctions from 1000 AD to 2000 AD are due to human activities, in particular destruction of plant and animal habitats. Raised rates of extinction are being driven by human consumption of organic resources, especially related to tropical forest destruction. While most of the species that are becoming extinct are not food species, their biomass is converted into human food when their habitat is transformed into pasture, cropland, and orchards. It is estimated that more than 40% of the Earth's biomass is tied up in only the few species that represent humans, livestock and crops. Because an ecosystem decreases in stability as its species are made extinct, these studies warn that the global ecosystem is destined for collapse if it is further reduced in complexity. Factors contributing to loss of biodiversity are: overpopulation, deforestation, pollution (air pollution, water pollution, soil contamination) and global warming or climate change, driven by human activity. These factors, while all stemming from overpopulation, produce a cumulative impact upon biodiversity.

There are systematic relationships between the area of a habitat and the number of species it can support, with greater sensitivity to reduction in habitat area for species of larger body size and for those living at lower latitudes or in forests or oceans. Some characterize loss of biodiversity not as ecosystem degradation but by conversion to trivial standardized ecosystems (e.g., monoculture following deforestation). In some countries lack of property rights or access regulation to biotic resources necessarily leads to biodiversity loss (degradation costs having to be supported by the community).

A September 14, 2007 study conducted by the National Science Foundation found that biodiversity and genetic diversity are dependent upon each other—that diversity within a species is necessary to maintain diversity among species, and vice versa. According to the lead researcher in the study, Dr. Richard Lankau, "If any one type is removed from the system, the cycle can break down, and the community becomes dominated by a single species."

At present, the most threathened ecosystems are those found in sweet water. The marking of sweet water ecosystems as the ecosystems most under threat was done by the Millennium Ecosystem Assessment 2005, and was confirmed again by the project "Freshwater Animal Diversity Assessment", organised by the biodiversity platform, and the French Institut de Recherche pour le Développement (MNHNP).

Exotic species

The rich diversity of unique species across many parts of the world exist only because they are separated by barriers, particularly large rivers, seas, oceans, mountains and deserts from other species of other land masses, particularly the highly fecund, ultra-competitive, generalist "super-species". These are barriers that could never be crossed by natural processes, except for many millions of years in the future through continental drift. However humans have invented ships and airplanes, and now have the power to bring into contact species that never have met in their evolutionary history, and on a time scale of days, unlike the centuries that historically have accompanied major animal migrations.

The widespread introduction of exotic species by humans is a potent threat to biodiversity. When exotic species are introduced to ecosystems and establish self-sustaining populations, the endemic species in that ecosystem, that have not evolved to cope with the exotic species, may not survive. The exotic organisms may be either predators, parasites, or simply aggressive species that deprive indigenous species of nutrients, water and light. These exotic or invasive species often have features, due to their evolutionary background and new environment, that make them highly competitive; able to become well-established and spread quickly, reducing the effective habitat of endemic species.

As a consequence of the above, if humans continue to combine species from different ecoregions, there is the potential that the world's ecosystems will end up dominated by relatively a few, aggressive, cosmopolitan "super-species".

F1. PRINCIPLES OF PLANT BREEDING

Plant breeding is a science based on principles of genetics and cytogenetics. It aims at improving the genetic make up of the crop plants. The following objectives of plant breeding are,

1. Higher yield
2. Improved quality
3. Diseases and insect resistance
4. Change in maturity duration
5. Agronomic characteristics
6. Photo insensitivity
7. Synchronous maturity
8. Non-shattering characteristics
9. Determinate growth
10. Dormancy
11. Moisture stress and salt tolerance
12. Elimination of toxic substances
13. Winter hardiness

Some well-known achievements are development of semi-dwarf wheat and rice varieties, mobilization of Indian canes, and production of hybrid and composite varieties of maize, Jowar and bajra.

PLANT INTRODUCTION

Plant introduction consists of taking a genotype or a group of genotypes of plants into new environments where they were not being grown before. This introduction may involve new varieties of a crop already grown in the area, wild relatives of the crop species or a totally new crop species for the area. Often the materials are introduced from other countries or continents. But movement of crop varieties from one environment into another within a country is also introduction. Some examples of within the country introduction are popularization of grape cultivation in Haryana, of wheat in West Bengal and of rice in Punjab. Introduction may be classified into two categories, primary and secondary.

Primary introduction: When the introduced variety is well suited to the new environment. It is released for commercial cultivation without any alteration in the original genotype, this constitutes primary introduction. It is less common particularly in countries having well organized crop improvement programme.

Example: Introduction of semi-dwarf wheat (*Triticum aestivum*) varieties, Sonora 64, Lerma Roja (pronounced as "Lerma Roho') and of semi dwarf Rice (O. sativa) varieties Taichung native 1, IR 8, IR 28, IR 36, IR 64,. IR 66, IR 72 are some examples of primary introduction in this country.

Secondary introduction: The introduced variety maybe subjected to selection to isolate a superior variety. Alternatively, it may be hybridized with local varieties to transfer one or few characters from this variety to the local ones. These processes are known as secondary introduction. It is much more common than primary introduction.

Example: Kalyansoa and Sonalika wheat varieties selected from materials introduced from CIMMYT, Mexico. Semi dwarf wheat and rice varieties developed through hybridization with introduced varieties etc. TKM 9, ADT 36, ADT 39, ADT 43, TRY 1, TRY (R) 2, ASD 18 rice varieties.

Main objective of plant introduction

i. To obtain an entirely new crop plant e.g Maize, potato, tomato
ii. To serve as new varieties. E.g. IRRI varieties.
iii. To be used in crop improvement
iv. To save the crop from diseases and pests. Eg. Coffee from leaf rust. Hevea Rubber from leaf diseases.
v. For scientific studies
vi. Aesthetic value.

Procedure for plant introduction

The chief function of plant introduction is to make available variation to be utilized in breeding programme. Introduction of the following steps:

1. Procurement
2. Quarantine
3. Cataloguing
4. Evaluation
5. Multiplication
6. Distribution.

Plant introduction agencies in India

1. NBPGR, New Delhi (Routed only through NBPGR)
2. Forest Research Institute, Dehra Dun
3. Botanical Survey of India.
4. Central Research Institute for various crops.

Acclimatization: Generally, the introduced varieties perform poorly because they are often not adapted to the new environment. Sometimes, the performance of a variety in the new environment improves with the number of generations grown there. The process that leads to the adaptation of a variety to a new environment is known as "Acclimatization". Acclimatization is brought about by a faster multiplication of those genotypes, that are better adapted to the new environment. Thus acclimatization is essentially natural selection.

The extent of acclimatization is determined by

i. the mode of pollination
ii. the range of genetic variability present in the original population and
iii. the duration of life cycle of the crop.

As a result, cross-pollination is much more helpful in acclimatization than self-pollination.

Merits of plant introduction

i. It provides entirely new crop plants
ii. Provide superior varieties either directly, after selection or hybridization.
iii. Introduction and exploration are the only feasible means of collection germplasm and to protect variability from genetic erosion.
iv. It is very quick and economical method of crop improvement, particularly when the introduction are released as varieties either directly or after a simple selection.
v. Plants may be introduced in new disease free areas to protect them from damage e.g. Coffee and Rubber.

Demerits of plant introduction

The disadvantages of plant introduction are associated with the introduction of weeds, diseases and pests.

Weeds: *Aregemme mexicana, Eichhornia crassipes. Phylaris minor* are the some of the noxious weeds introduced in India.

Diseases: Late blight of potato was introduced from Europe flag smut of wheat was introduced from Australia, Bunchy top Coffee rust came from Ceylon of Banana.

Insect pests: Potato tuber moth came from Italy in 1900 Wooly aphids of apple and fluted scale of citrus were also introduced in India along with plant introductions.

Ornamentals turned weeds: Water hyacinth, *Lantana camara* were both introduced as ornamental plants, but they are now noxious weeds.

Threat to ecological balance: Some introduced species may disturb the ecological balance in their new home, and may cause serious damage to the ecosystem. e.g. Eucalyptus sp. introduced from Australia cause a rapid depletion of the sub soil water reserves. However, most of the cases of introduction of weeds, diseases and insect pests occurred during a period when quarantine was almost non existent. At present, plant introductions have to satisfy rigid quarantine laws. They are thoroughly examined for weeds disease and pest before their entry is permitted.

F2. BREEDING METHODS IN SELF POLLINATED CROPS

Various procedures like introduction, selection, hybridization, mutations are used for genetic improvement of crop plants. The choice of breeding method mainly depends upon four main factors.

1. Mode of pollination
2. Mode of Reproduction
3. Gene action
4. Breeding objectives in a crop species.

SELECTION

In self-pollinated crop, selection permits reproduction only in those plants that have the desirable characteristics. This is achieved by raising the next generation from seeds produced by the selected plants only. Seeds from the remaining plants are rejected. Selection is essentially based on the phenotype of plants.

The two requirements of selection are;

1. Variation must be present in the population
2. Variation must be heritable.

The purpose of selection in to isolate desirable plant type from a population. Selection is one of the two fundamental steps of any breeding programme. The two basic steps are:

PROGENY SELECTION

Evaluation of the worth of plants on the basis of the performance of their progenies is known as progeny test. Louis De Vilmorin developed the progeny test. Therefore it is also known as "Vilmorin Isolation principal" or Vimorin Principle. The progeny test in the basic step in every breeding method. The progeny test serves two valuable functions.

i. To determine the breeding behaviour of a plant i.e. whether it is homozygous or heterozygous.
ii. To find out, whether the character for which the plant was selected, is heritable i.e due to genotype.

Selections have to be based on phenotype. The relative contributions of genotype and environment to the phenotype of the selected plants can be determined through progeny test. If the phenotypic differences are due to differences in genotype, they will be present in the progeny as well. Otherwise they will be absent in the progeny.

1. **PURE LINE CONCEPT:** A pure line is the progeny of a single homozygous plant of a self-pollinated species. All the plants in a pure line have the same genotype. The phenotypic differences within a pure line are due to the environment and have no genetic basis. Therefore, variation within a pureline is not heritable.

GENETIC MAKEUP OF SELF-POLLINATED CROPS

Self-pollination increases homozygosity with a corresponding decrease in heterozygosity. Self-pollination is the most intense form of in breeding, since this case, the same individual functions as the male as well as the female parent.

The effect of self-pollination on homozygosity and heterozygosity may be illustrated by an example. Suppose an individual heterozygous for a single gene (Aa), in self pollinated in successive generations. Every generations of self-pollination will reduce the frequency of heterozygote Aa to 50 per cent of that in the previous generation. There is a corresponding increase in the frequency of the homozygotes, AA and aa. As a result, after 10 generations of selfing, virtually all the plants in the populations would be homozygous, ie. AA and aa. On the other hand the frequency of heterozygote Aa would be one only 0.095 per cent, which is negligible.

When a number of genes are segregating together, each gene would become homozygous at the same rate as Aa. Thus the number of genes segregating does not affect the percentage of homozygosity. The term homozygosity denotes the frequency of genes in homozygous condition in the population. Similarly, linkage between genes does not affect the percentage of homozygosity in the population.

No. of generations of selfing	Frequency (%)			Frequency %	
	AA	Aa	aa	Homozygotes	Heterozygotes
O	O	100	O	O	100
1	25	50	25	50	50
2	25 + 12.5	25	25 12.5	75	25
3	37.5+ 6.25	12.5	37.5+6.25	87.5	12.50
4	43.75+3.125	6.25	43.75+3.125	93.73	6.25
5	46.875+1.562	3.125	46.875+1.562	96.874	3.125
10	49.948	0.097	49.948	99.896	0.097
n	2n-1/ 2n+1	2/2n+1	2n-1/ 2n+1	2n-1/2n	1/2n

Population of completely homozygous plants

$$= [2m-1/ 2m]^{n}$$

Where,
'm' is the number of generations of selfing.
'n' is the number of genes segregating.

Thus, self-pollination has two main effects on the populations; first all the plants in the population become completely homozygous and second the population is a mixture of several homozygous genotypes.

PURE LINE SELECTION

A pure line is the progeny of a single, homozygous, self-pollinated plant. In pure line selection, a large number of plants are selected from a self-pollinated crop and are harvested individually; individual plant progenies from than are evaluated, and the best progeny is released as pureline variety. Therefore, pureline selection is also known as individual plant selection. A pureline variety is a variety obtained from a single homozygous plant of a self-pollinated crop.

CHARACTERISTIC OF PURE LINES

1. All the plants within a pureline have the same genotype as the plant from which the pureline was derived.
2. The variation within a pureline is environment and non-heritable.
3. Purelines become genetically variable with time. The genetic variation is produced by mechanical mixtures, natural hybridization or mutation.

Merits of pureline selection

1. Pureline selection achieves the maximum possible improvement over the original variety. This is because the variety is the best pureline present in the population.
2. Pureline varieties are extremely uniform since all the plants in the variety have the same genotype. Such a uniform variety is more liked by the farmers and the consumers than a less uniform variety developed through mass selection.
3. Due to its extreme uniformity the variety is easily identified in seed certification programmes.

Demerits of pure line selection

1. The varieties developed through pureline selection generally do not have wide adaptation and stability in production possessed by the local varieties from which they are developed.
2. The procedure of pureline selection requires more line space and more expensive yield trials than mass selection.
3. The upper limit on improvement is set by the genetic variation present in the original population.
4. The breeder has to devote more time to pureline selection than o mass selection. This leaves less time for other breeding programmes.

MASS SELECTION

A large number of plants of similar phenotype are selected and their seeds are mixed together to constitute the new variety. The plants are selected on the basis of their appearance or phenotype. Therefore selection is done for easily observable characters like plant height, ear type, grain colour, grains size, disease resistance, tillering ability, lodging resistance, shattering resistance etc., Sometimes yield of the plant may be used a criterion of selection. If the population has variation for grain characteristics like seed colour and seed size, selection may be done for them before the seeds of selected plants are mixed together.

Generally, the plants selected in mass selection are not subjected to progeny test. Incase of self-pollinated crop, mass selection has two major applications.

1. Improvement of desi or local varieties.
2. Purification of the existing pureline varieties.

Mass selection has only a limited application for the improvement of self-pollinated crops. It is generally not used for the handling of segregating populations derived from hybridization. In cross-pollinated crops, mass selection leads to avoid inbreeding depression, loss in vigour and yield. Further because of the heterozygous nature of the population, several cycles of mass selection may effectively be practiced.

Mass selection procedure

I - Year

1. From a variable population, 200-2000 plants with similar but desirable traits are selected.
2. The seeds from selected plants are composited.

II - year

1. The composited seeds were planted in a preliminary yield trial along with standard check.
2. Phenotype of the selected population is critically evaluated.

III -VIth Year

Promising selections are evaluated in co-ordinated trials at several locations.

1. If outstanding, released as a new variety.

VIIth - Year

Seed multiplication for distribution.

Mass selection p in self-pollinated crops coupled with progeny testing

This method is more useful than the mass selection without progeny testing. It is commonly used for maintaining the purity of pureline varieties.

I - Year

1. Select 200-2000 plants of similar but superior phenotype.
2. Harvest seeds separately from each selected plant.

II - year

1. Grow individual plant progenies
2. Reject inferior or segregating progenies.
3. Bulk the seeds from remaining progenies.

III Year

3. Preliminary yield trials from the bulked seed, standard checks are included.
4. If superior the variety is included in multilocation trials.

V- VIIth - Year

1. Multilocation coordinated yield trials
2. If superior released as a new variety.

VIIIth Year

Seed multiplication for distribution.

Merits

1. The varieties developed through mass selection are likely to be more widely adapted than purelines. It is generally accepted that a mixture of closely related purelines is more stable in performance over different environments than a single pureline.
2. Extensive and prolonged yield trials are not necessary. This reduces the time and cost needed for developing a new variety.
3. Mass selection retains considerable genetic variability.

4. It is a less demanding method. The breeder can devote more time to other breeding programmes.

Demerits

1. The varieties developed through mass selection show the variation and are not as uniform as pureline varieties. Therefore such varieties are generally less liked than pure line varieties.
2. The improvement through mass selection is generally less than that through pureline selection. It is because at least some of the plant progenies which make up the new variety would be poorer than the best pureline that may be selected from among them.
3. In the absence of progeny test, it is not possible to determine the genotype of the selected plants. As suggested by Allard, progeny test may be included in mass selection programmes to overcome this defect.
4. Varieties developed by mass selection are more difficult to identify the purelines in seed certification programmes.
5. Mass selection utilizes the variability already present in a variety or population. Therefore only those varieties that show genetic variation can be improved through mass selection. Thus mass selection is limited by the fact that it cannot generate variability.

GENETIC STRUCTURE OF A CROSS POLLINATED CROPS

Cross-pollinated crops are highly heterosis due to free intermating among their plants. They are often referred as " Random mating" populations because each individual of the population has equal opportunity of mating with any other individuals of that population. Such a population is also known as "Mendalian population" or "Panictic population". A Mendalian population may be thought of having a gene pool consisting of all the gametes produced by the population. Thus the gene fool may be defined as the sum total of all the genes present in a population.

HARDY WEINBERG LAW

It is the fundamental law of population genetics and provides the basis for studying Mendalian population. This law was independently developed by hardy (1908) in England and Weinberg (1909) in Germany. The Hardy-Weinberg law states that, the gene and genotype frequencies in a Mendalian population remain constant generation after generation if there is no selection, mutation, migration or random drift.

The frequencies of the three genotypes for a locus wilt two alleles, say 'A' and 'a' would be p^2 (AA) 2 pq (Aa), q^2 (aa)

where, 'p' represents the frequency 'A'
'q' represents the frequency 'a'.
The sum of p and q is one. $p + q = 1$

Such a population would be at equilibrium, since the genotypic frequencies would be stable, that is would not change, from one generation to the next. The equilibrium is known as ' Hardy-Weinberg equilibrium'.

Let us consider a single gene with two alleles, A and a in a random making population.

There would be 4 genotypes, AA, Aa, aa suppose the population has 'N' individuals of which,

'D' individuals are AA
'H' individuals are Aa
'R' individuals are aa
So that D + H + R = N

The total number of alleles at this locus in the population would be 2N, since each individual has two alleles at a single locus.
The total number of 'A' alleles= 2 D + H
(A A) (A a)

The ratio (2D +H) 2 N is therefore the frequency of ' A ' allele in the population and is represented by 'p'. Similarly the ratio (2R +H) 2 N in the frequency of allele 'a' and is written as 'q' therefore

$P = (2D+H) / 2N = q = (2R+H) / 2N$
$= (D + 1/2 H) / N = (R + 1/2 H)/N$

Therefore. $p + q = \frac{D+H+R}{N} = \frac{N}{N} = 1$

$P = 1-q$ or $q = q-p$.

The values of p and q are known as gene frequency.

Gene frequency in the proportion of an allel 'A' or 'a' in the population.

Genotype frequency (Zygotic frequency) in the proportion of a genotype AA or Aa or aa.

FACTORS AFFECTING EQUILIBRIUM IN POPULATIONS

The equilibrium in random mating populations is disturbed by

1. **Migration:** It is the movement of individuals into a population from a different population. Migration may introduce new alleles into the populations or may change the frequencies of existing alleles.

2. Mutation: It is a sudden heritable change in an organism and is generally due to a structural change in a gene. Mutations may produce a new allele not present in populations or may change the frequencies of existing alleles.

3. Random drift: It is also called, as genetic drift is a random change in gene frequency due to sampling error.

4. Inbreeding: Mating between individuals sharing a common parent in their ancestry is known as inbreeding. It reduces the proportion of

heterozygotes and increases the frequency of homozygotes.

5. Selection: A differential reproduction rate of various genotypes is known as selection. Selection allows the selected genotypes to reproduce, while the undesirable genotypes are eliminated. Thus the breeder is able to improve the various characteristics by selecting for desirable types.

Selection is a random mating population is highly effective in increasing or decreasing the frequency of alleles, but is unable to either fix or eliminate them. However, in combination with a system of inbreeding, selection is highly efficient in the fixation and elimination of alleles.

Most of the characters of economic importance are quantitative characters and are governed by many genes. Such characters show a continuous distribution. Selection of the extreme phenotype increases the frequency of desirable alleles in the population.

The breeder has two basic tools to change the genetic composition of population.

1. Selection and
2. Mating system.

Mating Systems

1. **Random mating:** Each female gamete is equally likely to write with any male gamete and the rate of reproduction of each genotype is equal i.e. there is no selection. In such situations,

a. Gene frequencies remain constant
b. Variance for the character is constant and
c. The correlation between relatives or prepotency does not change.

2. **Genetic assortative mating:** The mating in between individuals that are more closely related by ancestry in random mating. This mating system is more commonly known as ' inbreeding'. It increases homzygosity and reduces heterozygosity. It is useful in development of inbreds, both partial and complete.

3. **Genetic disassortative mating:** Such individuals are mated which are less closely related by ancestry then would be under random mating. Thus in this system, totally unrelated individuals are mated. Examples of such a mating are inter varietal and interspecific crosses. This system would reduce homozygosity and increases heterozygosity.

4. **Phenotypic assortative mating:** Mating between individuals which are phenotypically more similar than would be expected under random mating is called phenotypic assortative mating. This is useful in the isolation of extreme population.

5. **Phenotypic disassortative mating:** Mating between phenotypically dissimilar individuals is referred to as phenotypic disassortative mating. It is very useful in making a population ' stable' i.e. maintaining variability. Suitable parents may be selected to remove their weakness. The progeny from such a mating would be more desirable than the parents. It is also useful when the desirable type is an intermediate one and the available parents have the extreme phenotypes. But the most notable use of this mating system is in maintaining variability in relatively smaller populations as it reduces inbreeding.

BREEDING METHODS IN CROSS-POLLIANTED CROPS

Populations of cross-pollinated crop species are highly heterozygous as well as heterogeneous. Their genetic makeup is such that they show variable inbreeding depression. Consequently, breeding methods for cross-pollinated crop aim at preventing in breeding. The breeding methods commonly used in cross-pollinated crops may be grouped into two broad categories;

i. Population improvement
ii. Hybrid and synthetic varieties.

In the case of population improvement, mass selection or its modifications are used to increase the frequency of desirable alleles, thus improving the characteristics of populations.

In case of hybrid and synthetic varieties, a variable number of strains are crossed to produce a hybrid population, the strains that are crossed are selected on the basis of their combining ability.

The population improvement methods may be grouped into two general classes'

i. Without progeny testing
ii. With progeny testing

Without progeny testing: Plants are selected on the basis of their phenotype and number of progeny test is carried out. E.g. Mass selection.

With progeny testing: The plants are initially selected on the basis of their phenotype, but the final selection of plants that contribute to the next generation is based on progeny test. This class of

population improvement includes: Progeny selection or Ear -to row method and Recurrent selection.

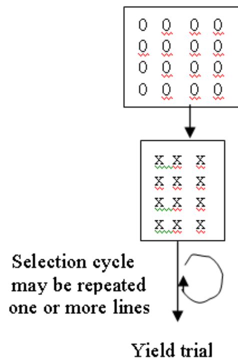
MASS SELECTION

It is the oldest breeding scheme available for cross-pollinated crops. In mass selection a number of plants are selected on the basis of their phenotype, and the open pollinated seed from them is bulked together to raise the next generation.

The selected plants are allowed to open pollinate i.e. to mate at random selection of plants is based on their phenotype and no progeny test in

conducted. The selection cycle may be repeated one or more times to increase the frequency of favourable alleles. Such a selection scheme in generally known as phenotypic recurrent selection. Care should be taken to select a sufficiently large number of plants in order to keep inbreeding to a minimum. The efficiency of mass selection primarily depends upon the number of genes controlling the character, gene frequencies and more importantly heritability.

i. Several plants selected on the basis of phenotype
ii. Open pollinated seed from the selected plants grown



i. Bulk seed from the selected plants grown
ii. Mass selection may be repeated.

Merits of mass selection

i. Work of the breeder is Kept to a minimum, since the selection are based on the phenotype of plants.
ii. The selection cycle is very short, i.e. only one generation
iii. It is highly efficient in improving characters that are easily identified visually and have high heritability. E.g. plant height, size of ear, date of maturity etc.,

Demerits

i. Selection of plants is based on the phenotype of individual plants. Superior phenotype is often a poor basis for the identification of superior genotype. The environment affects quantitative character.
ii. The selected plants are allowed to open pollinate so, the selected plants are pollinated by both superior and inferior plants present in the population. This reduces the effectiveness of selection.

Modifications of mass selection

Two defects of mass selection are;

i. Lack of control on the pollen source and
ii. Confusing effect of environment on the phenotype of individual plants.

The defects may be corrected as follows

1. Inferior plants in the field are destasselled and the remaining plants are allowed to open pollinate. This modification exercise some control on the pollen source, but the identification of inferior plants of necessity is based on only those characters which are expressed before flowering.
2. Pollen from all the selected plants is collected and bulked; this pollen is used to pollinate the selected plants. This ensures full control on the pollen source.
3. Stratified mass selection: This modification suggested by Gardner in 1961. It is also known as the 'grid method of mass selection'. The field from which selection is to be done is divided into several small plots. E.g. having 40-50 plants each. Equal numbers of superior plants are selected within in the plots, not among the plots. The seed from all the selected plants is composited to raise next generation that variation due to environment, including variation in soil fertility, will be much reduced with in the small plots than the whole field. Thus selection within the plots is expected to be more effective than that without any stratification. It has been able to increase the yielding ability of an open pollinated variety of maize, hays golden, by about 3% per cycle, for15 generations.

Effectiveness of mass selection: It has effectively improved characters with high heritability in maize e.g. ear height, lodging resistance, ear type, adaptiveness, oil and protein content, resistance to leaf blight and days to flowering.

HETEROSIS AND INBREEDING DEPRESSION

Cross-pollinated species and species reproducing asexually are highly are heterozygous. These species usually show a severe reduction in fertility and vigour due to inbreeding. Conversely hybridization between unrelated strains generally leads to an increased vigour and fertility.

Inbreeding: It is mating between individuals related by descent or ancestry. When the individuals are closely related. E.g. in brother sister mating or sib mating; the degree of inbreeding is high. The highest degree in breeding is achieved by selfing.

Selfing reduces heterozygosity by a factor of 1/2 in each generation. The degree of inbreeding increases in the same proportion.

Inbreeding depression: It may be defined as the reduction or loss in vigour and fertility as a result of inbreeding.

Effects of inbreeding

1. Appearance of Lethal and sub lethal alleles: Chlorophyll deficiency, rootless seedlings, defects in flower structure. These plants cannot be maintained and are lost from the population.
2. Reduction in vigour: Plants become shorter and weaker, reduction in size of various plant parts.

3. Reduction in Reproductive ability: Poor reproduction.
4. Separation of the population into distinct lines: The population rapidly separates into phenotypically distinct lines.
5. Increase in homozygosity: Variation within a line decreases rapidly. The lines, which are almost homozygous due to continued inbreeding and are maintained through close inbreeding, are known as inbred lines.
6. Reduction in yield: Inbreeding generally leads to a loss in yield. In maize the best inbred lines yield about half as much as the open pollinated varieties from which they were produced.

Degrees of inbreeding depression

The various species differ considerably in their response to inbreeding. They may be grouped into four broad categories.

1. High inbreeding depression: e.g. Alfalfa, carrot - do not survive resulting in lethality.
2. Moderate inbreeding depression: Maize, sorghum, cumbu. Reproduction in fertility and yield upto 50% production and maintenance of inbred lines are relatively easier in these species.
3. Low inbreeding depression: e.g. Onion, cucurbits, rye, and sunflower. Loss invigour and fertility is small. Reduction in yield is small or absent.
4. No inbreeding depression: Self-pollinated species do not show inbreeding depression, although they do show heterosis.

Homozygous and heterozygous balance

This concept was advanced by Mather, to explain the varied response of different species to inbreeding. The species carry a large number of lethal, sub vital and other unfavourable recessive genes. Which are of little immediate value to the species. The sum total of these unfavourable genes is known as "Genetic Load".

The harmful effects of such recessive alleles are masked by their dominant alleles as a result of which they are retained in the population. The population therefore develops a genetic organization, which favours heterozygosity. This type of genetic organization is known as heterozygous balance, because it promotes heterozygosity.

The self-fertilized species are naturally homozygous. They have no genetic load, because unfavourable recessive genes become homozygous and are eliminated from the population. These species therefore, develop a genetic organization, which is adapted to homozygosity i.e., which does not produce undesirable effects in the homozygous state. This type of genetic organization is known as homozygous balance.

OVER DOMINANCE HYPOTHESIS

This hypothesis was independently proposed by East and Shull in 1908. According to this, heterozygotes at atleast some of loci are superior to both the relevant homozygotes. Thus heterozygote 'Aa' consequently heterozygosity resulting from inbreeding produces inbreeding depression. It would therefore, be impossible to isolate inbreds as vigorous as F_1 hybrids.

In 1936, East proposed that at each locus there are several alleles e.g., 91, 92, 93, 94….. etc with increasingly different functions. Heterozygotes for more divergent alleles would be more heterotic than those involving less divergent ones.

For example 91 94 would be superior to be 91 92, 92 93, and 93 94. It is assumed that, the different alleles some where different functions. The hybrid is therefore able to perform the functions of both the alleles, which is not possible in the case of two homozygotes.

HETEROSIS

The term heterosis was first used by Shull in1914. Heterosis may be defined as the superiority of an F1 hybrid over both its parents in terms of yield or some other character. Generally heterosis is manifested as an increase in vigour, size, growth rate, yield or some other characteristics. But in some cases, the hybrid may be inferior to the weaker parent. This is also regarded as heterosis. Hybrid vigour describes only the superiority of hybrids over their parents.

LUXURIANCE

It is the increased vigour and size of interspecific hybrids. The principle difference between heterosis and luxuriance lies in the reproductive ability of the hybrids. Heterosis is accompanied with an increased fertility, while luxuriance is expressed by interspecific hybrids that are generally sterile.

Different heterosis

1. **Relative heterosis (or) Average heterosis:** The superiority of F1 is estimated over the average of two parents or the mid parent (But it is not useful in practical plant breeding).
2. **Heterobeltiosis:** Superiority of F1 over better parents.
3. **Standard heterosis:** Superiority of F1 over standard variety.

Manifestation of heterosis

Heterosis is the superiority of a hybrid over its parents. This superiority may be in yield, quality, diseases and insect resistance, adaptability, general size or the size of the specific parts, growth rate, enzyme activity etc.,

1. Increased yield: It is the most important objective of plant breeding. The yield may be measured in terms of grain, fruit, seed, leaf, tubers or the whole plant.
2. Increased reproductive ability: It is expressed in higher yield of seeds or fruits.

3. Increase in size and general vigour: The hybrids are generally more vigorous. E.g. Fruit size in tomato, head size in cabbage, cob size in maize, head size in Jowar etc.,
4. Better quality: In onion, many hybrids show better keeping quality, but not yield.
5. Earlier flowering and maturity: Hybrids are earlier in flowering and maturity than the parents. Many tomato hybrids are earlier than their parents.
6. Greater resistance to disease and pests
7. Greater adaptability: Hybrids are generally more adapted to environmental changes than inbreds.

GENETIC BASES OF HETEROSIS AND INBREEDING DEPRESSION

Heterosis and inbreeding depression are closely related phenomena. In fact they may be regarded as two opposite sides of the same coin. There are two main theories to explain heterosis and consequently inbreeding depression.

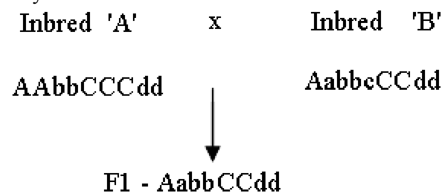
Dominance hypothesis: It was first proposed by Davenport in 1908. At each locus the dominant allele has a favourable effect, while the recessive allele has an unfavourable effect. In heterozygous state, the deleterious effects of recessive alleles are masked by their dominant alleles. Thus heterosis results from the masking of harmful effects of recessive alleles by their dominant alleles. The harmful effects of recessive alleles, which become homozygous due to inbreeding, on the other hand, produce inbreeding depression.

Heterosis in an F1 hybrid is a result of the masking of harmful effects of recessive alleles present in one parent by the dominant alleles present in the other plant and vice versa. Hybrids from parents with similar recessive and dominant alleles would show little or no heterosis, while those with different alleles would show heterosis. Generally parents of diverse origin are more likely to produce heterotic progeny than those of similar origin.

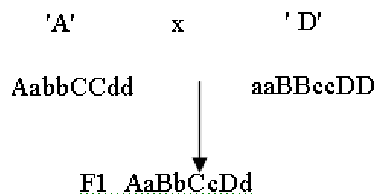
HYBRIDIZATION

Crossing of two plants or lines of dissimilar genotypes is known as hybridization. The seeds as well as the progeny resulting from the hybridization are known as hybrid or F1. The progeny of F1 obtained by selfing or intermating of F1 plants, and the subsequent generations are termed as segregating generations. The term ' Cross' is often used to denote the products of hybridization i.e. the F1 as well as the segregating generations.

Objectives: The chief objective of hybridization is to create genetic variation. When two genotypically different plants are crossed, the genes from both the parents are brought together is F1. Segregation and recombination produce many new gene combinations in F2 and later generation i.e. segregating generations. The aim of hybridization may be the transfer of one or few qualitative characters or use the F1 as hybrid variety.



No heterosis
Harmful effects of 'b' and 'd' genes are not masked



Heterosis

Combination breeding: The main aim of combination breeding is the transfer of one or more characters into a single variety, from other varieties. A familiar example of combination breeding is that of disease resistance. In combination breeding one of the parents must have in a sufficient intensity, the character under transfer while the other parent is generally a popular variety.

Transgressive breeding: Aims at improving yield or its contributing characters through transgressive segregation. Transgressive segregation is the production of plants in an F2 generation that are superior to both the parents for one or more characters such plants are produced by an accumulation of plus or favourable genes from both the parents as a consequence of recombination.

The parents involved in hybridization must combine well with each other, and should preferably be genetically diverse i.e. quite different. Each parent is expected to contribute different plus genes which when brought together by recombination give rise to transgressive segregant.

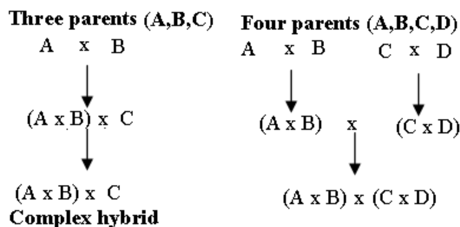
Types of hybridization

The plants or lines involved in hybridization may belong to the same variety, different varieties of the same species, different species of the same genus or species from different genera. Based on the taxonomic relationships of the two parents, hybridization may be classified into two broad groups.

1. Inter-varietal hybridization: The parents involved in hybridization belong to the same species. They may be two strains, varieties or races of the same species. It is also known as intra-specific hybridization. In crop improvement programmes, inter varietal hybridization is the

most commonly used. The inter varietal crosses may be simple or complex depending upon the number of parents involved.

Simple cross: Two parents are crossed to produce the F1. The F1 is selfed t produce F2.
Complex cross: More than two parents are crossed to produced the hybrid, which is then used to produced F2 such a cross is also known as convergent cross, because this crossing programme aims at converging i.e. bringing together genes from several parents into a single hybrid.



In breeding of highly improved self-pollinated crops like wheat and rice, complex crosses are common practices today. As crop improvement progresses the crop varieties would accumulate more and more favourable genes.

2. Distant hybridization: It includes cross between different species of the same genus or different genera. When two species of the same genus are crossed it is known as interspecific hybridization. But when they belong to two different genera, it is termed as inter generic hybridization. Generally the objective of such crosses is to transfer one or few simply inherited characters like disease resistance to a crop species.

For e.g.

i. C O 31. Rice variety was developed from the cross, *Oryza sativa* var indica x O. perennis.
ii. All the present day sugarcane varieties have been developed from complex crosses between *Saccharum officinarum* (Noble cane), *S.barberi* (Indian cane) and *Saccharum* sp. like *S. spontaneous*.
iii. The improvement in length of Indian cotton (*Gossypium arboreum*) has been brought about by crossing it with American cultivated cotton (G. hirsutum).
iv. Intergenric cross line (wheat) Triticum sp. x Secale cereale (Rye) - Triticale

Useful for developing new crop species like Triticale.

Procedure of hybridization

There are seven steps involved in hybridization

1. **Choice of parents:** The choice of parents mainly depends upon the objectives of breeding programme. In addition to other objectives, increased yields are always an objective of the breeder. Therefore, at least one of the parents involved in a cross should be a well adapted and proven variety in the area, for which the new variety in being developed. The other variety should be having characters that are absent in this variety. Some parents produce superior F1s and F2s, while other do not. This property of the parent is known as "combining ability". The combining ability of the parents may serve as a useful guide in selection of parents for hybridization programme. Thus the choice of parents is the basic step in a hybridization programme and often more than anything else, determines its success or failure.

2. **Evaluation of parents:** If the performance of parents in the area, where breeding is to be done is not known, it should be determined, particularly for the characters they are expected to contribute and for disease resistance. New strains should also be checked for mechanical mixture and for heterozygosity. If it is suspected to be heterozygosity, it may be necessary to self-pollinate a parent for one or more generations.

3. **Emasculation:** The removal of stamens or anthers or the killing of pollen grains of a flower without affecting in any way the female reproductive organ is known as emasculation. The purpose of emasculation is to prevent self-fertilization in the flowers of the female parent. In dioecious plants, male plants are removed, while in monoecious species the male flowers are removed to prevent self-pollination. But emasculation is essential in bisexual flowers. The various techniques of emasculation are, hand emasculation, suction method, hot weather emasculation, alcohol treatment, cold treatment, genetic emasculation and use of gametocides.

4. **Bagging:** Immediately after emasculation, the flowers or the inflorescence are enclosed in suitable bags of appropriate size to prevent random cross-pollination. In cross-pollinated crops, like maize, the male flowers are also bagged to maintain the purity of pollen used for pollination. Butter paper bags are the most commonly used.

5. **Tagging:** The emasculated flowers are tagged just after bagging. The tags are attached to the flower or the inflorescence with the help of thread. The following information is recorded on the tags with the carbon pencil.

a. Date of emasculation b. Date of pollination c. Name of the female and male parent.

6. **Pollination:** The two important operations that determine the amount of seed set in hybridization are emasculation and pollination. In case of pollination, mature fertile and viable pollen should be placed on a receptive stigma to bring about fertilization. It

is advisable that fresh pollen from mature anthers should be used for pollination.

7. **Harvesting and storing the F1 seeds:** The crossed head or pods should be harvested and threshed. The seeds should be dried and properly stored to protect them from storage pests. The seeds from each cross should be kept separately and preferably kept with labels.

Introgression: Transfer of a few genes from one species into the full diploid chromosome complement of another species.

POLY PLOIDY IN PLANT BREEDING

The somatic chromosomes number of any species, whether diploid or polyploid is designated as ' 2n' and the chromosomes number of gametes in denoted as 'n'. An individual carrying the gametic chromosome number 'n' is known as 'haploid'. A monoploid on the other hand, has the basic chromosome number X. In diploid species n=X. One 'X' constitutes a genome or chromosome complement.

Individuals carrying chromosome number other than diploid number are known as heteroploids.

The change in chromosome number may involve once or few chromosomes of the genome. This is known as aneuploidy.
Aneuploidy - One or a few for chromosome extra of missing from 2n (2n -1 or few)

Nullisomic - One chromosome pair missing (2n-2)
Monosomic- One chromosome missing (2n-1)
Double monosomic-One chrosome from each of two different pairs missing (2n-1-1)
Trisomic - (2n+1)
Double Trisomic (2n+1+1)
Tetrasonic (extra one pair) (2n+2)

Application in crop improvement
1. Aneuploids are useful in studies on the effects of loss or gains of an entire chromosome or a chromosome are on the phenotype of an individual.
2. Aneuploids are useful in locating a linkage group and a gene to a particulars chromosome.
3. Study of Aneuploids has shown the homeology between A, B and D genomes of wheat.
4. Aneuploids are useful in identifying the chromosomes involved in translocations.
5. They are useful in the production of substitution lines. It is useful for transfer of genes carried by specific chromosomes of a variety into another one.

Autopolyploid

Euploidy is more commonly known as ployploidy. When all the genomes present in a polyploid species are identical, it is known as 'autopolyploid'. In case of allopolyploids, two or more distinct genomes.

In autopolyploidy are included monoploidy, triploidy tetraploidy and higher levels of ploidy. Autoploidy are directly or indirectly through chromose doubling.

Origin and production of doubled chromosome numbers
1. Due to treatment with physical agents
2. Regeneration invitro
3. Colchicine treatment
4. Chemical agents

Application of autoploidy in crop improvement

Monoploids and haploids

Monoploids are weaker than diploids and are little agricultural value directly.
1. They are used for developing homozygous diploid lines, following chromosome doubling is two years. This greatly reduces the time and labour required for the isolation of inbred and pure lines.
2. They may be useful in the isolation of mutants.
3. Haploid derived diploids may be expected to be more efficient than that based on zygote derived diploid.

Haploids occur spontaneously in low frequencies may be induced from pollen grains through allus formation or embryo production and by chromosome elimination in certain interspecific crosses.

Triploids: These are by hybridization between tertraploid and diploid strains. They are generally highly sterile. This feature is useful in the production of seedless watermelons. In certain species they may be more vigorous than the normal diploids.

Seedless watermelons are grown commercially in Japan. They are produced by crossing tetraploid (4x used as female) and diploid (2X, used as male) lines, since the reciprocal cross (2X x 4X) is not successful. Triploid plants do not produce true seeds almost all the seeds are small. For good fruit setting, pollination is essential. For this purpose diploid lines are planted in the ratio 1 diploid: 5 triploid plants.

Triploid sugarbeet (*Beta vulgaris*): Produce large roots and more sugar per unit area than diploids, while tetraploids produce smaller roots and lower yield than diploids. Apparently, 3X is the optimum level of ploidy in sugar beets. Triploid seeds maybe produced is one of the following two ways.
1. Using 4 x plants as female and 2X as male
2. Using 2x as female and 4x as male

Commercial triploid sugarbeet seed is produced by inter planting 4 x and 2x lines in the ratio of 3:1 and the seeds from both 4x and 2x plants is harvested. This seed consists of about 75% triploid

(3x) seeds. Triploid sugarbeet may give 10-15% higher yields than diploids.

Triploid tea: Produces larger shoots and more biomass yield, more cured leaf per unit area and more drought tolerant to drought than diploid.

Tetraploid: Autoploids have been produced in large number of crops species. They may be useful in one of the following ways; in breeding, improving quality, overcoming self-incompatibility, making distant crosses and used directly as varieties.
Some autotetraploids may be superior in some quality characters to their respective diploids. E.g. Tetraploid maize has 43% more carotenoid pigment and vitamin A activity than the diploid.
Some tetraploids may be hardier than diploids.
Auto tetraploidy in able to overcome self-incompatibility in certain cases like tobacco. Certain distant crosses are not successful at the diploid level, but are relatively successful at the autotetraploid level e.g. Brassica.

Autotetraploids are larger in size and are more vigorous than diploids. Autotetraploid varieties of forage crops have been considerably successful. Many ornamentals are autotetraploids with increased flower size with long flowering duration. Autotetraploids have been explored in several crop species but the most successful case in that rye, barley and jowar, where larger grains increased protein content and higher yields are the objective.

Allopolyploidy: Allopolyploids have genomes from two or more species. Several of our crop plants are allopolyploids.

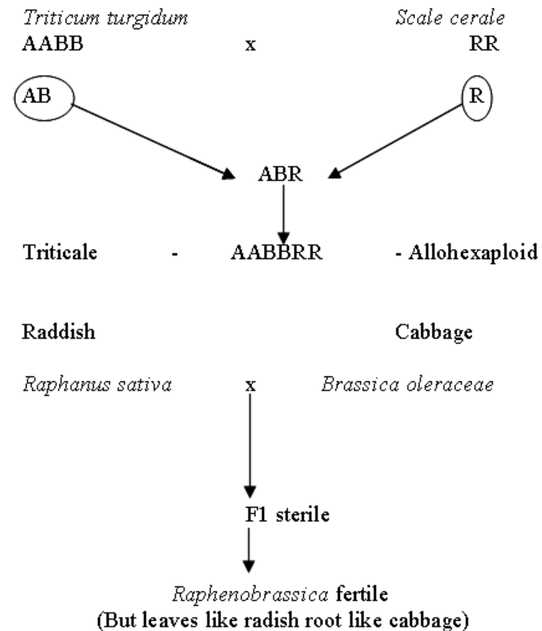
Origin: The present day allopolyploids were most likely produced by chromosome doubling in F1 hybrids between two distinct species belonging to same genus or to different genera. Experimental production of allopolyplids in achieved by doubling the chromosome number of distant hybrids with the help of Colchicine or some other agent. The productions of allopolyploids involve two steps.

1. Production of F1 distant hybrid
2. Chromosome doubling

Experimental production of an allopolyploid

The aim of producing Raphanobrassica was to synthetic a crop species that would combine the root or radish (R. Sativus) and the leaves of the cabbage. Raheno Brassica did combine the characteristic root and shoot systems of the two parental species, but in opposite direction, that is it had leaves like radish and roots like cabbage. On the other hand, Tritical has combined the favourable features of the two parental species i.e the hardiness of rye and yielding ability of wheat.

In general allopolyploids are more vigorous than diploids, but this also is not true in all the cases. Another feature of allopoloyploids is that many of them are Apomictic.



Applications of Allopolyploids in crop improvement: Allopoloyploids have been more successful as crop species than autoploids. Many of our present day crop species are allopoloyploids. It is estimated that, about one third of the angiosperms are polyploids and by far the vast majority of them are allopolyploids.

i. Utilization a bridging species: Amphidiploids serve as a bridge in the transfer of character from one species to related species generally from a wild species to a cultivated species. e.g. Nicotiana, Gossypium
ii. Creation of new crop species: Triticale in the most successful synthetic allopolyploids produced by crossing wheat with Rye.
iii. Widening the genetic base of existing allopolyploids
The genetic base of some natural allopoloids may be narrow and it can be eividenced by introduced more vairability. E.g *Brassica napus*.

Limitations of allopolyploidy

1. The effects of allopolyploidy cannot be predicted. The allopolyploids have some features from both the parental species, but these features may be the undesirable ones. E.g. Raphanobrassica or the desirable ones e.g. Triticale.
2. Newly synthesized alloploids have many defects e.g. low fertility, cytogenetic and genetic instability, other undesirable features etc.,
3. The synthetic allopolyploids have to be improved through extensive breeding at the polyploids level. This involves considerable time, labour and other resources.
4. Only a small proportion of allopolyploids is promising. The synthetic polyploid can be improved through extensive breeding.

UNIT-12: APPLIED BIOLOGY

CLONAL SELECTION

Some agricultural crops and a large number of horticultural crops are asexually propagated. Some common asexually propagated crops are sugarcane, potato, sweet potato colocasia, Discorea (gams), Mentha, Ginger, turmeric, banana etc., almost all the fruit trees.

Segregation and recombination produce new gene combinations due to which the progeny differ from their parents in genotype and phenotype. A sexual reproduction, on the other hand, produces progeny exactly identical to their parents in genotype because the progeny are derived from vegetative cells through mitosis. It preserves the genotype of an individual indefinitely. Any genotype is preserved and maintained through asexual reproduction.

Characteristics of Asexually propagated crops

1. A great majority of them are perennials e.g. sugarcane, fruit trees etc.,
2. Many of them show reduced flowering and seed set.
3. They are invariably cross pollinated
4. These crops are highly heterozygous ands show severe inbreeding depression
5. A vast majority of asexually propagated crops are either polyploids eg., sugarcane, potato.
6. Many species are interspecific hybrids eg. Banana and sugarcane.
7. This crop consists of a large number of clones that is progeny derived from a single plant through asexual reproduction.

CLONE: A clone is a group of plants produced from a single plant through asexual reproduction. Thus asexually propagated crops consists of a large number of clones These crops are also known as clonal crops. All the members of a clone have the same genotype as the parent plant. As a result, they are identical with each other in genotype. Consequently the phenotypic differences within a clone do not have a genetic basis and are purely due to the environmental effects.

Characteristics of a clone

1. All the individuals belonging to a single clone are identical in genotype.
2. The phenotype variation with in a clone is due to the environment only
3. The phenotype of a clone is due to the effects of genotype (G) the environment (E) and the G x E interaction over the population mean (r)
   Phenotype (P) = M+G+E+ GE
4. Theoretically clones are immortal i.e. a clone can be maintained indefinitely through asexual reproduction. But clones usually degenerate due to viral or bacterial infections.
5. Clones are generally, highly heterozygous and show severe loss in vigour due to inbreeding.

Genetic variation within clones: It may arise due to somatic mutation, mechanical mixture and occasional sexual reproduction.

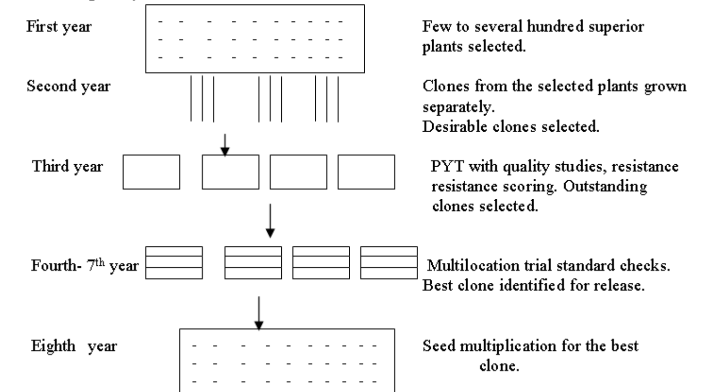
Clonal degeneration: Theoretically, clones are immortal. The loss in vigour and productivity of clones with time is known as clonal degeneration. The clonal degeneration may result from (1) mutation (2) viral diseases and (3) bacterial diseases.
Mutation: It is a recurrent process, it may become a problem over a long period of time
Viral diseases: They are easily transmitted through vegetative propagules. Viruses are perhaps responsible for more cases of clonal degeneration than any other single cause.
Bacterial diseases: By the result of bacterial infections making clonal degeneration.

Methods of improvement of Asexually propagated crops
A single outstanding plant selected form a population forms the basis of a new variety. The breeding behaviour or genotypes of the plant is not important, Since there would be no further sexual reproduction. The outstanding plant may be selected from an old unimproved variety, an improved variety that has become variable or from a population produced by crossing two or more clones. The procedure of selection used for such crops is known as "clonal selection". Since the selected plants are used to produce new clones.

In the earlier stages of clonal selection, when selection is based on single plants or single plots, the emphasis is on the elimination of weak and undesirable plants or clones. The breeder cannot be reasonably hope to identify superior genotype at this stage. In the later stages when replicated trials are the basis of selection, the emphasis is to identify and select the superior clones.

Merits of clonal selection

1. It is the only method of selection applicable to clonal crops. It avoids inbreeding depression and preserves the gene combination present in clones.
2. Clonal selection can be combined with hybridization to generation necessary variability for selection.
3. The selection scheme is useful in maintaining the purity of clones.



Demerits of clonal selection

1. This method utilizes the natural variability already present in the population. It is not devised to generate variability.
2. Sexual reproduction is necessary for the creation of variability through hybridization.

WIDE HYBRIDIZATION

Hybridization between individuals from different species, belonging to the same genus or two different genera, is termed as distant or wide hybridization

When individuals being crossed belong to species from two different genera, it is referred as intergeneric hybridization. When individuals from two distinct species of the same genes are crossed it is known as interspecific hybridization.

BARRIERS FOR CROSSSING

Several wild species are not crossable with the commerical cultivars due to various isolation barriers. The isolation barrier may be pre-zygotic that prevents fertilization and zygote formation or postzygotic in which fertilization takes place, hybrid zygotes are formed but they are inviable or give rise to weak or sterile hybrids.

Pre zygotic barriers

1. Failure of pollen germination
2. Slow growth of the pollen tube
3. Inability of the pollen tube to reach the ovary
4. Arrest of pollen tube in the style, ovary and ovule.

These are due to genic differences or differences in ploidy between species.

Post –Zygotic barriers

1. Hybrid inviability and weakness leading to chromosome elimination, lethality and embryo abortion.
2. Hybrid sterility
3. Hybrid breakdown with weak or sterile individuals in F2 owing to recombination of the gene complements of the parental species.

Techniques to overcome isolation barriers

Pre zygotic barriers can be overcome by the following techniques.

i. Mechanical removal of style followed by pollination of the exposed stylar end
ii. Bud pollination.
iii. Use of growth hormones such as GA3, IAA, NAA etc.,
iv. Invitro fertilization
v. Protoplast fusion
vi. Chromosome doubling before hybridization.
vii. Adopting bridging species technique

Post zygotic barriers can be overcome by

i. Chromosome doubling (Amphidiploidy)
ii. Back crossing
iii. Embryo rescue
iv. Tissue culture techniques.

Breeding procedure for wide hybridization

1. **Backcross breeding:** When interspecific crosses between two species of varying ploidy level are made invariably the hybrids are sterile. By chromosome doubling with application of Colchicine, amphidiploids can be produced. Such amphidiploids are fertile. Cultivated tobacco, Nicotiana tabacum (2n=24) which crossed to N. glutinosa (2n=12) produced sterile F1 and by chromosome doubling an amphidiploid N. digluta (2n=36) was produced. This was reasonably fertilize with N. tabacum. By repeated backcrossing, a mosaic resistant line with 2n=24 was developed.

2. **Amphidiploidy:** The manmade cereal Triticale is an intergenic allopolyploid combining Triticum aestivum (Wheat 2n=42) and Secale cereale (rye –2n-14). Rapanobrassica was synthesised by crossing Raphanus sativus, radish (2n-18) and Brassica olereaceae cabbage (2n-20).

3. **Bridging species Technique:** When direct crosses between two species are difficult, a third species is used in such crosses. Hexaploid wheat, Triticum aestivum (2n-42) does not cross with diploid species. When T. dicoccoides (2n=28) is crossed to Aegilops umbellulata (2n=14) and an amphidiploid was produced it crossed with T. aestivum (2n=42). Nicotiana sylvestrin (2n=24) is the bridging species to transfer nematode resitance from N. repanda (2n-48) to N. tacbaccum (2n=48).

4. **Alien-addition and Alien substitution lines:** By crossing two unrelated species of different ploidy level and doubling the chromosome number of the sterile F1, fertile amphidiploids are obtained. The amphidiploid in backcrossed to the cultivated species repeatedly twice or thrice and them selfed. In the selfed progeny, plants with one chromozome pair from the donor species in addition to the normal diploid chromosome of the parent species may be present and the y are called alien –addition lines. In certain other plants, one chromosome pair of the donor species may substitute one chromosome pair of the parent species when they are called alien substitution lines. By adopting the above methods, mosaic resistance from Nicotiana glutinosa (2n=24) was transferred to N. tabaccum (2n-48) by alien addition (2n=48+2) and alien substitution (2n=48-2+2).

MUTATION BREEDING

Mutation is a sudden heritable change in a characteristic of an organism. Mutations produced by changes in the base sequences of genes are known as gene or point mutations. The term mutations was introduced by Hugo de Vries in 1900

Spontaneous mutation: Mutations occur in natural populations (without any treatment by man) at a low rate. These are known as spontaneous mutations. The frequency of natural mutations is generally one in ten lacs.

Induced mutation: Mutations may be artificially induced by a treatment with certain physical or chemical agents. Such mutations are known as induced mutations, and the agents used for producing them are termed as mutagen. The utilization of induced mutations for crop improvement in known as mutation breeding. Induced mutations have a great advantage over the spontaneous ones, they occur at a relatively higher frequency so that it is practical to work with them.

Characteristics of mutations

1. Mutations are generally recessive, but dominant mutations also occur.
2. Mutations are generally harmful to the organism, but a small proportion (0.1 percent) of them are beneficial.
3. Mutations are random i.e., they may occur in any gene. However some genes show higher mutations rate than others.
4. Mutations are recurrent, that is the same mutations may occur again and again.
5. Induced mutations commonly show pleiotropy, often due to mutations in closely linked genes.

Mutagen: Agents used for induction of mutations are known as mutagens. The mutagens are classified into two groups, physical and chemical mutagens.

Physical mutagen: The mutations inducing radiation's are of two kinds.
i. Ionizing radiation ii. Non ionizing radiation.

Non-ionizing radiation: When compounds absorb energy from non-ionizing radiations, their electrons are raised to higher energy levels (excitation). It results in increased reactivity of the affected molecules leading to mutations.

The only one non-ionizing radiation capable of inducing mutations in ultra violet light. U-V radiation can be obtained from a mercury vapour lamp. U V rays have much longer wave lengths (about 2500 Angstroms)

Chemical mutagens

1. Alkylating agents – eg., EMS (Ethyl Methane Sulphonate) MMS (Methyl Methane Sulphonate)
2. Acridine dyes eg., Ethidium Bromide, acriflavine proflavine
3. Base analogue – eg. 5 Bromouracil, 5 – Chlorouracil
4. Others - eg., Nitrous acid, hydoxyl amine , sodium azide.

Ionizing radiation: Alpha, Beta and gamma rays of radio active substances, Neutrons and X rays are examples of ionizing radiation. When ionizing radiations passes through matter, atoms, absorb energy from them and lose electrons. When an atom becomes ionized, molelcule of which it is a part undergoes chemical change. If the molelcule is a gene and if this changed gene duplicate its new pattern, the result of the change is a mutation.

Gamma garden: The gamma garden of the Indian Agricultural Research Institute, New Delhi is a three-acre plot. In the centre of this field, there is a large source of radioactive cobalt (CO^{60}) and plants in pots are kept at varying distances from the source, irradiated and studied. It is used for irradiating whole plants during different stages and for varying durations. Gamma rays are of shorter wavelength than X-rays and hence are penetrating. Gamma rays are commonly measured in terms of Roentgen units (r).

Mutagenesis: Treating a biological material with a mutagen in order to induce mutations is known as mutagenesis. Exposure of a biological material to radiation (x-rays, gamma rays etc.,) is known as irradiation.

Part of the plant to the treated: Seeds, pollen grains, or vegetative propagules (buds and cuttings) may be used for mutagenesis. Chemical mutagens are best used with seeds.

Dose of the mutagen: Mutagen treatments reduce germination, growth rate, vigour and fertility (pollen as well as ovule). An optimum dose in the on which produces the maximum frequency of mutations and causes the minimum killing. LD 50 in that dose of a mutagen which would kill 50 percent of the treated individuals. LD 50 value varies with the crop species and with the mutagen used. A preliminary experiment is generally conducted to determine the suitable mutagen dose. Dose of the mutagen may be varied by varying the intensity or the treatment time. Intensity in the case of chemical mutagens may be varied by changing the concentration of mutagens.

Mutagen treatment: The selected plant part is exposed to the desired mutagen dose. The case of chemical mutagens, seeds are usually presoaked for a few hours, to initiate metabolic activities, exposed to the desired mutagen and then washed in running tap water to remove the mutagen present in them. The treated seeds are immediately planted in the field to raise the M1 generation. M2, M3, M4 etc are the subsequent generations derived from M1, M2, M3 etc., plants through selfing.

DOSIMETRY

The dose of X ray and gamma rays is measured roentgen (r) units which is defined as the quantity of radiation whose associated corpuscular emission per 0.001293 gm of air produces in air, ions carrying one esu of electricity per cc of air at NTP. The roentgen is expressed in mt. (0.001 r) and Kr (1000 r).

Radiation Absorbed Dose (RAD): One rad corresponds to the absorption of energy of 100 ergs / gm of tissue.

Radiation equivalent physical (REP): It has been introduced to measure the ionization by X, B and r particles. It corresponds to the amount of any kind of radiation producing the same number of ion pairs of energy in tissue of water as are produced by one r of x or gamma radiation.

Curie (C): It is defined as the activity of a radioactive isotope in which 3.7 x 10^{10} disintegration takes place per second.

Molar (M): One molar solution is the one molecular weight of the chemical in one litre of water.

F3. ANIMAL BREEDING

Animal breeding is the selective mating of animals to increase the possibility of obtaining desired traits in the offspring. It has been performed with most domesticated animals, especially cats and dogs, but its main use has been to breed better agricultural stock. The more modern techniques involve a wide variety of laboratory methods, including the modification of embryos, sex selection, and genetic engineering. These procedures are beginning to supplant traditional breeding methods, which focus on selectively combining and isolating livestock strains. In general, the most effective strategy for isolating traits is by selective inbreeding; but different strains are sometimes crossed to take advantage of hybrid vigor and to forestall the negative results of inbreeding, which include reduced fertility, low immunity, and the development of genetic abnormalities.

The genetic basis of animal breeding

Breeders engage in genetic "experiments" each time they plan a mating. The type of mating selected depends on the goals. To some breeders, determining which traits will appear in the offspring of a mating is like rolling the dice—a combination of luck and chance. For others, producing certain traits involves more skill than luck—the result of careful study and planning. Breeders have to understand how to manipulate genes within their breeding stock to produce the kinds of dogs they want. They have to first understand dogs as a species, then dogs as genetic individuals.

Once the optimal environment for raising an animal to maturity has been established (i.e., the proper nutrition and care has been determined) the only way to manipulate an animal's potential is to manipulate its genetic information. In general, the genetic information of animals is both diverse and uniform: diverse, in the sense that a population will contain many different forms of the same gene (for instance, the human population has 300 different forms of the protein hemoglobin); and uniform, in the sense that there is a basic physical expression of the genetic information that makes, for instance, most goats look similar to each other.

In order to properly understand the basis of animal breeding, it is important to distinguish between genotype and phenotype. Genotype refers to the information contained in an animal's DNA, or genetic material. An animal's phenotype is the physical expression of its genotype. Although every creature is born with a fixed genotype, the phenotype is a variable influenced by many factors in the animal's environment and development. For example, two cows with identical genotypes could develop quite different phenotypes if raised in different environments and fed different foods.

The close association of environment with the expression of the genetic information makes animal breeding a challenging endeavor, because the physical traits a breeder desires to selectively breed for cannot always be attributed entirely to the animal's genes. Moreover, most traits are due not just to one or two genes, but to the complex interplay of many different genes.

DNA consists of a set of chromosomes; the number of chromosomes varies between species (humans, for example, have 46 chromosomes). Mammals (and indeed most creatures) have two copies of each chromosome in the DNA (this is called diploidy). This means there are two copies of the same gene in an animal's DNA. Sometimes each of these will be partially expressed. For example, in a person having one copy of a gene that codes for normal hemoglobin and one coding for sickle-cell hemoglobin, about half of the hemoglobin will be normal and the other half will be sickle-cell. In other cases, only one of the genes can be expressed in the animal's phenotype. The gene expressed is called dominant, and the gene that is not expressed is called recessive. For instance, a human being could have two copies of the gene coding for eye color; one of them could code for blue, one for brown. The gene coding for brown eyes would be dominant, and the individual's eyes would be brown. But the blue-eyes gene would still exist, and could be passed on to the person's children.

Most of the traits an animal breeder might wish to select will be recessive, for the obvious reason that if the gene were always expressed in the animals, there would be no need to breed for it. If a gene is completely recessive, the animal will need to have two copies of the same gene for it to be expressed (in other words, the animal is homozygous for that particular gene). For this reason, animal breeding is usually most successful when animals are selectively inbred. If a bull has two copies of a gene for a desirable recessive trait, it will pass one copy of this gene to each of its offspring. The other

copy of the gene will come from the cow, and assuming it will be normal, none of the offspring will show the desirable trait in their phenotype. However, each of the offspring will have a copy of the recessive gene. If they are then bred with each other, some of their offspring will have two copies of the recessive gene. If two animals with two copies of the recessive gene are bred with each other, all of their offspring will have the desired trait.

There are disadvantages to this method, although it is extremely effective. One of these is that for animal breeding to be performed productively, a number of animals must be involved in the process. Another problem is that undesirable traits can also mistakenly be selected for. For this reason, too much inbreeding will produce sickly or unproductive stock, and at times it is useful to breed two entirely different strains with each other. The resulting offspring are usually extremely healthy; this is referred to as "hybrid vigor." Usually hybrid vigor is only expressed for a generation or two, but crossbreeding is still a very effective means to combat some of the disadvantages of inbreeding.

Another practical disadvantage to selective inbreeding is that the DNA of the parents is altered during the production of eggs and sperm. In order to make eggs and sperm, which are called gametes, a special kind of cell division occurs called meiosis, in which cells divide so that each one has half the normal number of chromosomes (in humans, each sperm and egg contains 23 chromosomes). Before this division occurs, the two pairs of chromosomes wrap around each other, and a phenomenon known as crossing over takes place in which sections of one chromosome will be exchanged with sections of the other chromosome so that new combinations are generated. The problem with crossing over is that some unexpected results can occur. For instance, the offspring of a bull homozygous for two recessive but desirable traits and a cow with "normal" genes will all have one copy of each recessive gene. But when these offspring produce gametes, one recessive gene may migrate to a different chromosome, so that the two traits no longer appear in one gamete. Since most genes work in complicity with others to produce a certain trait, this can make the process of animal breeding very slow, and it requires many generations before the desired traits are obtained—if ever.

Economic considerations

There are many reasons why animal breeding is of paramount importance to those who use animals for their livelihood. Cats have been bred largely for aesthetic beauty; many people are willing to pay a great deal of money for a Siamese or Persian cat, even though the affection felt for a pet has little to do with physical appearance. But the most extensive animal breeding has occurred in those areas where animals have been used to serve specific practical purposes. For instance, most dog breeds are the result of a deliberate attempt to isolate traits that would produce better hunting and herding dogs (although some, like toy poodles, were bred for traits that would make them desirable pets). Horses have also been extensively bred for certain useful qualities; some for size and strength, some for speed. But farm animals, particularly food animals, have been the subject of the most intensive breeding efforts.

The physical qualities of economic importance in farm animals vary for each species, but a generalized goal is to eliminate the effects of environment and nutrition. An ideal strain of milk cow, for instance, would produce a large amount of high-quality milk despite the type of food it is fed and the environment in which it is reared. Thus, animals are generally all bred for feed efficiency, growth rate, and resistance to disease. However, a pig might be bred for lean content in its meat, while a hen would be bred for its laying potential. Many cows have been bred to be hornless, so they cannot inadvertently or deliberately gore each other.

Although maximum food production is always a major goal, modern animal breeders are also concerned about nutritional value and the ability of animals to survive in extreme environments. Many parts of the world are sparsely vegetated or have harsh climatic conditions, and a high efficiency producer able to endure these environments would be extremely useful to the people who live there. In addition, many people of industrialized countries are concerned not about food availability but about the quality of this food; so breeders seek to eliminate the qualities that make meat or milk or eggs or other animal products unhealthy, while enhancing those qualities that make them nutritious.

Modern methods in biotechnology

Although earlier animal breeders had to confine themselves to choosing which of their animals should mate, modern technological advances have altered the face of animal breeding, making it both more selective and more effective. Techniques like genetic engineering, embryo manipulation, artificial insemination, and cloning are becoming more and more refined. Some, like artificial insemination and the manipulation of embryos to produce twins, are now used habitually. Others, such as genetic engineering and cloning, are the subject of intense research and will probably have a great impact on future animal breeding programs.

Artificial insemination

Artificial insemination is the artificial introduction of semen from a male with desirable traits into females of the species to produce pregnancy, and is useful because a far larger number of offspring can be produced than would be possible if the animals were traditionally bred. Because of this, the value of the male as breeding stock can be determined much more rapidly, and the use of many different

females will permit a more accurate evaluation of the hereditability of the desirable traits. In addition, if the traits produced in the offspring do prove to be advantageous, it is easier to disperse them within an animal population in this fashion, as there is a larger breeding stock available. One reason artificial insemination has been an extremely important tool is that it allowed new strains of superior stock to be introduced into a supply of animals in an economically feasible fashion.

The process of artificial insemination requires several steps. Semen must be obtained and effectively diluted, so that the largest number of females can be inseminated consistent with a high probability of pregnancy. The semen must be properly stored so that it remains viable. The females must be tested before the sample is introduced to ensure they are fertile, and following the procedure, they must be tested for pregnancy to determine its success. All these factors make artificial insemination more expensive and more difficult than traditional breeding methods, but the processes have been improved and refined so that the economic advantages far outweigh the procedural disadvantages, and artificial insemination is the most widely applied breeding technique.

Embryo manipulation

In order to understand the techniques of embryo manipulation, it is important to understand the early stages of reproduction. When the egg and sperm unite to form a zygote, each of the parents supply the zygote with half of the chromosomes necessary for a full set. The zygote, which is a single cell, then begins to reproduce itself by the cellular division process called mitosis, in which each chromosome is duplicated before separation so that each new cell has a full set of chromosomes. This is called the morula stage, and the new cells are called blastomeres. When enough cells have been produced (the number varies from species to species), cell differentiation begins to take place. The first differentiation appears to be when the blastocyst is formed, which is an almost hollow sphere with a cluster of cells inside; and the differentiation appears to be between the cells inside, which become the fetus, and the cells outside, which become the fetal membranes and placenta. However, the process is not entirely understood at the present time and there is some variation between species; so it is difficult to pinpoint the onset of differentiation, which some scientists believe occurs during blastomere division.

During the first stages of cell division, it is possible to separate the blastomeres with the result that each one develops into a separate embryo. Blastomeres with this capability are called totipotent. The purpose of this ability of a single blastomere to produce an entire embryo is probably to safeguard the process of embryo development against the destruction of any of the blastomeres. In theory, it should be possible to produce an entire embryo from each blastomere (and blastomeres are generally totipotent from the four to eight cell stage), but in practice it is usually only possible to produce two embryos. That is why this procedure is generally referred to as embryo splitting rather than cloning, although both terms refer to the same thing (cloning is the production of genetically identical embryos, which is a direct result of embryo splitting).

Interestingly enough, although the embryos produced from separated blastomeres usually have fewer cells than a normal embryo, the resulting offspring fall within the normal range of size for the species.

It is also possible to divide an embryo at other stages of development. For instance, the time at which embryo division is most successful is after the blastocyst has formed. Great care must be taken when dividing a blastocyst, since differentiation has already occurred to some extent, and it is necessary to halve the blastocyst very precisely.

Another interesting embryonic manipulation is the creation of chimaeras. These are formed by uniting two different gametes, so that the embryo has two distinct cell lineages. Chimaeras do not combine the genetic information of both lineages in each cell. Instead, they are a patchwork of cells containing one lineage or the other. For this reason, the offspring of chimaeras are from one distinct genotype or the other, but not from both. Thus chimaeras are not useful for creating new animal populations beyond the first generation. However, they are extremely useful in other contexts. For instance, while embryo division as described above is limited in the number of viable embryos that can be produced, chimaeras can be used to increase the number. After the blastomeres are separated, they can be combined with blastomeres of a different genetic lineage. It has been found that with the additional tissue, the survival rate of the new embryos is more favorable. For some reason only a small percentage of the resulting embryos are chimaeric; this is thought to be because only one cell lineage develops into the cells inside the blastocyst, while the other lineage forms extra-embryonic tissue. It is believed that the more advanced cells are more likely to form the inner cells.

Another application of chimaeras could be for breeding endangered species. Because of the different biochemical environments in the uterus, and the different regulatory mechanisms for fetal development, only very closely related species are able to bear each other's embryos to term. For example, when a goat is implanted with a sheep embryo or the other way around, the embryo is unable to develop properly. This problem can perhaps be surmounted by creating chimaeras in which the placenta stems from the cell lineage of the host species. The immune system of an animal attacks tissue it recognizes as "non-self," but it is possible that the mature chimaeras would be

compatible with both the host species and the target species, so that it could bear either embryo to term. This has already proved to be true in studies with mice.

A further technique being developed to manipulate embryos involves the creation of uniparental embryos and same-sex matings. In the former case, the cell from a single gamete is made to go through mitosis, so that the resulting cell is completely homozygous. In the latter case, the DNA from two females (parthogenesis) or two males (androgenesis) is combined to form cells that have only female- or male-derived DNA. These zygotes cannot be developed into live animals, as genetic information from male and female derived DNA is necessary for embryonic development. However, these cells can be used to generate chimaeras. In the case of parthogenetic cells, these chimaeras produce viable gametes. The androgenetic cells do not become incorporated in the embryo; they are used to form extra-embryonic tissue, and so no gametes are recovered.

Aside from these more ambitious embryo manipulation endeavors, multiple ovulation and embryo transfer (MOET) could soon become a useful tool. MOET is the production of multiple embryos from a female with desirable traits, which are then implanted in the wombs of other females of the same species. This circumvents the disadvantages of breeding from a female line (which are that a female can only produce a limited number of offspring due to the time investment and physical rigors of pregnancy). At the present time, MOET is still too expensive for commercial application, but is being applied experimentally.

Genetic engineering

Genetic engineering is being implemented to create animals that have had a new gene inserted directly into their DNA. These animals are called transgenic. The procedure involves microinjection of the desired gene into the nucleus of fertilized eggs. It has been found that in many cases, but with varying rates of success, the new gene is reproduced in all developing cells, and the gene can be transcribed (which means the information contained in the gene can be read and utilized by the cell). This is a startling breakthrough in animal breeding endeavors, because it means a specific trait can be incorporated into a population in a single generation, rather than the several generations this takes when conventional breeding techniques are used.

However, there are some serious limitations to the procedure. The first of these has to do with the manner in which many genes work together to produce most traits. In fact, there are very few traits a breeder would like to include in an animal population that involve only one or two genes. Although it might some day be possible to incorporate any number of genes into an embryo's DNA, the complex interplay of genes is not understood very well, and the process of identifying all of the genes related to a desired trait is costly and time-consuming.

Another problem in the production of transgenic animals is that they pass their modified DNA on to their offspring with varying success rates and unpredictable results. In some cases, the new gene is present in the offspring but it is not utilized. The new gene may also be altered or rearranged in some way, probably during the process of gamete production.

These factors have made it difficult to successfully produce a transgenic strain of animals. However, with further research into the mechanism by which the gene is incorporated into the genome, and by successfully mapping the target animal genome and identifying the genes responsible for various traits, genetic engineering will no doubt become a major tool for improving animal strains.

Sex selection

It would be extremely useful if a breeder were able to predetermine the sex of each embryo produced, because in many cases one sex is preferred. For instance, in a herd of dairy cows or a flock of laying hens, females are the only commercially useful sex. When the owner of a dairy herd has inseminated a cow at some expense, this issue becomes more crucial. In some cases, an animal is being bred specifically for use as breeding stock; in this case, it is far more useful to produce a male that can be bred with multiple females than a female, which can only produce a limited number of offspring.

Whether or not an animal is male or female is determined by its sex chromosomes, which are called X and Y chromosomes. An animal with two X chromosomes will develop into a female, while an animal with one X and one Y chromosome will become a male. In mammals, the sex of the offspring is almost always determined by the male parent, because the female can only donate an X chromosome, and it is the presence or absence of the Y chromosome that causes maleness (this is not true in, for instance, birds; in that case it is the female who has two different sex chromosomes). The problem in sex selection is to separate the Y-carrying sperm from the X-carrying sperm. Thus far, attempts to do so have been largely unsuccessful or too expensive for commercial application, but the economic advantages make this an area of intense research, and it is quite probable that an efficient and cost-effective method will soon be developed.

F4. Marker assisted selection

Marker assisted selection or marker aided selection (MAS) is a process whereby a marker (morphological, biochemical or one based on DNA/RNA variation) is used for indirect selection of a genetic determinant or determinants of a trait of interest (i.e. productivity, disease resistance, abiotic stress tolerance, and/or quality). This process is used in plant and animal breeding.

Marker types

A marker may be:

- **Morphological** - First markers loci available that have obvious impact on morphology of plant. Genes that affect form, coloration, male sterility or resistance among others have been analyzed in many plant species. Examples of this type of marker may include the presence or absence of awn, leaf sheath coloration, height, grain color, aroma of rice etc. In well-characterized crops like maize, tomato, pea, barley or wheat, tens or even hundreds of such genes have been assigned to different chromosomes.
- **Biochemical-** A gene that encodes a protein that can be extracted and observed; for example, isozymes and storage proteins.
- **Cytological** - The chromosomal banding produced by different stains; for example, G banding.
- **Biological-** Different pathogen races or insect biotypes based on host pathogen or host parasite interaction can be used as a marker since the genetic constitution of an organism can affect its susceptibility to pathogens or parasites.
- **DNA-based and/or molecular**- A unique (DNA sequence), occurring in proximity to the gene or locus of interest, can be identified by a range of molecular techniques such as RFLPs, RAPDs, AFLP, DAF, SCARs, microsatellites etc.

Sax in 1923 first reported association of a simply inherited genetic marker with a quantitative trait in plants when he observed segregation of seed size associated with segregation for a seed coat color marker in beans (*Phaseolus vulgaris* L.). Rasmusson in 1935 demonstrated linkage of flowering time (a quantitative trait) in peas with a simply inherited gene for flower color.[citation needed]

Gene vs marker

The gene of interest is directly related with production of protein(s) that produce certain phenotypes whereas markers should not influence the trait of interest but are genetically linked (and so go together during segregation of gametes due to the concomitant reduction in homologous recombination between the marker and gene of interest). In many traits genes are discovered and can be directly assayed for their presence with a high level of confidence. However, if a gene is not isolated marker's help is taken to tag a gene of interest. In such case there may be some false positive results due to recombination between marker of interest and gene (or QTL). A perfect marker would elicit no false positive results.

Important properties of ideal markers for MAS

An ideal marker:

- Easy recognition of all possible phenotypes (homo- and heterozygotes) from all different alleles
- Demonstrates measurable differences in expression between trait types and/or gene of interest alleles, early in the development of the organism
- Has no effect on the trait of interest that varies depending on the allele at the marker loci
- Low or null interaction among the markers allowing the use of many at the same time in a segregating population
- Abundant in number
- Polymorphic

Demerits of morphological markers

Morphological markers are associated with several general deficits that reduce their usefulness including:

- the delay of marker expression until late into the development of the organism
- dominance
- deleterious effects
- pleiotropy
- confounding effects of genes unrelated to the gene or trait of interest but which also affect the morphological marker (epistasis)
- rare polymorphism
- frequent confounding effects of environmental factors which affect the morphological characteristics of the organism

To avoid problems specific to morphological markers, the DNA-based markers have been developed. They are highly polymorphic, simple inheritance (often codomimant), abundantly occur throughout the genome, easy and fast to detect, minimum pleiotropic effect and detection is not dependent on the developmental stage of the organism. Numerous markers have been mapped to different chromosomes in several crops including rice, wheat, maize, soybean and several others. Those markers have been used in diversity analysis, parentage detection, DNA fingerprinting, and prediction of hybrid performance. Molecular markers are useful in indirect selection processes, enabling manual selection of individuals for further propagation.

Selection for major genes linked to markers

The major genes which are responsible for economically important characteristics are frequent in the Plant Kingdom. Such characteristics include disease resistance, male sterility, self-incompatibility, others related to shape, color, and architecture of whole plants and are often of mono- or oligogenic in nature. The marker loci which are tightly linked to major genes can be used for selection and are sometimes more efficient than direct selection for the target gene. Such vantages in efficiency may be due for example, to higher expression of the marker mRNA in such cases that the marker is actually a gene. Alternatively, in such cases that the target gene of interest differs between two alleles by a difficult-to-detect single nucleotide polymorphism, an external marker (be it another gene or a polymorphism that is easier to detect, such as a short tandem repeat) may present as the most realistic option.

Situations that are favorable for molecular marker selection

There are several indications for the use of molecular markers in the selection of a genetic trait.

In such situations that:

- the selected character is expressed late in plant development, like fruit and flower features or adult characters with a juvenile period (so that it is not necessary to wait for the organism to become fully developed before arrangements can be made for propagation)
- the expression of the target gene is recessive (so that individuals which are heterozygous positive for the recessive allele can be crossed to produce some homozygous offspring with the desired trait)
- there is requirement for the presence of special conditions in order to invoke expression of the target gene(s), as in the case of breeding for disease and pest resistance (where inoculation with the disease or subjection to pests would otherwise be required). This advantage derives from the errors due to unreliable inoculation methods and the fact that field inoculation with the pathogen is not allowed in many areas for safety reasons. Moreover, problems in the recognition of the environmentally unstable genes can be eluded.
- the phenotype is affected by two or more unlinked genes (epistatis). For example, selection for multiple genes which provide resistance against diseases or insect pests for gene pyramiding.
- The cost of genotyping (an example of a molecular marker assay) is reducing while the cost of phenotyping is increasing[citation needed] particularly in developed countries thus increasing the attractiveness of MAS as the development of the technology continues.

Steps for MAS

Generally the first step is to map the gene or quantitative trait locus (QTL) of interest first by using different techniques and then use this information for marker assisted selection. Generally, the markers to be used should be close to gene of interest (<5 recombination unit or cM) in order to ensure that only minor fraction of the selected individuals will be recombinants. Generally, not only a single marker but rather two markers are used in order to reduce the chances of an error due to homologous recombination. For example, if two flanking markers are used at same time with an interval between them of approximately 20cM, there is higher probability (99%) for recovery of the target gene.

QTL mapping techniques

In plants QTL mapping is generally achieved using bi-parental cross populations; a cross between two parents which have a contrasting phenotype for the trait of interest are developed. Commonly used populations are recombinant inbred lines (RILs), doubled haploids (DH), back cross and F2. Linkage between the phenotype and markers which have already been mapped is tested in these populations in order to determine the position of the QTL. Such techniques are based on linkage and are therefore referred to as "linkage mapping".

Quantitative trait locus (QTL)

Typically, QTLs underlie continuous traits (those traits that vary continuously - the trait could have any value within a range - e.g., height) as opposed to discrete traits (traits that have two or several character values - e.g., eye colour or smooth vs. wrinkled peas used by Mendel in his experiments).

Moreover, a single phenotypic trait is usually determined by many genes. Consequently, many QTLs are associated with a single trait.

A quantitative trait locus (QTL) is a region of DNA that is associated with a particular phenotypic trait - these QTLs are often found on different chromosomes. Knowing the number of QTLs that explains variation in the phenotypic trait tells us about the genetic architecture of a trait. It may tell us that plant height is controlled by many genes of small effect, or by a few genes of large effect.

Another use of QTLs is to identify candidate genes underlying a trait. Once a region of DNA is identified as contributing to a phenotype, it can be sequenced. The DNA sequence of any genes in this region can then be compared to a database of DNA for genes whose function is already known.

In a recent development, classical QTL analyses are combined with gene expression profiling i.e. by DNA microarrays. Such expression QTLs (e-QTLs) describe cis- and trans-controlling elements for the expression of often disease-associated

genes. Observed epistatic effects have been found beneficial to identify the gene responsible by a cross-validation of genes within the interacting loci with metabolic pathway- and scientific literature databases.

QTL mapping

QTL mapping is the statistical study of the alleles that occur in a locus and the phenotypes (physical forms or traits) that they produce. Because most traits of interest are governed by more than one gene, defining and studying the entire locus of genes related to a trait gives hope of understanding what effect the genotype of an individual might have in the real world.

Statistical analysis is required to demonstrate that different genes interact with one another and to determine whether they produce a significant effect on the phenotype. QTLs identify a particular region of the genome as containing a gene that is associated with the trait being assayed or measured. They are shown as intervals across a chromosome, where the probability of association is plotted for each marker used in the mapping experiment.

The QTL techniques were developed in the late 1980s and can be performed on inbred strains of any species.

To begin, a set of genetic markers must be developed for the species in question. A marker is an identifiable region of variable DNA. Biologists are interested in understanding the genetic basis of phenotypes (physical traits). The aim is to find a marker that is significantly more likely to co-occur with the trait than expected by chance, that is, a marker that has a statistical association with the trait. Ideally, they would be able to find the specific gene or genes in question, but this is a long and difficult undertaking. Instead, they can more readily find regions of DNA that are very close to the genes in question. When a QTL is found, it is often not the actual gene underlying the phenotypic trait, but rather a region of DNA that is closely linked with the gene.

For organisms whose genomes are known, one might now try to exclude genes in the identified region whose function is known with some certainty not to be connected with the trait in question. If the genome is not available, it may be an option to sequence the identified region and determine the putative functions of genes by their similarity to genes with known function, usually in other genomes.

Another interest of statistical geneticists using QTL mapping is to determine the complexity of the genetic architecture underlying a phenotypic trait. For example, they may be interested in knowing whether a phenotype is shaped by many independent loci, or by a few loci, and do those loci interact. This can provide information on how the phenotype may be evolving.

Analysis of variance

The simplest method for QTL mapping is analysis of variance (ANOVA, sometimes called "marker regression") at the marker loci. In this method, in a backcross, one may calculate a t-statistic to compare the averages of the two marker genotype groups. For other types of crosses (such as the intercross), where there are more than two possible genotypes, one uses a more general form of ANOVA, which provides a so-called F-statistic. The ANOVA approach for QTL mapping has three important weaknesses. First, we do not receive separate estimates of QTL location and QTL effect. QTL location is indicated only by looking at which markers give the greatest differences between genotype group averages, and the apparent QTL effect at a marker will be smaller than the true QTL effect as a result of recombination between the marker and the QTL. Second, we must discard individuals whose genotypes are missing at the marker. Third, when the markers are widely spaced, the QTL may be quite far from all markers, and so the power for QTL detection will decrease.

Interval mapping

Lander and Botstein developed interval mapping, which overcomes the three disadvantages of analysis of variance at marker loci. Interval mapping is currently the most popular approach for QTL mapping in experimental crosses. The method makes use of a genetic map of the typed markers, and, like analysis of variance, assumes the presence of a single QTL. Each location in the genome is posited, one at a time, as the location of the putative QTL.

Composite interval mapping (CIM)

In this method, one performs interval mapping using a subset of marker loci as covariates. These markers serve as proxies for other QTLs to increase the resolution of interval mapping, by accounting for linked QTLs and reducing the residual variation. The key problem with CIM concerns the choice of suitable marker loci to serve as covariates; once these have been chosen, CIM turns the model selection problem into a single-dimensional scan. The choice of marker covariates has not been solved, however. Not surprisingly, the appropriate markers are those closest to the true QTLs, and so if one could find these, the QTL mapping problem would be complete anyway

Single step MAS and QTL mapping

In contrast to two-step QTL mapping and MAS, a single-step method for breeding typical plant populations has been developed. In such an approach, in the first few breeding cycles, markers linked to the trait of interest are identified by QTL mapping and later the same information in used in the same population. In this approach, pedigree structure are created from families that are created by crossing number of parents (in three-way or

four way crosses). Both phenotyping and genotyping is done using molecular markers mapped the possible location of QTL of interest. This will identify markers and their favorable alleles. Once these favorable marker alleles are identified, the frequency of such alleles will be increased and response to marker assisted selection is estimated. Marker allele(s) with desirable effect will be further used in next selection cycle or other experiments.

Use of MAS for backcross breeding

A minimum of five or six-backcross generations are required to transfer a gene of interest from a donor (may not be adapted) to a recipient (recurrent – adapted cultivar). The recovery of the recurrent genotype can be accelerated with the use of molecular markers. If the F1 is heterozygous for the marker locus, individuals with the recurrent parent allele(s) at the marker locus in first or subsequent backcross generations will also carry a chromosome tagged by the marker.

Marker assisted gene pyramiding

Gene pyramiding has been proposed and applied to enhance resistance to disease and insects by selecting for two or more than two genes at a time. For example in rice such pyramids have been developed against bacterial blight and blast. The advantage of use of markers in this case allows to select for QTL-allele-linked markers that have same phenotypic effect.

G1. Bioremediation

Bioremediation can be defined as any process that uses microorganisms, fungi, green plants or their enzymes to return the environment altered by contaminants to its original condition. Bioremediation may be employed to attack specific soil contaminants, such as degradation of chlorinated hydrocarbons by bacteria. An example of a more general approach is the cleanup of oil spills by the addition of nitrate and/or sulfate fertilisers to facilitate the decomposition of crude oil by indigenous or exogenous bacteria.

1.1 Overview and applications: Naturally-occurring bioremediation and phytoremediation have been used for centuries. For example, desalination of agricultural land by phytoextraction has a long tradition. Bioremediation technology using microorganisms was reportedly invented by George M. Robinson. He was the assistant county petroleum engineer for Santa Maria, California. During the 1960's, he spent his spare time experimenting with dirty jars and various mixes of microbes.

Bioremediation technologies can be generally classified as in situ or ex situ. In situ bioremediation involves treating the contaminated material at the site while ex situ involves the removal of the contaminated material to be treated elsewhere. Some examples of bioremediation technologies are bioventing, landfarming, bioreactor, composting, bioaugmentation, rhizofiltration, and biostimulation.

Not all contaminants, however, are easily treated by bioremediation using microorganisms. For example, heavy metals such as cadmium and lead are not readily absorbed or captured by organisms. The assimilation of metals such as mercury into the food chain may worsen matters. Phytoremediation is useful in these circumstances, as many plants are able to bioaccumulate these toxins in their above-ground parts, which are then harvested for removal. The heavy metals in the harvested biomass may be further concentrated by incineration.

1.2 Genetic engineering approaches: The use of genetic engineering to create organisms specifically designed for bioremediation has great potential. The bacterium Deinococcus radiodurans (the most radioresistant organism known) has been modified to consume and digest toluene and ionic mercury from highly radioactive nuclear waste.

Advantages: There are a number of cost/efficiency advantages to bioremediation, which can be employed in areas that are inaccessible without excavation. For example, hydrocarbon spills (specifically, petrol spills) or certain chlorinated solvents may contaminate groundwater, and introducing the appropriate electron acceptor or electron donor amendment, as appropriate, may significantly reduce contaminant concentrations after a lag time allowing for acclimation. This is typically much less expensive than excavation followed by disposal elsewhere, incineration or other ex situ treatment strategies, and reduces or eliminates the need for "pump and treat", a common practice at sites where hydrocarbons have contaminated groundwater.

1.3 Monitoring bioremediation : The process of bioremediation can be monitored indirectly by measuring the Oxidation Reduction Potential or redox in soil and groundwater, together with pH, temperature, oxygen content, electron acceptor/donor concentrations, and concentration of breakdown products (e.g. carbon dioxide). Table 1 shows the (decreasing) biological breakdown rate as function of the redox potential.

Table 1: Biological breakdown rate as function of redox potential

Process	Reaction	Redox potential (Eh in mV)
aerobic:	$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$	600 — 400
anaerobic:		
denitrification	$2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + 6H_2O$	500 — 200
manganese IV reduction	$MnO_2 + 2e^- + 4H^+ \rightarrow Mn^{2+} + 2H_2O$	400 — 200
iron III reduction	$Fe(OH)_3 + e^- + 3H^+ \rightarrow Fe^{2+} + 3H_2O$	300 — 100
sulfate reduction	$SO_4^{2-} + 8e^- + 10H^+ \rightarrow H_2S + 4H_2O$	0 — -150
fermentation	$2CH_2O \rightarrow CO_2 + CH_4$	-150 — -220

This, by itself and at a single site, gives little information about the process of remediation.

- it is necessary to sample enough points on and around the contaminated site to be able to determine contours of equal redox potential. Contouring is usually done using specialised software, e.g. using Kriging interpolation.
- if all the measurements of redox potential show is that electron acceptors have been used up, it's in effect an indicator for total microbial activity. Chemical analysis is also required to determine when the levels of contaminants and their breakdown products have been reduced to below regulatory limits.

G2. Phytoremediation

Phytoremediation describes the treatment of environmental problems (bioremediation) through the use of plants.

The word's etymology comes from the Greek (phyto) = plant, and Latin "remedium" = restoring balance, or remediating. Phytoremediation consists in depolluting contaminated soils, water or air with plants able to contain, degrade or eliminate metals, pesticides, solvents, explosives, crude oil and its derivatives, and various other contaminants, from the mediums that contain them.

It is clean, efficient, inexpensive and non-environmentally disruptive, as opposed to processes that require excavation of soil.

Various phytoremediation processes

A range of processes mediated by plants are useful in treating environmental problems:

- **Phytoextraction** - uptake and concentration of substances from the environment into the plant biomass.
- **Phytostabilization** - reducing the mobility of substances in the environment, for example by limiting the leaching of substances from the soil.
- **Phytotransformation** - chemical modification of environmental substances as a direct result of plant metabolism, often resulting in their inactivation, degradation (phytodegradation) or immobilization (phytostabilization).
- **Phytostimulation** - enhancement of soil microbial activity for the degradation of contaminants, typically by organisms that associate with roots. This process is also known as rhizosphere degradation.
- **Phytovolatilization** - removal of substances from soil or water with release into the air, sometimes as a result of phytotransformation to more volatile and / or less polluting substances.
- **Rhizofiltration** - filtering water through a mass of roots to remove toxic substances or excess nutrients. The pollutants remain absorbed in or adsorbed to the roots.

Phytoextraction

Phytoextraction (or phytoaccumulation) uses plants to remove contaminants from soils, sediments or water into harvestable plant biomass. Phytoextraction has been growing rapidly in popularity world-wide for the last twenty years or so. Generally this process has been tried more often for extracting heavy metals than for organics. At the time of disposal contaminants are typically concentrated in the much smaller volume of the plant matter than in the initially contaminated soil or sediment. 'Mining with plants', or phytomining, is also being experimented with.

The plants absorb contaminants through the root system and store them in the root biomass and/or transport them up into the stems and/or leaves. A living plant may continue to absorb contaminants until it is harvested. After harvest a lower level of the contaminant will remain in the soil, so the growth/harvest cycle must usually be repeated through several crops to achieve a significant cleanup. After the process, the cleaned soil can support other vegetation.

Two versions of phytoextraction:

- natural hyper-accumulation, where plants naturally take up the contaminants in soil unassisted, and
- induced or assisted hyper-accumulation, in which a conditioning fluid containing a chelator or another agent is added to soil to increase metal solubility or mobilization so that the plants can absorb them more easily.

In many cases natural hyperaccumulators are metallophyte plants that can tolerate and incorporate high levels of toxic metals.

Examples of phytoextraction from soils:

- Arsenic, using the Sunflower (Helianthus annuus), or the Chinese Brake fern ("Pteris spp"], a hyperaccumulator. Chinese Brake fern stores arsenic in its leaves.
- Cadmium and zinc, using alpine pennycress (Thlaspi caerulescens), a hyperaccumulator of these metals at levels that would be toxic to many plants. On the other hand, the presence of copper seems to impair its growth (see table for reference).
- Lead, using Indian Mustard (Brassica juncea), Ragweed (Ambrosia artemisiifolia), Hemp Dogbane (Apocynum cannabinum), or Poplar trees, which sequester lead in its biomass.
- Salt-tolerant (moderately halophytic) barley and/or sugar beets are commonly used for the extraction of Sodium chloride (common salt) to reclaim fields that were previously flooded by sea water.
- Uranium, using sunflowers, as used after the Chernobyl accident.
- Mercury, selenium and organic pollutants such as polychlorinated biphenyls (PCBs) have been removed from soils by transgenic plants containing genes for bacterial enzymes.

Phytostabilization

Phytostabilization focuses on long-term stabilization and containment of the pollutant. For example, the plant's presence can reduce wind erosion, or the plant's roots can prevent water erosion, immobilize the pollutants by adsorption or accumulation, and provide a zone around the roots where the pollutant can precipitate and stabilize. Unlike phytoextraction, phytostabilization mainly focuses on sequestering pollutants in soil near the roots but not in plant tissues. Pollutants become less bioavailable and livestock, wildlife, and human exposure is reduced. An example application of this sort is using a vegetative cap to stabilize and contain mine tailings.

Phytotransformation

In the case of organic pollutants, such as pesticides, explosives, solvents, industrial chemicals, and other xenobiotic substances, certain plants, such as Cannas, render these substances non-toxic by their metabolism. In other cases, microorganisms living in association with plant roots may metabolize these substances in soil or water. These complex and recalcitrant compounds cannot be broken down to basic molecules (water, carbondioxide etc) by plant molecules, and hence the term phytotransformation represents a change in chemical structure without complete breakdown of the compound.

The term "Green Liver Model" is used to describe phytotransformation, as plants behave similar to the human liver when dealing with these xenobiotic compounds (foreign compound /pollutant). After uptake of the xenobiotics, plant enzymes increase the polarity of the xenobiotics by adding functional groups such as hydroxyl groups (-OH). This is known as Phase I metabolism, similar to the way the human liver increases the polarity of drugs and foreign compounds (Drug Metabolism.

While in the human liver, enzymes like Cytochrome P450s are responsible for the initial reactions, in plants enzymes such as nitroreductases carry out the same role. In the second stage of phytotransformation, known as Phase II metabolism, plant biomolecules such as glucose and amino acids are added to the polarized xenobiotic to further increase the polarity (known as conjugation). This is again similar to the processes occurring in the human liver wherein glucuronidation (addition of glucose molecules by the UGT (e.g. UGT1A1) class of enzymes) and glutathione addition reactions occur on reactive centers of the xenobiotic.

Phase I and II reactions serve to increase the polarity and reduce the toxicity of the compounds, although many exceptions to the rule are seen at least in the case of the human liver. The increased polarity also allows for easy transport of the xenobiotic along aqueous channels. In the final stage of phytotransformation (Phase III metabolism), a sequestration of the xenobiotic occurs within the plant. The xenobiotics polymerize in a lignin-like manner and get a complex structure which is sequestered in the plant. This ensures that the xenobiotic is safely stored in the plant, and does not affect the functioning of the plant. However, preliminary studies have shown that these plants can be toxic to small animals (such as snails) and hence plants involved in phytotransformation may need to be maintained in a closed enclosure. The human liver differs from plants in Phase III metabolism, since the liver can transport the xenobiotics into the bile for eventual excretion. Since plants have no excretory mechanisms, they sequester the modified xenobiotics. Hence, the plants reduce toxicity (with exceptions) and sequester the xenobiotics in phytotransformation. Trinitrotoluene phyto-transformation has been extensively researched a transformation pathway has been proposed.

The role of genetics

Breeding programs and genetic engineering are powerful methods for enhancing natural phytoremediation capabilities, or for introducing new capabilities into plants. Genes for phytoremediation may originate from a micro-organism or may be transferred from one plant to another variety better adapted to the environmental conditions at the cleanup site. For example, genes encoding a nitroreductase from a bacterium were inserted into tobacco and showed faster removal of TNT and enhanced resistance to the toxic effects of TNT.

Advantages:

- the cost of the phytoremediation is lower than that of traditional processes both in situ and ex situ
- the plants can be easily monitored
- the possibility of the recovery and re-use of valuable metals (by companies specializing in “phytomining”)
- it is the least harmful method because it uses naturally occurring organisms and preserves the natural state of the environment.

Limitations:

- phytoremediation is limited to the surface area and depth occupied by the roots.
- slow growth and low biomass require a long-term commitment
- with plant-based systems of remediation, it is not possible to completely to prevent the leaching of contaminants into the groundwater (without the complete removal of the contaminated ground which in itself does not resolve the problem of contamination)
  the survival of the plants is affected by the toxicity of the contaminated land and the general condition of the soil.
- possible bio-accumulation of contaminants which then pass into the food chain, from primary level consumers upwards.

Hyperaccumulators and biotic interactions

A plant is said to be a hyperaccumulator if it can concentrate the pollutants in a minimum percentage which varies according to the pollutant involved (for example: more than 1000 mg/kg of dry weight for nickel, copper, cobalt, chromium or lead; or more than 10,000 mg/kg for zinc or manganese. Most of the 215 metal-hyperaccumulating species included in their review hyperaccumulate nickel. They listed 145 hyperaccumulators of nickel (around 300 Ni accumulators are known; see Hyperaccumulators table – 2 : Nickel and its notes), 26 of cobalt, 24 of copper, 14 of zinc, four of Lead, and two of Chromium. This capacity for accumulation is due to hypertolerance, or phytotolerance: the result of adaptative evolution from the plants to hostile environments along multiple generations. Boyd and Martens list 4 biotic interactions that may be affected by metal hyperaccumulation, to which can be added the biofilm as a particular aspect of micorrhizae:

- protection
- Interferences with neighbour plants of different species
- Mutualism (Mycorrhizal associations or micorrhizae, and Pollen and seed dispersal)
- Commensalism
- The biofilm

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Protection

More and more evidence show that the metals in hyperaccumulating plants give them some protection from various bacteria, fungi and / or insects. For instance, with foliar Ni concentrations as low as 93 mg/kg, the larval weight of Spodoptera exigua (Lepidoptera: Noctuidae) (beet army worm) is reduced and time to pupation extended.

The defense against viruses is not always supported. Davis et al. (2001) have compared two close species *S. polygaloides* Gray (Ni hyperaccumulator) and *S. insignis* Jepson (non-accumulator), inoculating them with Turnip mosaic virus. They showed that the presence of nickel weakens the plant's response to the virus.

Circumvention of plants' elemental defences by their predators may occur in three ways:
(1) selective feeding on low-metal tissues,
(2) use of a varied diet to dilute metal-containing food (likely more efficient in large-sized herbivores), and
(3) tolerance of high dietary metal content.

Interferences with neighbour plants of different species

Its likelihood between hyperaccumulators and neighbouring plants was suggested but no mechanism was proposed. Gabrielli et al., and Wilson & Agnew (1992), suggested a decrease in competition experienced by the hyperaccumulators for the litterfall from hyperaccumulators' canopy.

This mechanism mimics allelopathy in its effects, although technically due to redistribution of an element in the soil rather than to the plant manufacturing an organic compound. Boyd et Martens call it ‘‘elemental allelopathy’’ - without the autoxicity problem met in other types of allelopathy (Newman 1978).

Mutualism

Two types of mutualism are considered here, mycorrhizal associations or mycorrhizae, and animal-mediated pollen or seed dispersal.

1 - Mycorrhizal associations or mycorrhizae

There are two types of mycorrhizal fungi: ectomycorrhizae and endomycorrhizae. Ectomycorrhizae form sheaths around plant roots, endomycorrhizae enter cortex cells in the roots.

Mycorrhizae are the symbiotic relationship between a soil-borne fungus and the roots of a plant. Some hyperaccumulators may form mycorrhizae and, in some cases, the latter may have a role in metal treatment. In soils with low metal levels, vesicular arbuscular mycorrhizae enhance metal uptake of non-hyperaccumulating species. On the other hand, some mycorrhizae increase metal tolerance by decreasing metal

uptake in some low-accumulating species. Mycorrhizae thus assists Calluna in avoiding Cu and Zn toxicity. Most roots need about 100 times the amount of carbon than do the hyphae of its associated ectomycorrhizae in order to develop across the same amount of soil. It is therefore easier for hyphae to acquire elements that have a low mobility than it is for plant roots. Caesium-137 and strontium-90 both have low mobilities.

Mycorrhizal fungi depend on host plants for carbon, while enabling host plants to absorb the soil's nutrients and water with more efficiency. In mycorrhizae, nutrient uptake is enhanced for the plants while they provide energy-rich organic compounds to the fungus. Although certain plant species that are normally symbiotic with mycorrhizal fungi can exist without the fungal association, the fungus greatly enhances the plant’s growth. Hosting mycorrhizae is much more energy effective to the plant than producing plant roots.

The Brassicaceae family reportedly forms few mycorrhizal associations. But Hopkins (1987) notes mycorrhizae associated with Streptanthus glandulosus Hook. (Brassicaceae), a non-accumulator. Some fungi tolerate easily the generally elevated metal contents of serpentine soils. Some of these fungal species are mycorrhizal. High levels of phosphate in the soil inhibit mycorrhizal growth.

The uptake of radionuclides by fungi depends on its nutritional mechanism (mycorrhizal or saprotrophic). Pleurotus eryngii absorbs Cs best over Sr and Co, while Hebeloma cylindrosporum favours Co. But increasing the amount of K increases the uptake of Sr (chemical analogue to Ca) but not that of Cs (chemical analogue to K). Moreover, the uptake of Cs decreases with Pleurotus eryngii (mycorrhizal) and Hebeloma cylindrosporum (saprotrophic) if the Cs content is increased, but that of Sr increases if its content is increased – this would indicate that the uptake is independent from the nutritional mechanism.

2 - Pollen and seed dispersal

Some animals obtain food from the plant (nectar, pollen, or fruit pulp). Animals feeding from hyperaccumulors high in metal content must either be metal-tolerant or dilute it with a mixed diet. Alternatively hyperaccumulators may rely on abiotic vectors or non-mutualistic animal vectors for pollen or seed transport, but we lack information on seed and pollen dispersal mechanisms for hyperaccumulating plants.

Various scienctists have studied metal contents of entire flowers and/or fruits. They have recorded elevated metal levels in these. Some plants may thus have a mechanism by which metal or other contaminants is excluded from their reproductive structures.

Commensalism

This is an interaction benefiting one organism while being of neutral value to another. The most likely one with hyperaccumulators would be epiphytism. But this is most noticeable in humid habitats, whereas only a few detailed field studies of hyperaccumulators have been conducted in such habitats, and those studies (mostly to do with humid tropical forests on serpentine soils) pay little or no attention to that point.

Proctor et al. (1988) studied the tree *Shorea tenuiramulosa*, which can accumulate up to 1000 mg Ni/kg dry weight in leaf material. They estimated covers of epiphytes on the boles of trees in Malaysia, but did not report values for individual species. Boyd et al. (1999) studied the occurrence of epiphytes on leaves of the Ni hyperaccumulating tropical shrub Psychotria douarrei (Beauvis.). Epiphyte load increased significantly with increasing leaf age, up to 62% for the oldest leaves. An epiphyte sample of leafy liverworts removed from P. douarrei, was found to contain 400 mg Ni /kg dry weight (far less than the host plant, whose oldest and most heavily epiphytized leaves contained a mean value of 32,000 mg Ni/kg dry weight). High doses of Ni therefore do not prevent colonization of Psychotria douarrei by epiphytes.

Chemicals that mediate host-epiphyte interactions are most likely to be located in the outermost tissues of the host. Also, most of the metal accumulates in epidermal or subepidermal cell walls or vacuoles. These findings suggest that epiphytes would experience higher metal levels when growing on hyperaccumulator leaves. But Severne (1974) measured the release of metal via leaching of leaves from the Ni hyperaccumulator Hybanthus floribundus (Lindl.) F. Muell. (Violaceae) from western Australia; he concluded that its leaves do not easily leach Ni.

In theory another commensal interaction could exist, if the high metal content of the soil under hyperaccumulator plants was needed for another plant species to establish itself. No evidence is known showing such effect.

The biofilm

A biofilm is a layer of organic matter and microorganism formed by the attachment and proliferation of bacteria on the surface of the object. Biofilms are characterised by the presence of bacterial extracellular polymers glycocalyx that create a thin visible slimy layer on solid surface.

H. Biosensors

A biosensor is an analytical device which employs a biological material to specifically interact with an analyte; this interaction produces some detectable physical change which is measured and converted into an electrical signal by a transducer. Finally, the electrical signal is amplified, interpreted and displayed as analyte concentration in the solution/preparation. An analyte is a compound whose concentration is to be determined, in this case, antibodies, lactins, whole cells, entire organs or tissue slices are also used.

The nature of interaction between the analyte and the biological material used in the biosensor may be of two types: (i) the analyte may be converted into a new chemical molecule (by enzymes; such bio-sensors are called catalytic bio-sensors), and (ii) the analyte may simply bind to the biological material prevent on the biosensor (e.g., to antibodies, nucleic acids; these biosensors are known as affinity biosensors)

A successful biosensor must have at least some of the following features:

- (i) it should be highly specific for the analyte
- (ii) the reaction used should be as independent of factors like stirring, pH, temperature etc. as is manageable,
- (iii) the response should be linear over a useful range of analyte concentrations,
- (iv) the device should be tiny and bio-compatible in case it is to be used for analyses within the body,
- (v) the device should be cheap, small and easy to use and
- (vi) it should be durable, i.e., should be capable of repeated use.

A biosensor has two distinct type components:

- (i) biological, e.g., enzyme antibody etc., and
- (ii) physical, e.g., transducer, amplifier etc. (Fig. 1).

The biological component of biosensor performs two key functions:

- (i) it specifically recognizes the analyte and
- (ii) interacts with it in such a manner which produces some physical change detectable by the transducer.

These properties of the biological component impart on the biosensor its specificity, sensitivity and the ability to detect and measure the analyte. The biological component is suitably immobilized on to the transducer. (Enzymes are usually immobilized by glutaraldehyde on a porous sheet like lens tissue paper or nylon net fabric; the enzyme membrane so produced is affixed to the transducer.) Generally, correct immobilization of enzymes enhances their stability, which may be rather dramatic in some cases. As a result, many enzyme immobilized system can be used more than 10,000 times over a period of several months.

The biological component interacts specifically to the analyte which produces a physical change close to the transducer surface. This physical change may be

- (i) heat released or absorbed by the reaction (measured by calorimetric biosensors),
- (ii) production of an electrical potential due to changed distribution of electrons (potentiometric biosensors),
- (iii) moment of electrons due to redox reaction (amperometric biosensors),
- (iv) light produced or absorbed during the reaction (optical biosensors), or
- (v) change in mass of the biological component as a result of the reaction (acoustic wave biosensors).

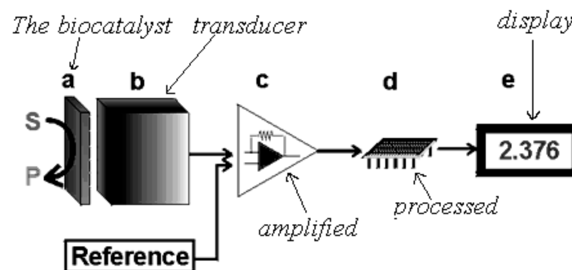


Fig. 1 The main components of a biosensor
The biocatalyst (a) converts the substrate to product. This reaction is determined by the transducer (b) which converts it to an electrical signal. The output from the transducer is amplified (c) processed (d) and displayed (e).

The transducer detects and measures this change and converts it into an electrical signal. This signal is necessarily very small, and is amplified by an amplifier before it is fed into the microprocessor. The signal is then processed and interpreted, and is displayed in suitable units. Thus biosensors convert a chemical information flow into an electrical information flow, which involves the following steps.

- (i) The analyte diffuses from the solution to the surface of the biosensor.
- (ii) The analyte reacts specifically and efficiently with the biological component of the biosensor.
- (iii) This reaction changes the physico-chemical properties of the transducer surface.
- (iv) This leads to a change in the optical or electronic properties of the transducer surface.
- (v) The change in optical/electronic properties is measured converted into electrical signal which is amplified, processed and displayed.

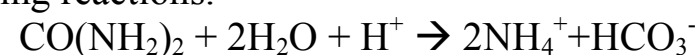
1. Type of Biosensors: The biosensors are of 5 types:

1.1. Calorimetric Biosensors: Many enzyme catalyzed reactions produce "heat (exothermic). Calorimetric biosensors measure the change in temperature of the solution containing the analyte following enzyme action and interpret in terms of the analyte concentration in the solution. The analyte solution in passed through a small (capacity 1 ml) packed bed column containing immobilized enzyme; the temperature of the solution is determined just before entry of the solution into the column and just as it is leaving the column using separate thermistors. This is the most generally applicable type of biosensor, and it can be used for turbid and strongly coloured solutions. The greatest disadvantage is to maintain the temperature of the sample stream at a constant, say $\pm$ 0.01°C, temperature. The sensitivity (10^{-4} M) and the range (10^{-4} -10^{-2} M) of such biosensors is

quite low for most applications. The sensitivity can be increased by using two or more enzymes of the pathway in the biosensor to link several reactions to increase the heat output. Alternatively, multifunctional enzymes may be used may used. An example is the use of glucose oxidase for determination of glucose.

5.1.2. Thermistor Containing Biosensor: Thermistor is used to record even a small temperature changes (between 0.1-0.001 ^{0}C) during biochemical reactions. By immobilizing enzymes like cholesterol oxidase, glucose oxidase, invertase, tyrosinase, etc. thermistors have been developed. Moreover, thermistors are also employed for the study of antigen-antibody with very high sensitivity (10-13 mol dm^{-3}) in Cathode case of thermometric Enzyme Linked Immunoabsorbant Assay (ELISA).

1.3. Potentiometric Biosensors: These biosensors use ion-selective electrodes to convert the biological reaction into electronic signal. The electrodes employed are most commonly pH meter glass electrodes (for cations), glass pH electrodes coated with a gas selective membrane (for CO_2, NH_3 or H_2S) or solid state electrodes. Many reactions generate or use up H^+ which is detected and measured by the biosensor; in such cases very weakly buffered solutions are used. Gas sensing electrodes detect and measure the amount of gas produced. An example of such an electrode is based on urease which catalyses the following reactions.



$$CO(NH_2)_2 + 2H_2O + H^+ \rightarrow 2NH_4^+ + HCO_3^-$$

This reaction can be measured by a pH sensitive, ammonium ion sensitivity, NH_3 sensitive or CO_2 sensitive electrode. Biosensors can now be prepared by placing enzyme coated membranes on the ion-selective gates of ion-selective field effect transistors; these biosensors are extremely small (<< 0.1 mm^2)

1.4. Amperometric Biosensors: These electrodes function by the production of a current when potential is applied between two electrodes the magnitude of current being proportional to the substrate concentration. The simplest ampreometric biosensors use the Clark oxygen electrode which determines the reduction of O_2 present in the sample (analyte) solution (These are the first generation biosensors). These biosensors are used to measure redox reactions, a typical example being the determination of glucose using glucose oxidase. Principle of a mediated biosensor is shown in Fig. 2

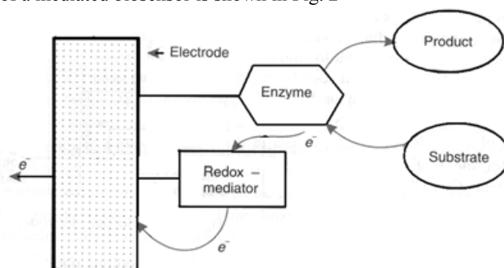


Fig. 2 . Mediated biosensor.

In this biosensor, a redox reaction catalysed by an enzymes is directly coupled to an electrode where enzyme is presented with the oxidizable substrate. The electrons are transferred from the substrate to the electrode via enzyme and redox mediator. In this biosensor the oxidase replaces the oxygen requirement of the enzymes.

A major problem of such biosensors is their dependence on the dissolved O_2 concentration in the analyte solution. This may be overcome by using mediators. e.g., ferrocenes; these molecules transfer the electrons generated by the reaction directly to the electrode rather than reducing the O_2 dissolve in analyse solution. (these are called second generation biosensors). The present-day electrodes, however, remove the electrons directly from the reduced enzymes (without the help of mediators), and are coated with electrically conducting organic salts.

Enzyme Electrodes: Enzyme electrodes are a new type of biosensors which have been designed for the amperometric assay of potentiometric assay of substrates such as urea, amino acid, glucose, alcohol, and lactic acid. The electrode consists of a given electrochemical sensor in close contact with a thin permeable enzyme membrane capable of reacting with the given substrates. The enzyme is embedded in the membrane and produce O_2, H^+, NH_4^+, CO_2 or other small molecules depending on enzymatic reactions. This is detected by the specific sensor. The magnitude of the response determines the concentration of substrates (Fig. 3).

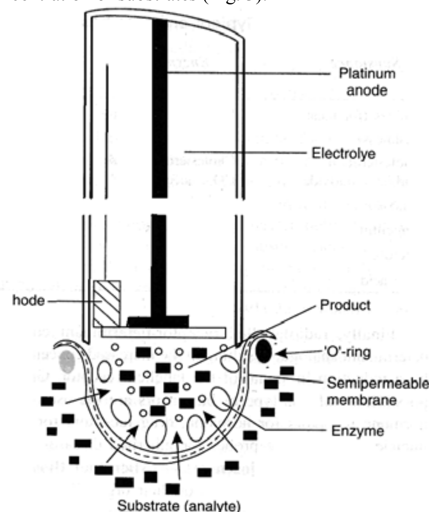


Fig. 3 A simple biosensor combining on electrochemical electrode and an enzyme immobilized on to a semipermeable membrane.

Table 2: Typical enzymes based biosensors

Substance	Enzymes	Response time	Range
Amines (for meat freshness)	Monoamine oxidase	4 min	50 - 200 μ mol dm^{-3}
Cholesterol	Cholesterol oxidase	2 min	10^{-2} - 3 x 10^{-5} mol dm^{-3}
Carbon monoxide	CO : acceptor	15 sec	0- 65 μ mol dm^{-3}
Glucose	Glucose oxidase	20 sec	2 x 10^{-3} - 3 x10^{-6} mol dm^{-3}
Penicillin	Penicillinases	25 sec	1 - 10 m mol dm^{-3}
Sucrose	Invertase	6 min	10^{-2} - 2 x 10^{-3} mol dm^{-3}
Uric acid	Uricase	30 min	5 x 10^{-3} - 5 x 10^{-5} mol dm^{-3}

1.5. Optical Biosensors: These biosensors measure both catalytic and affinity reactions. They measure a change in fluorescence or in absorbance caused by the products generated by catalytic reactions. Alternatively, they measure the changes induced in the intrinsic optical properties of the biosensor surface due to loading on it of dielectric molecules like protein (in case of affinity reactions). A most promising biosensor involving luminenscence uses firefly enzyme luciferase for detection of bacteria in food or clinical samples. The bacteria are specifically lysed to release ATP, which is used by luciferase in the presence of O_2 to produce light which is measured by the biosensor.

1.6. Bioaffinity Sensor: Bioaffinity sensors are developed recently. It measures the concentration of the determinants, i.e. substrates based on equilibrium binding. This shows a high degree of selectivity. These are of diverse nature because of the use of radiolabelled,

enzyme labelled or fluorescence-labelled substance. Principle of bioaffinity sensor is given in Fig. 4.

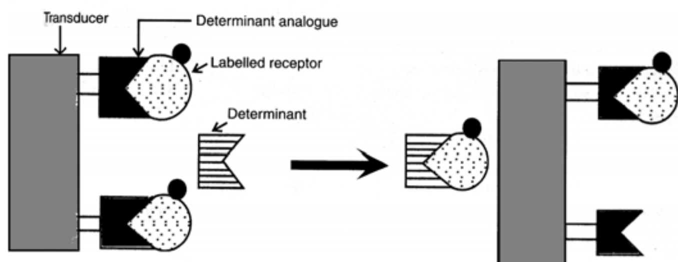


Fig. 4. Principle of bioaffinity sensor.

In this biosensor, a receptor is radiolabelled and allowed to bind with determinant analogue immobilized onto the surface of a transducer. When concentrations of a determinant are increased, the labelled receptor forms an intimately bound complex with determinant.

Finally, radiolabelled receptor-determinant complex is removed from the immobilized determinant analogue resulting in the increased concentrations of labelled receptor. This is measured by a reduction in signal of the labelled receptor. Gronow et at. (1988) have discussed that the possibilities of this type of biosensors are the use of lectin receptors for saccharide estimation, hormone receptors for hormone, drug receptors for drug, antibodies receptors for antigens and nucleic acid (as gene probe) for inherited diseases and fingerprinting.

1.7. Acoustic Wave Biosensors: These are also called piezoelectric devices. Their surface is usually coated with antibodies which bind to the complementary antigen present in the sample solution. This leads to increased mass which reduces their vibrational frequency; this change is used to determine the amount of antigen present in the sample solution.

1.8. Electrochemical Biosensors: This type of biosensor has been developed by using electronic devices such as field transmitter or light emitting diode; the former measures charge accumulation on their surface and the later photoresponse generated in a silica based chip as an alternating current. Hence, the field effect transmitor measures a biochemical reaction at the surface and induce into current. Moreover, the field effect transmitors can be modified to ion sensitive, enzyme sensitive or antibody sensitive ones by using selective ions, enzymes or antibodies respectively.

1.9. Whole Cell Biosensors - (Microbial Biosensors): In this device, either immobilized whole cell of microorganisms or their organelles are used. These react with a large number of substrates and show generally slow response. Immobilized *Azotobacter vinelandii* coupled with ammonia electrode shows sensitivity range between 10-5 and 8 x 10 mol dm-3. It measures the concentration of nitrate within 5-10 min^{-2}.

2. Applications of Biosensor.

In the beginning biosensor was applied in the field of medicine and industry. But in recent years, biosensors are becoming popular in many areas due to the small size, rapid and easy handling, low cost, and greater sensitivity and selectivity. Application of biosensor in some of the areas is described as below:

2.1 Uses in Medicine and Health: Biosensors have tremendous potential for its application in the field of medical science. In 1979, the first glucose analyser using biomolecule for the detection of blood glucose was commercialized by Yellow Springs Instruments Co., USA. A device, a minipump filled with insulin, has been constructed to deliver insulin to diabetics based on glucose levels of blood. When biosensor provides informations, the device delivers accurate amount of insulin required by the diabetics. Mitomycin, an aflatoxin, causes cancer in inborne infants. Therefore, mutagenicity of such chemicals can be detected by using the biosensor. Similarly, any other abnormal toxic substance produced in body due to infectious diseas; can also be detected.

2.2 Uses in Pollution Control: Biosensors are very helpful in environmental monitoring and pollution control, since they can be miniaturized and automated. As far as quality control of drinking water is concerned, the minitoring biosensors are successful in monitoring of pesticides in water. In Japan, a biosensor coupled with oxygen electrode and immobilized *Trichosporon cutaneum* is used for measuring biological oxygen demand (BOD).

The whole cell biosensor developed by immobilising Salmonella typhimurium and Bacillus subtilis in conjugation with oxygen electrode can be used to measure mutagenicity and carcinogenecity of several chemical compounds.

2.3 Uses in Industry: Generally, spectrophotometer and autoanalyzer are used to estimate the substrates utilized and the products formed in the fermented broth. In addition, there are a lot of problems associated with these. So the biosensors can be designed to measure the fermentation products to improve the feedback control, to carry out rapid sampling and rejection of below standard raw materials to improve the efficiency of workers. Isaokarube and coworkers of Tokyo University Research Centre of Advance Science & Technology have recently developed an ion sensitive field effect transistor (ISFET). This device is highly sensitive to change the ion concentration. Using this biosensor, it is possible to measure the odour, freshness and taste of foods. In determining fish freshness either ATPase, aminoxidase or putrescine oxidase is used. ATPase detects the presence of ATP in fish muscle. As ATP is not present in staled food, therefore, signals do not occur. Recently, a biosensor has been developed at Cransfield Institute of Technology, UK which measures cholesterol levels in butter. The enzyme cholesterol oxidase, when immobilized on the electrodes, reacts with cholesterol of food.

2.4 Biosensor in Military: The darker side of biosensor application is to provide support to military with such a biosensor that can detect toxic gases including chemical warfare agents. Such biosensors have advantages over the traditional methods of sensing of chemicals.

3. Biochips (Biological Computer)

Biochip is the result of merging of microchips technology with biotechnology. In future, there is the possibility of developing biological computers. Until the development of silicon microchips, setting up of computers was very costly and space occupying. But recently, one can have a computer to be fit on desk top. These affordable prices are mainly due to the development of silicon microchips which brought into a rapid revolution in technology. Further reduction in size of computers and improvement in computing powers will not be possible because the silicon microchip technology has certain limitations as below:

- There is inherent limit beyond which circuits cannot be squeezed onto a silicon chip. For example the width of

the circuit cannot be shorter than the wavelength of light. Light is used to etch out circuits during the manufacturing of silicon chips.

- Close placing beyond a limit of many electrical circuits on the same microchip results in 'electron tunneling' which creates short circuits ruining the whole system.
- After cramming together of a large number of circuits, heat is generated by the electric current. This may cause total failure of the system.

3.1 Principles of Biochips: One of the important features of macromolecules (e.g. proteins) is their self shaping into predetermined three dimensional structure. This property of proteins helps in biochip designing because the circuits can be crammed around three dimensional protein structure. While designing the biochips, a semi-conducting organic molecule is inserted into a protein framework; the whole unit is fixed onto a protein support (Fig. 5). In biochips the electrical signals can pass through the semi-conducting organic molecule in the same way as in silicon microchip. It has many advantages over silicon microchip as below:

- In biochip the width of electrical circuits should not be more than that of one protein molecule which is smaller than the smallest silicon microchip.
- The problem of electron tunnelling would be to certain extent less acute in biochip than silicon microchip.
- The protein molecule possess less electrical resistance, therefore, less heat will be generated during the course of production of electrical signals. Consequently, a large number of circuits can be placed together as it is not possible in silicon microchips.

3.2. Application of Biochips: There are many areas where biochip holds a great promise as given below:

- Biochips can respond to natural nerve impulses making looks more natural when implanted into the artificial limbs.
- It is also possible that they can also be used as a heart-heat regulator. This will solve the problems of users of costly pace makers.
- It can also help blind or deaf. It can be designed in such a way that can sense light and sound, and convert them to electrical signals. These signals after reaching brain stimulate sight and sound.

It can be designed in accordance with the need of military and protect the silicon-based computers. It can keep immune to the disastrous effects of electromagnetic waves which are generated due to nuclear explosion and protect the silicon-based computers.