Biotechnology

Textbook for Class XII

विद्यया ऽमृतमश्नुते
एन सी ई आर टी
NCERT
राष्ट्रीय शैक्षिक अनुसंधान और प्रशिक्षण परिषद्
NATIONAL COUNCIL OF EDUCATIONAL RESEARCH AND TRAINING

Reprint 2025-26

12150 – BIOTECHNOLOGY
Textbook for Class XII

ISBN 978-93-5292-402-8

First Edition
February 2023 Magha 1944

PD 5T BS

OFFICES OF THE PUBLICATION DIVISION, NCERT

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Guwahati 781 021 **Phone : 0361-2674869**

₹ 435.00

Printed on 80 GSM paper with NCERT watermark

Published at the Publication Division by the Secretary, National Council of Educational Research and Training, Sri Aurobindo Marg, New Delhi 110 016 and printed at Chandra Prabhu Offset Printing Works (P.) Ltd., C-40, Sector-8, Noida - 201 301 (U.P.)

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Foreword

NCERT prepares quality curricular material for its stakeholders at all levels of school education. *Biotechnology* is a new addition in the series of textbooks for students at the higher secondary stage. It is always considered important that a smooth transition of students occur from the secondary stage to the higher secondary stage. At the secondary stage, children pursue science as an integrated subject, whereas specific subject disciplines are offered at the higher secondary stage.

Biotechnology being an applied subject, involves the understanding of fundamentals of the components of Biology, Chemistry and Physics. Keeping this in mind, basic principles of organisms, cell and molecules have been discussed in the textbook of *Biotechnology* at initial level in Class XI. Thus, the learner can appreciate the basic aspects and principles with a focus on its applications. The applied aspects are dealt with in Class XII, where children have been given good exposure to understand as to how the basic cellular and molecular processes can be used for diverse applications for the welfare of society in general.

Such applications touch almost all aspects of human activities, like agriculture, health, food and nutrition, industry, and environment conservation. Keeping in view the cognitive domain of higher secondary students, attempts have been made to keep various aspects of applications of biotechnology in such a way that a smooth transition occurs from higher secondary to higher and technical level. As per the recommendations of the National Education Policy-2020, attempts have also been made to develop critical thinking analysing societal needs.

Being an applied subject, it is extremely crucial that children must develop skills to cope up with technological content of the subject. I do hope that the textbook would be up to the expectations of the stakeholders. Biotechnological researches have a great potential for exploring and establishing various enterprises with the industrial and commercial applications, therefore, an appropriate understanding of entrepreneurial skills among children pursuing the course is relevant. A chapter has been dedicated to this aspect as well. It is expected that this course of Biotechnology would be a perfect bridge between the secondary stage science and similar disciplines at higher and professional level.

I am confident that the development team has taken due care while preparing the manuscript about correctness, accuracy and appropriateness of the content. However, NCERT believes in the continuous improvement of our curricular materials, therefore, feedback and suggestions provided by different stakeholders would be of great help for further improving its quality and utility.

New Delhi
September 2022

Director
National Council of Educational
Research and Training

Preface

Biotechnology, by definition, is an applied science and its applications are widespread. It is becoming increasingly evident that the role of biotechnology is increasing day by day. In the field of agriculture, biotechnological applications have helped in improving many crop varieties from the perspective of increased productivity, pest resistance, drought and salinity tolerance. Production of human growth hormone and insulin, diagnosis of various diseases whether genetic or infectious and development of a number of vaccines including the one against COVID-19 have become possible only because of the advancements in the area of biotechnology. Even in the field of environment protection and conservation, biotechnological tools have tremendously contributed through bioremediation of toxic substance on one hand to detection of toxic substance through biosensor and elimination of toxic substances from soil on the other. Last but not the least, advancements in the area of bioinformatics provide a tool which has predictive potentialities from the point of view of prediction of disease a person is likely to suffer in future and drug discovery. It is worth mentioning that new researches are pouring in at a very fast speed and therefore, the understanding of the subject has to be fundamental and critical to address future challenges.

In Class XI, students of biotechnology have already been exposed to the basic understanding of biomolecules, cell organisation with cellular processes, fundamentals of genetic and molecular principles, and various tools and techniques employed in the biotechnological study. Course content of the subject for Class XII largely dwells around the application of molecular and cellular principles besides employing different microbes for various beneficial usage. Also, appropriate emphasis has been given on the aspect of recent innovations and development happening in the area. Another important feature of the book is the component on entrepreneurship which would develop an appropriate understanding as to how a biotechnology-based enterprise can be established.

Attempt has been made to keep a continuity of the content of biotechnology for Class XII with that of the fundamentals studied in earlier class. There are five units in the book divided into thirteen chapters. Unit I with four chapters deals with the details of recombinant DNA (rDNA) technology and gene cloning in which the role of host and vector for transfer of gene or a segment of DNA for various applications have been detailed first. In Chapter 4, a few of the common and important applications of rDNA technology have been described. Unit II focusses on the aspect of genome engineering in which various advancements of DNA sequencing technology, genome editing, comparative genomics and protein engineering have been described. Unit III has five chapters in which the fundamentals and application of various culture techniques, be it culture of microbes, plant and animal tissues or stem cells have been prescribed. The usage of microbial and cell culture in most of the cases lead to the bioprocessing of various products. Accordingly, Chapter 10 of the Unit III deals

with this applied technology of bioprocessing and biomanufacturing. Unit IV with one chapter emphasises on the applied aspect of microorganisms and technology for the treatment of wastewater and sewage besides bioremediation of toxic substances especially pesticides. Unit V has two chapters, one on recent innovations in the field of Biotechnology and the other on various aspects about entrepreneurship skill and its development.

It is expected that the entire course of Biotechnology would be helpful for students in developing a critical understanding of the subject, its application, future prospects besides developing entrepreneurial skills.

I express my deep sense of gratitude and appreciation to U. N. Dwivedi, *Former Professor*, Department of Biochemistry and *Former Vice Chancellor* of University of Lucknow, for providing leadership in this endeavour of NCERT. This task would not have been accomplished without the contribution of the entire development team and their efforts are highly appreciated.

The department welcomes the comments as well as suggestions for bringing out further improvement in the textbook.

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Acknowledgements

The National Council of Educational Research and Training (NCERT) gratefully acknowledges the contribution of the individuals and organisations involved in the development of *Biotechnology* textbook for Class XII. The Council is grateful to Binay Panda, *Professor*, School of Biotechnology, Jawaharlal Nehru University, Delhi; Pawan K. Dhar, *Professor*, School of Biotechnology, Jawaharlal Nehru University, Delhi; Mohammad Mahfuzul Haque, *Professor*, Department of Biotechnology, Jamia Millia lslamia, Delhi; Vinod Kumar Srivastava, *Former Associate Professor*, Indira Gandhi Government College, Tezu, Arunachal Pradesh, for their contribution in the review of the manuscript.

The NCERT is highly thankful to Shweta Singh, *Joint Director*, Central Board of Secondary Education, Delhi; Anjulika Joshi, *PGT*, Mount Carmel School, Anand Niketan, Delhi; Sushil Kumar Dwivedi, *PGT*, Kendriya Vidyalaya, Aliganj, Lucknow; Payal Priyadarshini, *PGT*, Kendriya Vidyalaya, Delhi Cantt-3, Delhi; Pratibha Sharma, *PGT*, Kendriya Vidyalaya, JNU, Delhi and Ambika Nagrath, *PGT*, Army Public School, Dhaula Kuan, Delhi; Upasna Chopra, *PGT*, Apeejay School, Panchsheel Park, Delhi; Dinesh Kumar Jamwal, HOD, Air Force Bal Bharti School, Lodhi road, Delhi; Ankit, *PGT*, Ahlcon International School, Mayur Vihar Phase 1, Delhi; Neva, *PGT*, Delhi Public School, R.K. Puram, Delhi; for their valuable suggestions.

The NCERT is also thankful to Bharat Biotech Pvt. Ltd., Dr. Banwarilal of The Energy and Resources Institute (TERI), for providing relevant information that was required for finalising a few chapters.

The Council also acknowledges the academic contributions of Ritika Gupta and Preeti Sharma, *Junior Project Fellows*, in finalising the manuscript. Contributions of Shilpi Singh, *Lab Assistant*; Suman Prajapati and Pooja Sharma, *Graphic Designers* and Rekha Sharma, *DTP Operator* for typesetting are also acknowledged. Without their effort, it would not have been possible to bring out the manuscript. Cooperation from Rajendra Singh, *Assistant Program Coordinator*, and his staff for their help in organising workshops and office logistics for the same is especially thanked.

The efforts of Soumma Chandra, *Editor* (Contractual), Chanchal Chauhan, *Assistant Editor* (Contractual), Surender Kumar, (Incharge, DTP Cell, NCERT), and Mohammed Aariz *DTP Operator* (Contractual) of the Publication Division, NCERT, in bringing out the first edition of this book are also highly appreciated.

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THE CONSTITUTION OF INDIA

PREAMBLE

WE, THE PEOPLE OF INDIA, having solemnly resolved to constitute India into a [1]**[SOVEREIGN SOCIALIST SECULAR DEMOCRATIC REPUBLIC]** and to secure to all its citizens :

JUSTICE, social, economic and political;

LIBERTY of thought, expression, belief, faith and worship;

EQUALITY of status and of opportunity; and to promote among them all

FRATERNITY assuring the dignity of the individual and the [2][unity and integrity of the Nation];

IN OUR CONSTITUENT ASSEMBLY this twenty-sixth day of November, 1949 do **HEREBY ADOPT, ENACT AND GIVE TO OURSELVES THIS CONSTITUTION.**

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1. Subs. by the Constitution (Forty-second Amendment) Act, 1976, Sec.2, for "Sovereign Democratic Republic" (w.e.f. 3.1.1977)
2. Subs. by the Constitution (Forty-second Amendment) Act, 1976, Sec.2, for "Unity of the Nation" (w.e.f. 3.1.1977)

UNIT I

Recombinant DNA Technology

Herbert Boyer

Herbert Wayne 'Herb' Boyer (born July 10, 1936) was a researcher and entrepreneur in Biotechnology. Herb Boyer hails from Derry, Pennsylvania. Boyer went on to graduate at the University of Pittsburgh, where he specialised in microbial genetics. After preliminary experiments in 1973, the Cohen-Boyer team was able to cut open a plasmid loop from one species of bacteria, insert a gene from different bacterial species and close the plasmid. This created a recombinant—plasmid containing recombined DNA from two different sources. The team had created the first genetically modified organisms. He is the recipient of the 1990 National Medal of Science, co-recipient of the 1996 Lemelson–MIT Prize, and a co-founder of Genentech. He was a professor at the University of California, San Francisco (UCSF) and later served as the Vice President of Genentech from 1976 until his retirement in 1991.

Chapter 1

An Overview of Recombinant DNA Technology

This chapter gives an overview of recombinant DNA (rDNA) technology as to how the application of basic concepts of molecular biology, microbiology, genetics, biochemistry, etc., led to initial development of rDNA technology. Potential application of rDNA technology in the field of medicine and agriculture is also discussed in conceptual manner along with some noticeable examples of products developed through rDNA technology.

1.1 An Overview of Recombinant DNA Technology

In the last century when scientists discovered that nucleic acid (DNA) is the principal molecule responsible for the expression of characters, attempts were made to alter the genetic makeup of an organism by manipulating nucleic acid directly. Various methods used for directly manipulating nucleic acid/genome of an organism are collectively referred to as recombinant DNA (rDNA) technology or genetic engineering.

rDNA technology has been possible due to rapid progress in various fields of biology, which spans from

biochemistry, genetics, cytology, microbiology, molecular biology and others. Isolation and purification of nucleic acids followed by the understanding of their structures, properties, functions and finally their sequencing in the last century are the most important contributions, which laid the foundation of development of rDNA technology. The first breakthrough in this journey was to establish the fact that DNA of an organism not only carries its genetic information but also propagates it from one generation to another. The next hallmark was the determination of chemical and physical structure of DNA molecule and double helical structure of DNA. Further, replication, transcription and translation of DNA was understood in detail by scientists. Scientists were also able to develop various methods and techniques to isolate and purify DNA from various organisms. Several enzymes were simultaneously discovered using which one can precisely manipulate a DNA molecule. Thus, new enzymes, such as restriction enzymes (which act as scissors to cut the molecules of DNA) by Werner Arbor, Hamilton Smith and Daniel Nathan (during late 1960s and early 1970s) and ligase (which joins two DNA fragments) by Gellert, Lehman, Richardson and Hurwitz in the year 1967 were discovered.

During this period, scientists also noticed that foreign DNA fragments can be taken by bacteria from its surrounding environment where it can be integrated into its genome. With all this knowledge, scientists asked a question that is it possible to transfer the gene of interest from one organism to another organism to get its product? Stanley Cohen had the expertise in introducing plasmid DNA into *Escherichia coli* (*E. coli*) and subsequent propagation and cloning of plasmids in the bacteria. On the contrary, Herbert Boyer had the expertise to cleave the double stranded DNA to produce single stranded ends with identical termini using restriction enzymes. Both visualised the potential of combining the two discoveries to what would later become rDNA technology or genetic engineering.

rDNA technology has immensely contributed in the diagnosis and treatment of various diseases including genetic disorders and to improve and develop disease free high yielding crops. The contribution of rDNA technology in shaping our life can be judged from the given examples. Earlier several tons of animal pancreatic glands were needed to get a few milligrams of insulin to treat diabetes, or thousands of animal pituitary glands were required to isolate growth hormone to treat dwarfism. Therefore, these products were available in limited quantity as well as at a high cost. Nevertheless, such purified therapeutic proteins from animal source exhibited immunogenic reactions in humans. Needless to say, scientists circumvent the above obstacles by producing human insulin and growth hormone in bacterial system using rDNA technology. Production of interferon to treat cancer, plasminogen activator and urokinase to dissolve blood clots are a few examples of the contribution of rDNA technology to human society.

In the last few decades, by employing rDNA technology, scientists have been able to introduce specific targeted modifications in plant genome to get genetically modified crops. Thus, in this way, crops have been developed that offer resistance to diseases, thereby helping farmers to be free from worry about damage of their crops. Similarly, drought resistant or salinity tolerant crops were also developed so that farmers can grow them in adverse environment. Such modifications in genetic system of plants or crops by rDNA technology not only improve the quality of production but also enhance the value of products.

Days are not far, when a variety of important therapeutic proteins, peptides and hormones will be produced from plants employing rDNA technology. Such products will have many advantages over animal-based products in terms of costs and contamination. In general, animal-based products are costlier and require extra care to be free of virus and other animal protein contaminants.

Landmark discoveries that led to the development of modern biotechnology (based on rDNA technology is given in Box 1).

Selected developments in the history of biotechnology

1917	Karl Ereky coined the term 'Biotechnology'
1944	Avery, MacLeod and McCarty demonstrated that 'DNA is the genetic material'
1952	Joshua Lederberg discovered 'Plasmids'
1953	Watson and Crick proposed 'Double Helical structure of DNA'
1960s	Werner Arber, Matthew Meselson discovered ' Type I restriction enzymes'
1967	Gellert, Lehman, Richardson and Hurwitz discovered 'ligase enzymes'
1970	Hamilton O. Smith and Thomas J. Kelly discovered ' Type II restriction enzymes'
1972	Paul Berg assembled the first 'Recombinant DNA' from a bacterium into the virus
1973	Stanley N. Cohen and Herbert Boyer developed 'DNA cloning and rDNA technology'
1975	Georges J.F. Köhler and César Milstein described the 'Hybridoma Technology' for production of monoclonal antibodies
1982	FDA approved world's first recombinant DNA Therapeutic Product 'Humulin' developed by Eli Lilly and Genentech
1983	Kary Mullis developed 'Polymerase Chain Reaction'
1984	Sir Alec Jeffreys invented 'DNA Fingerprinting'
1986	The first recombinant vaccine 'Recombivax HB' for Hepatitis B was approved for human use
1990	'Human Genome Project' officially initiated which was coordinated by the U.S. Department of Energy (DOE) and the National Institutes of Health (NIH)
1994	The first genetically engineered crop 'Flavr Savr' tomato was introduced that was produced by Calgene in 1992
1996	Keith Campbell and Ian Wilmut cloned the first mammal 'Dolly' the sheep from somatic cell using nuclear transfer technique.

1996	Researchers at Monsanto developed 'Bt cotton' and first commercially released it in China and the United States in 1996, followed by its introduction in India in 2003
2000	Ingo Potrykus and Peter Beyer developed 'Golden Rice'
2003	The Human Genome Project (HGP) was completed
2004	'Avastin', an anti-VEGF monoclonal antibody for cancer treatment was developed
2006	A recombinant vaccine 'Gardasil' against human papillomavirus (HPV) received FDA approval
2006	Nobel prize awarded for discovery of RNA interference 'Gene Silencing' by double stranded RNA
2010	Robert Edwards awarded Nobel Prize for the development of human '*in vitro* fertilization' (IVF) therapy
2012	Shinya Yamanaka and John B. Gurdon discovered that mature differentiated cells can be reprogrammed into 'Induced Pluripotent Stem Cells'
2019	Nobel prize awarded for discovery of 'CRISPR-Cas9' genome editing tool
2020	Recombinant vaccines against COVID-19 was developed.
2023	Nobel prize awarded for the mRNA vaccine against covid–19

In the next chapters of the present Unit, the various components and applications of rDNA technology are discussed in detail.

SUMMARY

- The methods used for manipulating nucleic acid/genome of an organism are collectively referred to as recombinant DNA (rDNA) technology or genetic engineering.
- The fundamental theme of rDNA technology is the isolation and propagation of a desired DNA molecule (gene) from a source with an aim to have its product in ample quantity. This technique is called gene cloning.

EXERCISES

1. Discuss in brief how recombinant DNA technology was initially developed?
2. Briefly discuss the application of rDNA technology in crop improvement and therapeutics.
3. Who discovered the Plasmid?
   (a) Paul Berg
   (b) Sir Alec Jeffreys
   (c) Joshua Lederberg
   (d) Kary Mullis
4. Plasminogen activator and Urokinase are used as:
   (a) Antiviral agent
   (b) Blood clot dissolving drug
   (c) Sugar lowering agent
   (d) Cholesterol lowering agent
5. **Assertion:** Restriction endonuclease cuts DNA and isolated mostly from bacteria.
   Reason: Restriction endonuclease is a type of nuclease.
   (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.
   (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.
   (c) Assertion is true but reason is false.
   (d) Both assertion and reason are false.
6. **Assertion:** *E. coli* divides in 20 minutes while replicates its DNA in about 60 minutes.
   Reason: *E. coli* follows multifork replication mechanism.
   (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.
   (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.
   (c) Assertion is true but reason is false.
   (d) Both assertion and reason are false.

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Chapter 2 Host–Vector System

Recombinant DNA technology is a two component system involving a compatible host and a vector. A variety of hosts as well as vectors are available for gene cloning. In this chapter, students will be exposed to the fundamentals of different kinds of prokaryotic and eukaryotic host and vectors used in rDNA technology.

2.1 Two Key Components of Recombinant DNA Technology

As discussed in the previous chapter (Chapter 1), rDNA technology refers to joining two different DNA molecules with an aim to isolate, propagate, characterise and manipulate the genes for various applications. This technique involves two major steps (Fig. 2.1). In the first step, the desired DNA molecule, generally termed as **insert** (target gene), is isolated from the source. In the second step, this target gene is inserted into a convenient carrier DNA molecule called **vector**. The vector containing the insert is called **recombinant DNA (rDNA)**. Subsequently, the rDNA is introduced into an organism referred to as **host**. Using

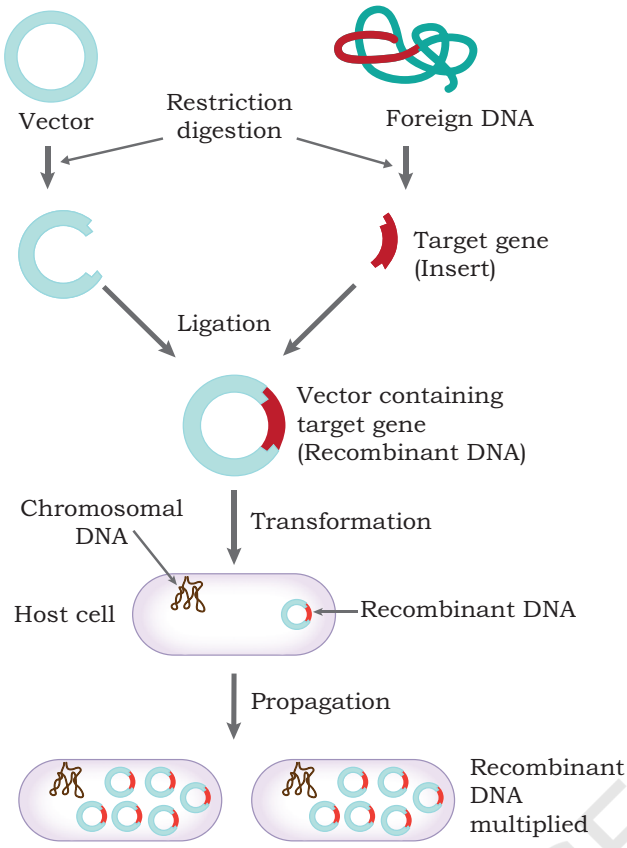


Fig. 2.1: Schematic representation of gene cloning

genetic machinery of the host, the rDNA undergoes propagation and expression. This whole process of rDNA technology is covered under the term 'gene cloning'. Thus, gene cloning may be considered as a two component system: a compatible host and a vector, where the vector provides essential sequences required for its replication in a compatible host, which provides various replication functions (enzymes and proteins).

2.2 Host

A large number of host organisms, both prokaryotic and eukaryotic are used for gene cloning (Fig. 2.2). A host should allow easy entry of the rDNA into the cell and should not consider the recombinant DNA as a foreign DNA and degrade it. The host must supply all the required enzymes and proteins for smooth replication of the vector DNA along with insert. A wide variety of genetically defined strains are available as hosts.

Among prokaryotic hosts, *E. coli* is the most extensively used. Typical *E. coli* is a rod-shaped Gram-negative bacterium commonly found in the lower intestine of warm-blooded organisms. It is able to reproduce and grow rapidly, doubling its population about every 20 mins. K12 strain of *E. coli* is one of the most commonly used hosts in gene cloning. Other prokaryotic hosts have also been developed. For example, *Bacillus subtilis* constitutes an important alternative host, where the aim is secretion of a protein encoded by a cloned gene. Among eukaryotic hosts, the most widely used is yeast.

2.3 Vector

In principle, any molecule of DNA, which can replicate itself inside a host cell, can work as a vector for gene cloning. However, in order for a plasmid to act as vector, it must

fulfil the following features:

1. For easy incorporation into the host cell, a vector should itself be small in size and be able to integrate a large size of the insert.
2. The vector should have an **origin of replication** or ***ori***, so that the vector is capable of autonomous replication inside the host organism.
3. The vector needs to have **unique restriction sites**. If it possesses too many restriction sites, then it would be fragmented into several pieces.
4. The vector needs to have at least one **selectable marker**. The selectable markers are required to screen out transformants [For example: resistance to antibiotics such as tetracycline (tet^R) and ampicillin (amp^R)].

A variety of both prokaryotic as well as eukaryotic vectors are given in (Fig. 2.2).

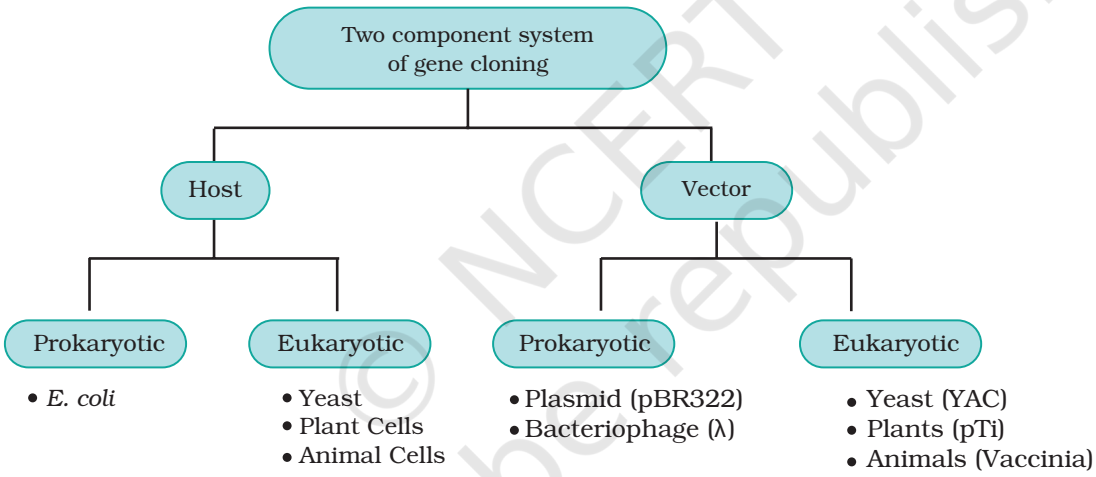


Fig. 2.2: Two component system of gene cloning showing various kinds of eukaryotic and prokaryotic hosts and vectors

2.3.1 Plasmid as a vector

Plasmids are circular, double-stranded (ds) and extra chromosomal DNAs capable of autonomous replication. They occur naturally in many bacteria, archaea, and even in eukaryotes, such as yeast. Size of plasmids ranges from a few thousand base pairs to more than 100 kilobase pairs (kbp). Like the host-cell chromosomal DNA, plasmid DNA is replicated before every cell division. During cell division, atleast one copy of the plasmid DNA is segregated to each daughter cell, assuring

continued propagation of the plasmid through successive generations of the host cell. Some plasmids can integrate into chromosomes. Such plasmids are called **episomes**.

Several naturally occurring plasmids contain genes that provide some benefit to the host cell. For example, some bacterial plasmids encode enzymes that deactivate antibiotics, such as ampicillin, tetracycline and chloramphenicol. Such drug-resistance providing plasmids, called **R-plasmids** constitute a major category of plasmids used for gene cloning. Another category of plasmids produce a variety of toxins called 'colicins' which kill other bacteria (called **Col plasmids**). Some plasmids contain 'transfer genes' encoding proteins that can form a macromolecular tube, or pilus, through which a copy of the plasmid can be transferred to other host cells of the same or related bacterial species called **F-plasmids**. Similarly, based on the copy number plasmids can also be classified as follows:

(a) High or multi copy plasmids: Replication and segregation of these plasmids are free from control of replication of the bacterial chromosomal DNA that allows for many copies of these plasmids per cell (10–30 copies) [Fig. 2.3(a)].

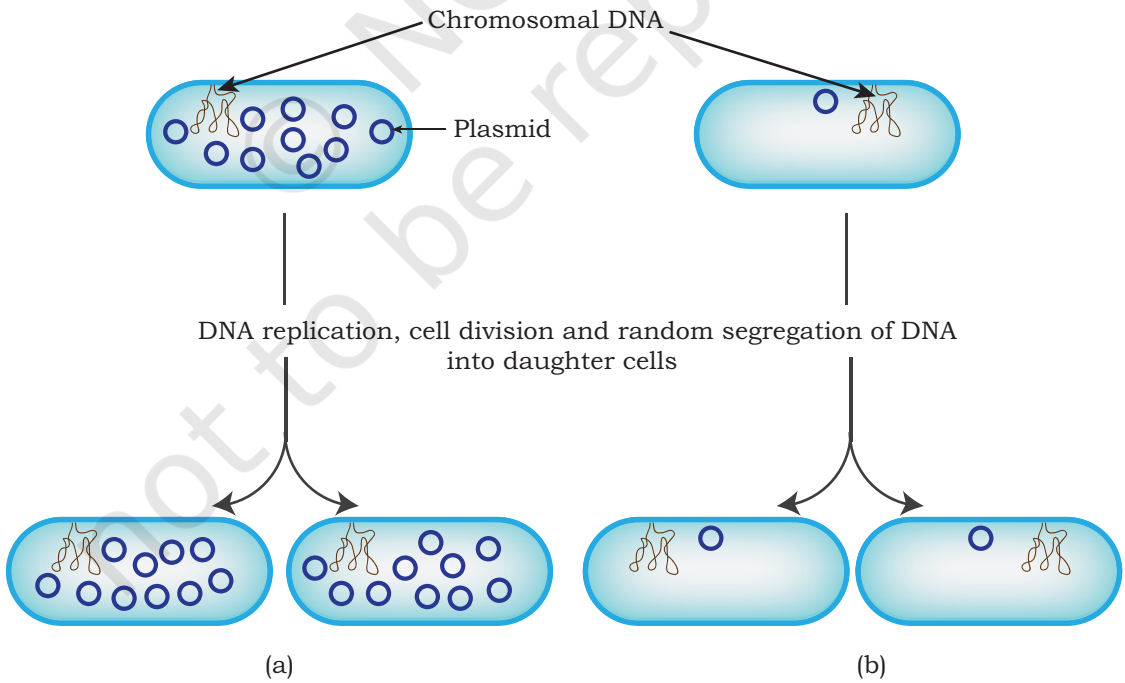


Fig. 2.3: (a) High copy plasmid (b) Low copy plasmid

Replication of these plasmids is referred to as **relaxed replication**. Use of a high copy number plasmid gives rise to higher yields and increased strength of the signals during screening of recombinants.

(b) Low or single-copy plasmids: Replication and segregation of these plasmids are under the same control as replication of the bacterial chromosomal DNA. As a result of this, the copy number of these plasmids are restricted to only one or few copies per cell [Fig. 2.3(b)]. Replication of these plasmids is referred to as **stringent replication**.

Development of plasmid based vectors

In order to use plasmids as a vector and to enhance their utility either for general purposes or to suit particular experimental designs, plasmids originally found in nature, are modified, shortened, reconstructed and recombined under both — *in vivo* as well as *in vitro* to incorporate the properties indicated above into them. For example, the plasmid based vector pBR313, which replicates in relaxed fashion (giving rise to high copy number), contains two selectable markers *tet*^{R} and *amp*^{R} (selectable markers were derived from other two naturally occurring plasmids) and carried a number of unique restriction sites (cloning sites). However, the size of this plasmid was too large (9 kb). It was observed that more than half of its DNA was not essential for its role as a vector. Therefore, the first phase of this plasmid vector development led to the construction of vector pBR322, whose size was reduced to 4,361 bp by deleting most of the non-essential sequences of pBR313. The vector pBR322 became the most widely used vector for gene cloning (Fig. 2.4).

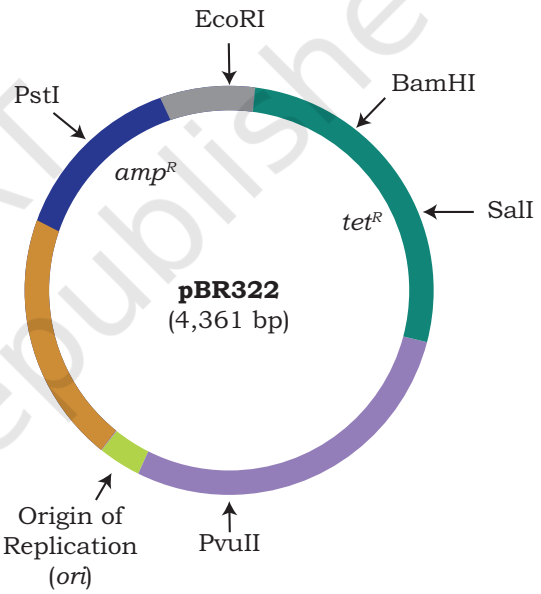


Fig. 2.4: Vector map of pBR322 showing the origin of replication (ori), unique cloning sites for restriction enzymes (PstI, EcoRI, BamHI, SalI, PvuII) *and antibiotic selectable marker genes (amp*^{R} *and tet*^{R}*)*

Subsequent development included the introduction of synthetic cloning sites termed as **multiple cloning site (MCS)** or **poly cloning site** or **polylinker**. MCS is a short synthetic DNA fragment containing a large number of unique restriction sites. This enables the insertion of a foreign DNA cleaved at any of these restriction sites into that region

(Fig. 2.5). Further development involved the introduction of easy and convenient selection markers. Thus, for example, selection of the recombinants on the basis of blue/white selection was introduced (for detail refer section 3.5; Chapter 3), which facilitated the selection of recombinants utilising β-galactosidase (*lacZ*) of *E. coli*. A common example of such an improved cloning vector is pUC19 vector as shown in Fig. 2.5. Many other plasmids are used as vectors for gene cloning in *E. coli*.

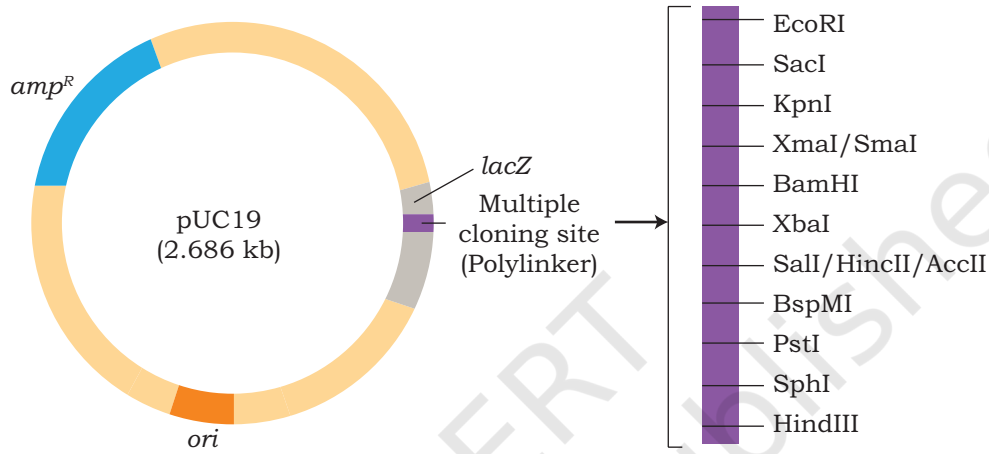


Fig. 2.5: Vector map of pUC19 showing origin of replication sequence (ori), ampicillin resistance gene (amp^R) and part of β-galactosidase gene (lacZ)

2.3.2 Bacteriophage as vectors

Another important category of vectors for gene cloning is derived out of the genome of bacteriophages (phages) i.e., viruses which infect the bacterial cells. In general, bacteriophage vectors are more efficient than plasmids for **cloning large inserts**, and **screening large numbers of bacteriophage plaques** (lysed bacterial cells due to phage infection) than screening bacterial colonies for a specific desired insert. The bacteriophage lambda (λ) and M13 are the two most common phages, whose genome has been frequently used to make cloning vectors for *E. coli* host. They are described in the following section.

Lambda (λ) Phage Vector

The bacteriophage lambda, a virus that infects *E. coli*, has been widely used as a cloning vector. The virus is easy to

propagate, and therefore, has long been a model system. It consists of a head (capsid) and a tail (Fig. 2.6). The head contains phage genome. The phage particles bind to the surface of *E. coli* and insert its genome *via* tail into the cytoplasm of the bacterial cell. λ phage is a virus with both lytic and lysogenic alternatives to its life cycle (Fig. 2.7). Usually, a 'lytic cycle' is followed, where the λ DNA is replicated and new phage particles are produced within the cell. This causes cell lysis, releasing the newly formed phage particles. However, under certain conditions, the phage DNA may integrate itself into the host cell chromosome in the lysogenic pathway. In this state, the λ DNA is called a prophage and stays resident within the host's genome without causing apparent harm to the host.

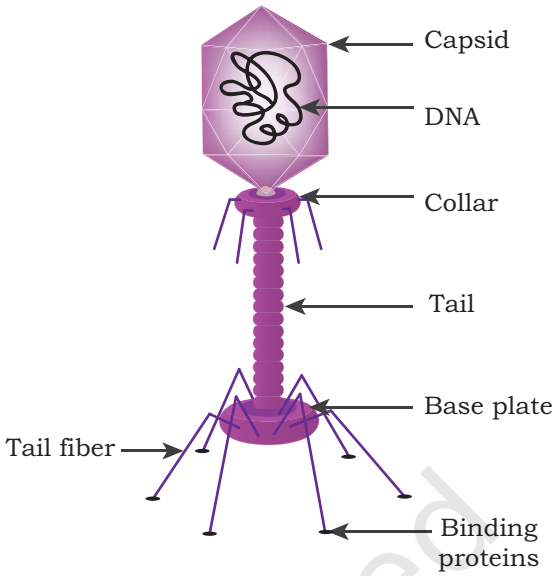


Fig. 2.6: Lambda (λ) bacteriophage

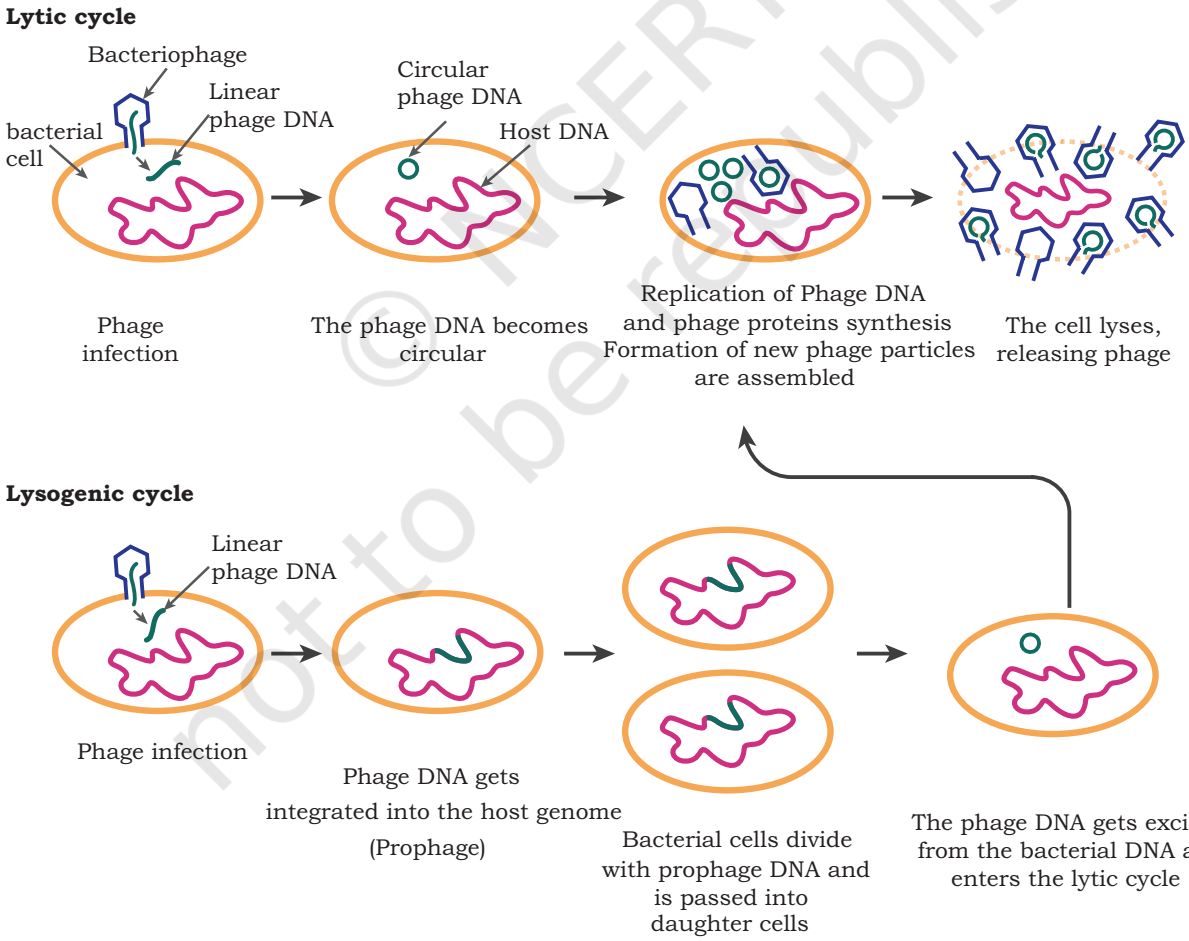


Fig. 2.7: Lytic and lysogenic life cycles of lambda phage

The λ phage genome is linear double stranded DNA containing 48,490 base pairs. Each end has a 12 bases single stranded segment known as ***cos*** sequences (cohesive ends). The two cohesive ends are complementary to each other. The λ genome has a replication origin site, genes for viral head and tail proteins, enzymes of replication involved in lytic and lysogenic cycles. The λ genome remains linear within the phage head [Fig. 2.8 (a)]. Once inside the *E. coli* host cell, the *cos* sequences pair up and cohesive ends are ligated together by host enzyme forming the circular version of lambda genome [Fig. 2.8 (b)]. The sealed cohesive end is called *cos* site [Fig. 2.8 (b)]. The 12 nucleotides long single stranded DNA at both ends pair with each other to form double stranded DNA. Therefore, in its circular form, the phage genome is 48,502 base pairs in length. The phage λ genome can be inserted into host chromosome and is then called a prophage (Fig. 2.7).

During multiplication, the phage genome undergoes rolling circle replication in which a template strand rotates, churning out a chain of many copies of λ genomes to form a long concatemer joined end to end and separated by ***cos*** sites. The concatemer is cleaved at the *cos* sites to yield one unit of phage DNA. This is followed by the packaging of single copy of λ genome into each empty head followed by the attachment of tail and release of newly formed phage particles by lysis of bacterial cells (Fig. 2.7).

Approximately, one-third of the lambda genome, the middle region of the λ genome (which contains genes required for lysogeny) is dispensable (non-essential) for successful lytic infection (Fig. 2.8). Therefore, in order to construct a vector for gene cloning out of the lambda phage DNA, all or part of the middle dispensable region is replaced with an insert segment of appropriate length such that the size of the recombinant DNA (rDNA) lies between 38 to 52 kbp for its efficient packing into the λ head.

Typical vectors coming out of lambda genome fall into two broad classes, namely 'insertion vectors' and 'replacement vectors'. Insertion vectors have a single restriction enzyme site (cloning site) for insertion of insert DNA, whereas replacement vectors have a pair of cloning sites flanking a segment of non-essential bacteriophage

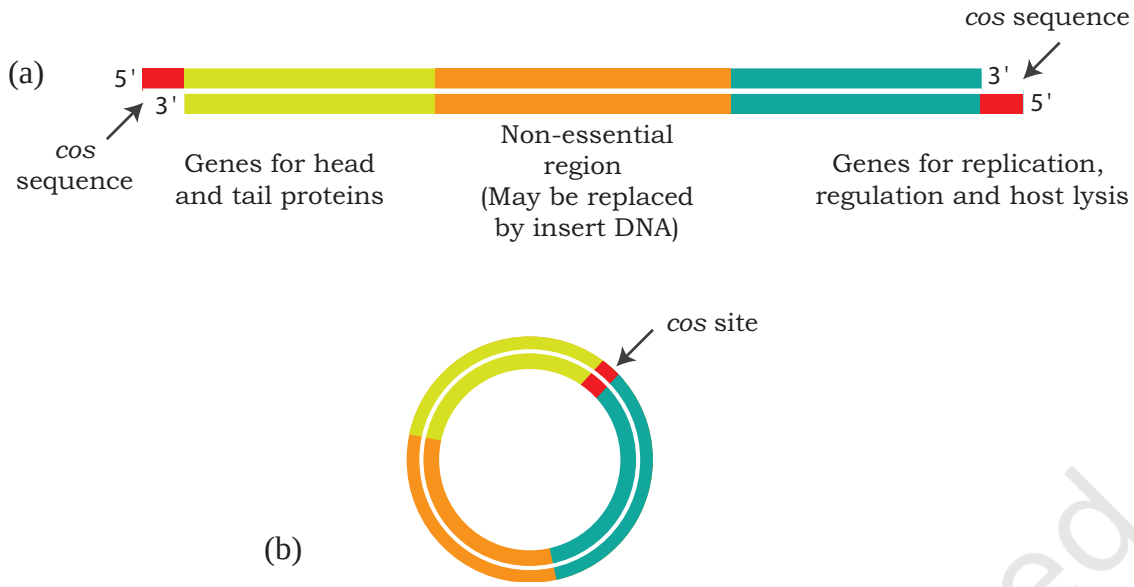


Fig. 2.8: Lambda genome (a) linear inside virus particle (b) circular after infection of bacterial cell

λ DNA (central stuffer region) that can be replaced by the insert DNA.

A large array of λ vectors has been constructed for different purposes. Some commonly used λ bacteriophage based vectors for *E. coli* are given in Table 2.1 below.

Table 2.1: Commonly used λ bacteriophage based vectors for *E. coli* as host.

S.No.	Vector name	Type of vector	Selection of recombinants	Maximum insert size (kbp)
1.	λgt10	Insertion vector	Lytic (plaque formation)	6
2.	λgt11	Insertion vector	Blue white	7.2
3.	λ EMBL3	Replacement vector	Lytic (plaque formation)	20

Bacteriophage M13

M13 is a filamentous bacteriophage of *E. coli*. Its genome consists of a single stranded circular DNA of about 6.4 kb nucleotides which is packaged in a tube like capsid (Fig. 2.9). Almost full genome encodes genes (I-X)required for coat protein, viral replication and viral assembly, except for a small non-essential region flanking *ori* called intergenic region. Bacteriophage M13 infects only *E. coli* cells harbouring F plasmid. These cells have

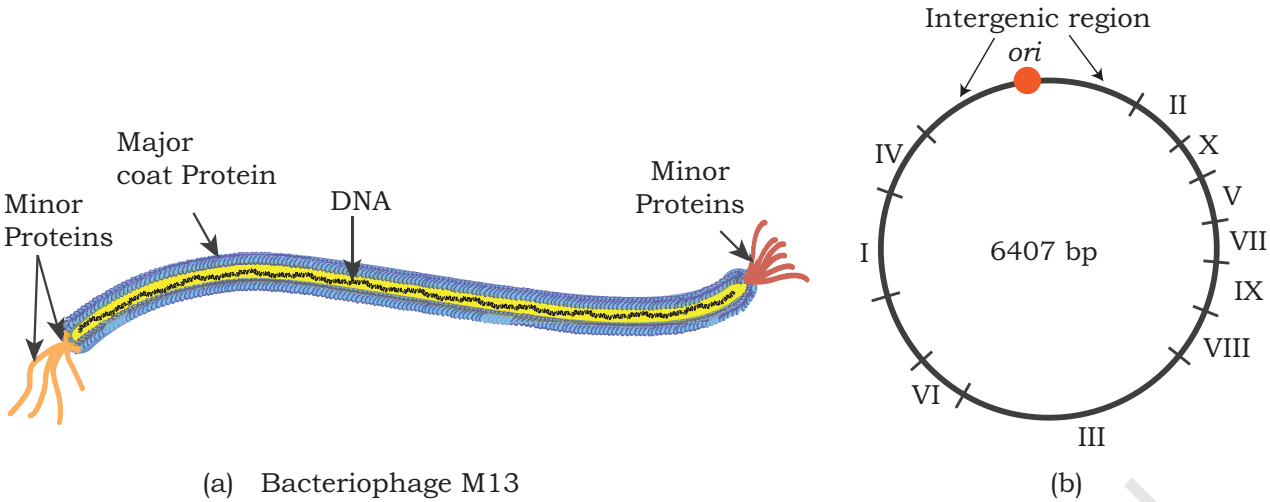


Fig. 2.9: M13 bacteriophage (a) single stranded genome (b) M13 genome indicating different genes I to X

a tube-like structure called 'pilus' extending from their membranes. This pilus is required for attachment and adsorption of the phage to the cell (Fig. 2.10).

During the life cycle of the M13 bacteriophage, the single stranded M13 phage DNA undergoes replication inside the host to generate a double stranded replicative form (RF) (Fig. 2.10). Subsequently, the mode of replication changes to generate a single stranded genomic DNA (+) from double stranded replicative form (Fig. 2.10). The

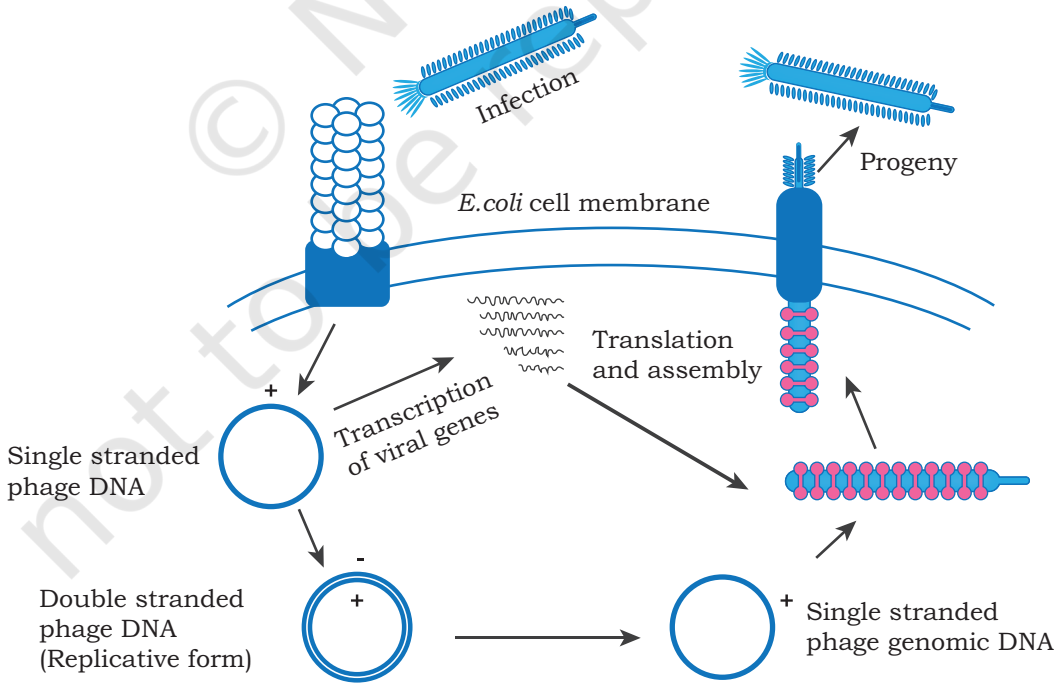


Fig. 2.10: Life cycle of bacteriophage M13

circular single stranded genomic DNA (+) is assembled with capsid proteins during their passage through the host cell without causing cell lysis. The double stranded RF DNA is readily isolated from infected cells for use as a vector, and the single stranded genome is abundantly available in the form of phage in the growth medium. Thus, for M13 vector construction, the double stranded RF of the M13 genome is used. The intergenic region is exploited for putting the insert so that it does not interfere with M13 replication. A large sized DNA (more than 42 kb) can be cloned by M13 bacteriophage. An example of M13 based vector for *E. coli* is M13mp18 which facilitates blue/white selection of the recombinants. You will study blue/white selection in Chapter 3 of this book.

2.3.3 Cosmid vector

A cosmid is a type of hybrid (combination) vector that replicates like a plasmid but can also be packaged *in vitro* into lambda phage coats. A typical cosmid has replication functions, unique restriction endonuclease sites, and selective markers contributed by plasmid DNA, combined with a λ DNA segment that includes the joined cohesive ends (*cos* sites) (Fig. 2.11). As little as 250 bp of λ DNA is sufficient to provide the *cos* junction, including the sequences required for binding to and cleavage by an enzyme called terminase. Advantage of most cosmid cloning vectors is that they accommodate DNA inserts as large as 45 kbp.

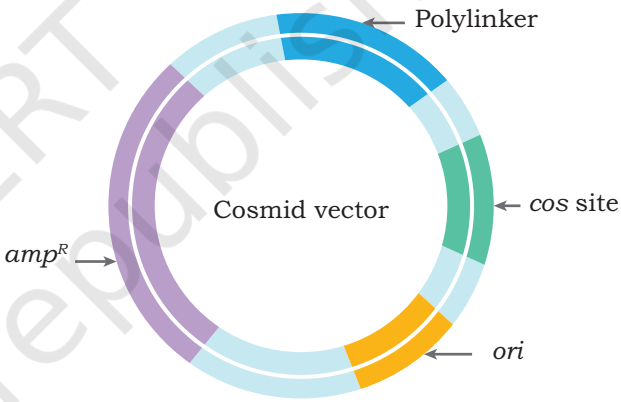


Fig. 2.11: A typical cosmid vector

2.3.4 Phasmids (Phagemids)

Phasmids are true combination (hybrid) vectors between phage and plasmid. They are linear duplex DNAs whose ends are lambda phage DNA that contain all the genes required for a lytic infection and whose middle region is linearised plasmid. Both the lambda phage and the plasmid replication functions are intact (Fig. 2.12). Phasmid recombinants are packaged *in vitro* before infection. Once inside an *E. coli* cell, the phasmid can replicate like a phage

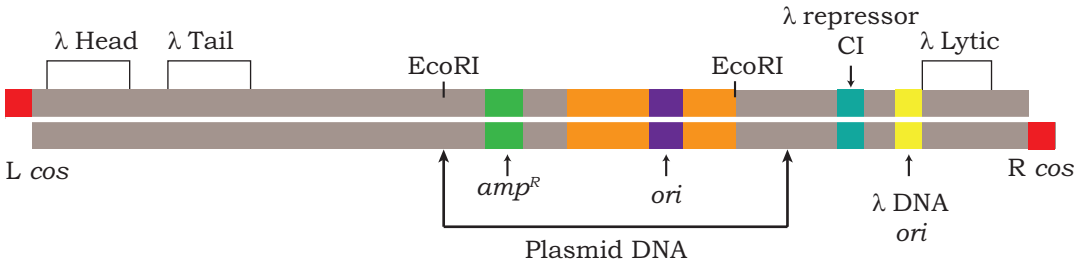


Fig. 2.12: A typical phasmid vector

and form plaques in a normal way. However, if the vector contains the gene that encodes the lambda repressor, then the phasmid replicates as a plasmid rather than as a phage.

2.4 Eukaryotic Host Vector System

Genome analysis of the higher organisms requires the cloning of much larger fragments because eukaryotic genes contain introns, which may be hundreds of kilobases in length and even if a eukaryotic gene without introns is to be inserted, its size exceeds the maximum length as an insert for a plasmid. Therefore, special vectors are required for such large DNA fragments. Consequently, 'artificial chromosomes' have been developed to carry huge lengths of eukaryotic DNA.

Among eukaryotic host vector system, the most common is the baker's yeast, *Saccharomyces cerevisiae*. Although yeast can reproduce sexually, the cells usually multiply asexually by budding. They grow as single cells in suspension and produce colonies in solid medium, much as in *E. coli*. A large collection of metabolic, biosynthetic, and cell cycle defective mutants are known and genetically mapped. Because of a long history of safe use of *S. cerevisiae* in food industry, it belongs to the category which is '**G**enerally **R**ecognised **A**s **S**afe' **(GRAS)** organisms.

S. cerevisiae also harbors a double stranded circular plasmid called 2 µm plasmid, which has been used to develop a number of vectors. One important category of vectors that has been designed for cloning large size DNA insert (200–500 kb) is known as **Yeast Artificial Chromosomes** (YACs). The YAC vector consists of two copies of a yeast telomeric sequence (telomeres are the

sequences at the ends of chromosomes), a yeast centromeric sequence, a yeast ARS (an autonomously replicating sequence where DNA replication begins), and appropriate selectable markers. YAC has two forms, a circular form for growing in bacteria, and a linear form for growing in yeast (Fig. 2.13). The circular form can be manipulated and grown like any other plasmid in bacteria since it has a bacterial origin of replication and an antibiotic resistance gene. In order to use this in yeast, the circular form is isolated and linearised such that the yeast telomere sequences are on each end.

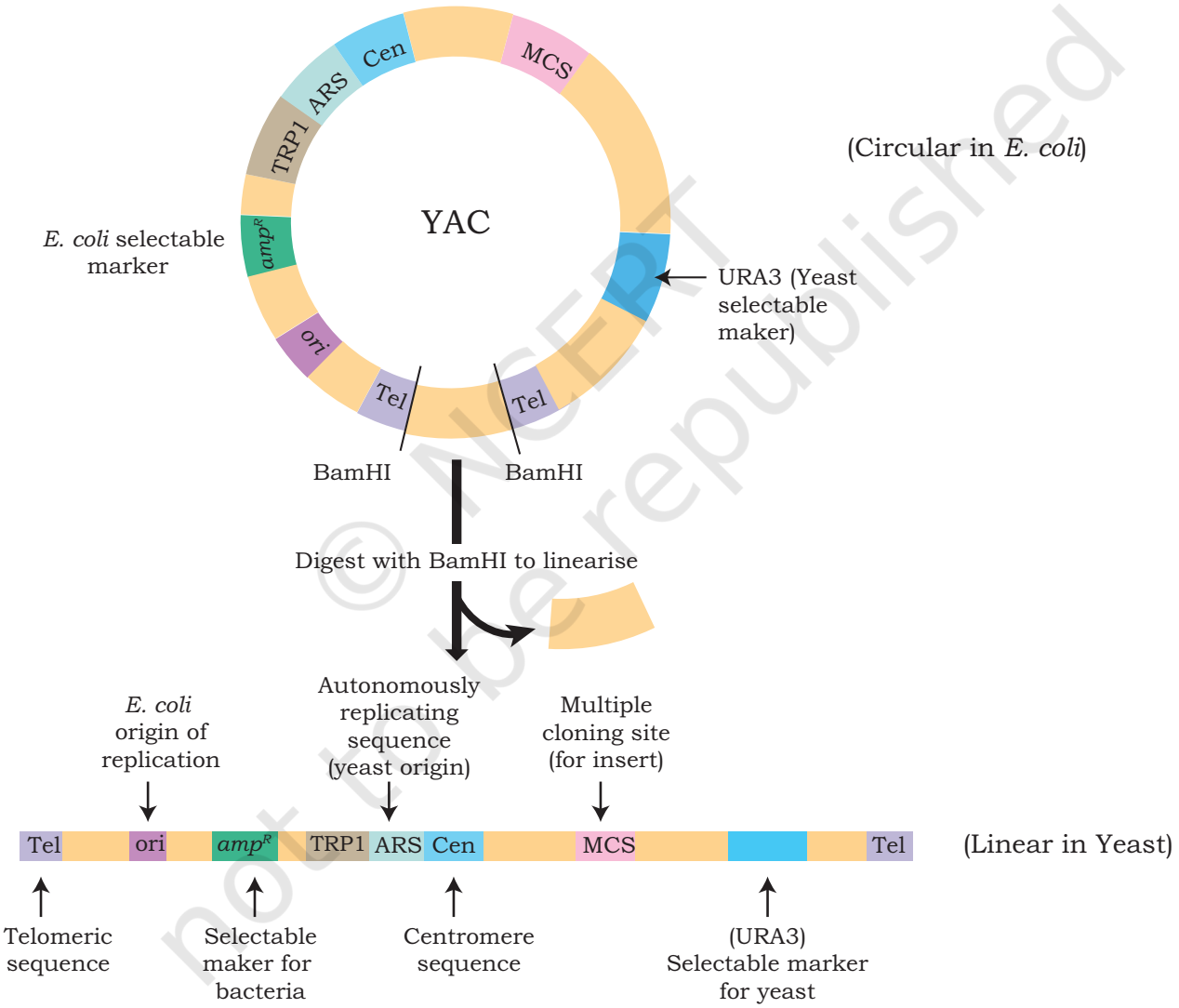


Fig. 2.13: Yeast Artificial Chromosome (YAC)

Vectors like YACs are sometimes called high capacity cloning vectors. Some other high capacity cloning vectors coming from bacterial plasmid and phage are called **Bacterial Artificial Chromosomes (BACs)** and **Phage Artificial Chromosomes (PACs)**.

A list of commonly used vectors for gene cloning having different capacities to take up different size inserts are shown in Table 2.2.

Table 2.2: DNA insert size with different cloning vectors

S. No.	Vectors	Insert size (kb)
1.	Plasmid	≤ 10kb
2.	Bacteriophage	8–25 kb
3.	Cosmids	23–40 kb
4.	PAC	100–300 kb
5.	BAC	≤ 300 kb
6.	YAC	200–500 kb

2.5 Expression Vectors

Till now we have discussed the different types of cloning vectors which are used to propagate DNA. Another type of vectors are expression vectors which act as vehicles for DNA insert and also allow the DNA insert (gene) to be expressed efficiently (Fig. 2.14). These may be plasmids or viruses based vectors. These vectors contain an efficient

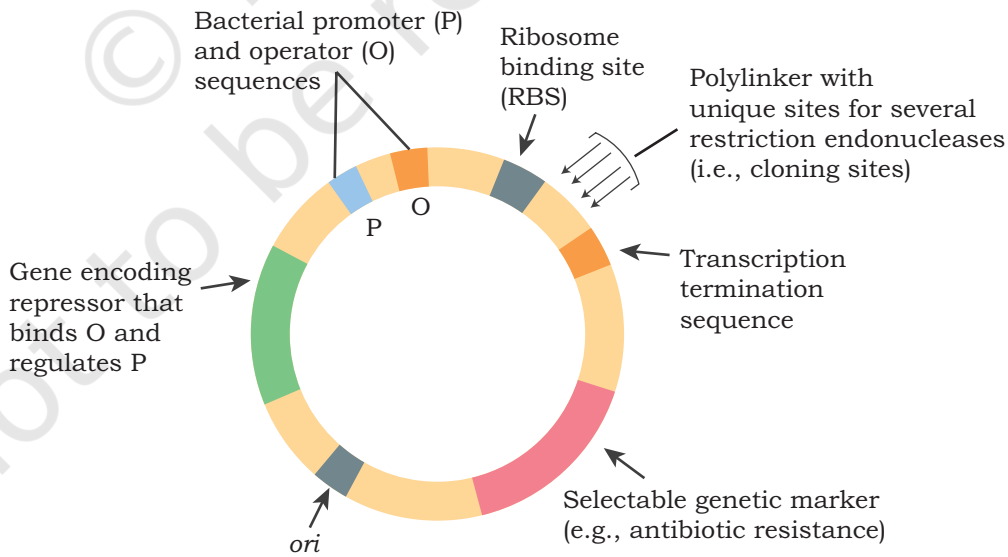


Fig. 2.14: An expression vector

promoter for expression of the cloned genes. These promoters are generally inducible in nature so that expression of the cloned gene can be regulated. Downstream of the promoter there are unique restriction sites for insertion of the gene to be expressed. Furthermore, in order to ensure correct termination of transcription of the cloned genes, a proper transcription termination sequence is provided by the vector near the 3' end of the gene. Thus, the cloned gene is inserted between a promoter towards the 5' end (upstream) and a terminator at the 3' end (downstream) of the cloned gene in an expression vector. This portion of the vector is called expression cassette. Such vectors are therefore, sometimes also called **sandwich expression vectors**.

Expression of a gene by recombinant strategies depends on the source of the gene as well as the host being used. Thus, for example, if a gene of eukaryotic origin is to be expressed in a prokaryotic host (or a vice versa) known as heterologous gene expression, as the gene being expressed is foreign to the host. Such heterologous expression requires that the eukaryotic gene being expressed: (i) does not contain intron(s) as the process of splicing is absent in the prokaryotic host, (ii) expressed protein does not require post translational modification (e.g. glyocosylation) for its biological activity (functionality).

2.6 Shuttle Vectors

Vectors developed for replication in two alternative hosts (either prokaryotic or eukaryotic) are called shuttle vectors. These vectors are themselves constructed by recombinant DNA techniques, and many different types have been made. Some of them shuttle between two prokaryotic species, others between a prokaryote (usually *E. coli*) and eukaryotic cells (including yeast, plants and animals).

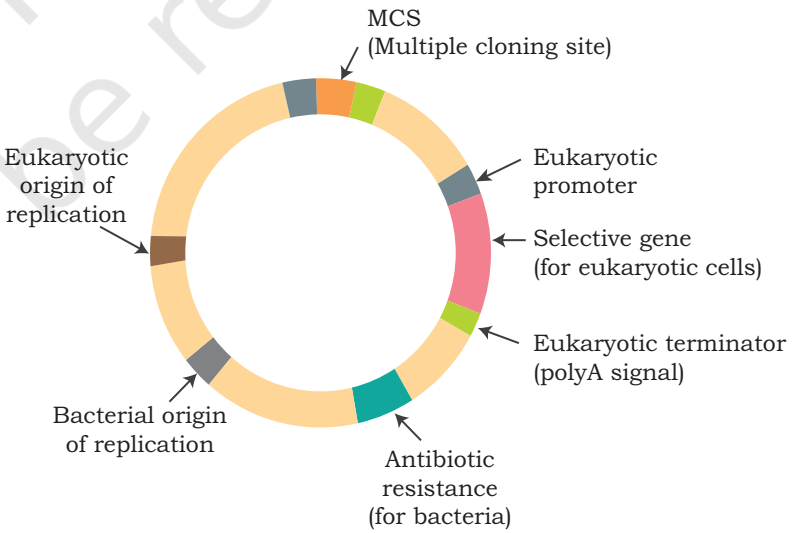


Fig. 2.15: Shuttle vector for E. coli and yeast

Indeed, most of the eukaryotic vectors are shuttles. Shuttle vectors contain two origin of replication, however, in a given host, only one origin is active at a time (Fig. 2.15).

SUMMARY

- rDNA technology is a two-component system: a compatible host and a vector combination, where the vector provides essential sequences required for its replication in a compatible host that provides various replication functions.
- The cloning vector should be small in size and have an origin of replication (*ori* site), unique restriction sites and selectable marker.
- Plasmids are circular, extra-chromosomal double stranded DNA (dsDNA) capable of autonomous replication.
- The bacteriophage lambda (λ) and M13 are the two most common phages whose genomes have been frequently used to make cloning vectors for *E. coli* host.
- The bacteriophage lambda, a bacterial virus that infects *E. coli*, has been widely used as a cloning vector.
- Typical vectors coming out of lambda genome fall into two broad classes, namely 'insertion vectors' and 'replacement vectors'.
- M13 is a filamentous bacteriophage of *E. coli* having genome consisting 6.4kb long circular DNA packaged in a tubular capsid.
- An example of M13 based vector for *E. coli* is M13mp18 that facilitates blue/white selection of recombinants.
- Cosmids are a type of hybrid (combination) vector that replicate like a plasmid but can be packaged *in vitro* into lambda phage coats.
- A typical cosmid has replication functions, unique restriction endonuclease sites, and selective markers contributed by plasmid DNA, combined with a lambda DNA segment that includes the joined cohesive ends (*cos* sites).
- Phasmids are true combination vectors between phage and plasmid. They are linear duplex DNAs whose ends are lambda segments that contain all the genes required for a lytic infection and the middle segment is linearised plasmid.

- Among eukaryotic host vector system, the most common is the baker's yeast, *Saccharomyces cerevisiaes,* from which YAC's have been derived through genetic engineering.
- A YAC cloning vector consists of two copies of a yeast telomeric sequence (telomeres are the sequences at the ends of chromosomes), a yeast centromeric sequence, a yeast ARS (an autonomously replicating sequence) and appropriate selectable markers.
- Expression vectors facilitate efficient-expression of cloned genes. They contain inducible promoters, unique insertion sites, and termination sequences. In heterologous expression, genes must lack introns and not rely on post-translational modifications.
- Shuttle vectors are versatile DNA constructs that replicate in two hosts (prokaryotic and eukaryotic). They are made using recombinant DNA methods and can switch between active replication origins in a host.

EXERCISES

1. Describe the importance of host vector system in rDNA technology.
2. What are the major characteristics of a vector?
3. What is plasmid and what are its different types?
4. Discuss the strategy applied for the development of (pBR322) plasmid cloning vectors.
5. Briefly describe the structure of lambda bacteriophage and also discuss the role of lambda phage based vectors.
6. Discuss the M13 based vectors and its application.
7. Differentiate between cosmids and phagemids.
8. Why is a vector required for cloning of a gene?
9. A plasmid capable of getting integrated into host chromosome is called:
   (a) Col plasmid
   (b) Episome
   (c) Ti plasmid
   (d) R plasmid
10. Why the replication of single copy plasmid called stringent replication?

11. Identify the incorrect match pair from the following:

(i)	Multicopy plasmid	(a)	Stringent replication
(ii)	Col plasmid	(b)	Kills bacteria
(iii)	pBR322	(c)	Plasmid
(iv)	Prophage	(d)	Phage genome inserted into a host genome

12. How can a large size eukaryotic gene insert be cloned?
13. **Assertion:** An ideal vector should have selectable marker.

    Reason: Selectable markers are required to screen out transformation.

    (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.

    (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.

    (c) Assertion is true but reason is false.

    (d) Both assertion and reason are false.
14. **Assertion:** Cosmid is a hybrid vector.

    Reason: Cosmid has properties of both plasmids and lambda phage vector.

    (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.

    (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.

    (c) Assertion is true but reason is false.

    (d) Both assertion and reason are false

Chapter 3 Gene Cloning

Gene cloning is a customary procedure to use a gene for its product in the biotechnology industry and various other purposes. Traditionally, it engages the transfer of a DNA fragment containing the gene of interest to a host cell by a vector so that many copies of the gene will be available for its characterisation and future application. Technological breakthrough in the field of genetic engineering have made it possible to analyse DNA, isolate a specific gene from a genome, enzymatically inserting it into an autonomously replicating vector (e.g. plasmid) to generate rDNA molecule and ultimately introducing into host (e.g., bacteria) to produce a virtually unlimited number of copies (clones) of it. This chapter will expose students to all the procedures involved in gene cloning.

3.1 Identification of Candidate Gene

Over the past decades, rDNA technology has been utilised to produce crops that are resistant to pests, diseases, herbicides and pathogens. This is possible by manipulating the specific gene of interest of one organism followed by its transfer into the genome of another organism, which upon expression results in the desired product or activity.

The first and most formidable problem in this process is to identify the candidate gene in the genome of an organism.

Identification of a gene to be cloned depends upon its significance with regard to its role in biomedical, economical and evolutionary fields. This information on a gene comes from its biochemical and physiological studies. For example, the cause of diseases (diabetes in human beings due to deficiency of insulin) or defect in the metabolic pathways (iron deficiency leading to chlorosis in plants) or resistance to environment (salinity tolerance in plants) or resistance to infection (both in plants and animals) or economically important genes (milk protein, blood clotting factors, etc.) are candidate genes for the improvement of human health and needs. Once a gene of interest is identified, it is explored in new sources and the same is cloned as mentioned in the subsequent sections.

Searching a gene of interest is not an easy task. This will be clear from the following example. As you know, a haploid human genome contains approximately 3.2 billion bp. Therefore, searching a gene of interest having a size of 3000 to 3500 bp, which is one-millionth of the genome; is perhaps more difficult than looking for a needle in the haystack.

There are a few methods developed to achieve this task. One of these methods is to deduce the DNA sequence of the gene coding for a specific polypeptide chain based on its amino acid sequence. Another way to synthesize candidate gene is to isolate the mRNA of the desired gene from specific tissue, then synthesizing single stranded cDNA by using reverse transcriptase enzyme and converting that in to double stranded cDNA as candidate gene, which can be cloned (as discussed in the subsequent section).

3.2 Isolation of Nucleic Acids

The first and foremost requirement for any molecular biology experiment is isolation of nucleic acids from organisms. Extraction of nucleic acids is encountered by two big challenges. First one is related to their availability in cells as DNA and RNA, both of which are present in very

small amounts in cells in comparison to other biological macromolecules, such as proteins, carbohydrates and lipids. Second, the enormous length of nucleic acids, particularly makes it susceptible to cleavage when exposed to harsh physical stress. In addition, the chemical bonds by which different components of nucleic acids are joined with each other and various groups present in them make nucleic acid vulnerable to chemical agents.

Four important steps are involved during the extraction of nucleic acids. The first step involves the effective rupture of cell membrane or walls to release the nucleic acids and other cellular molecules. The second step involves the protection of nucleic acids from their respective degrading

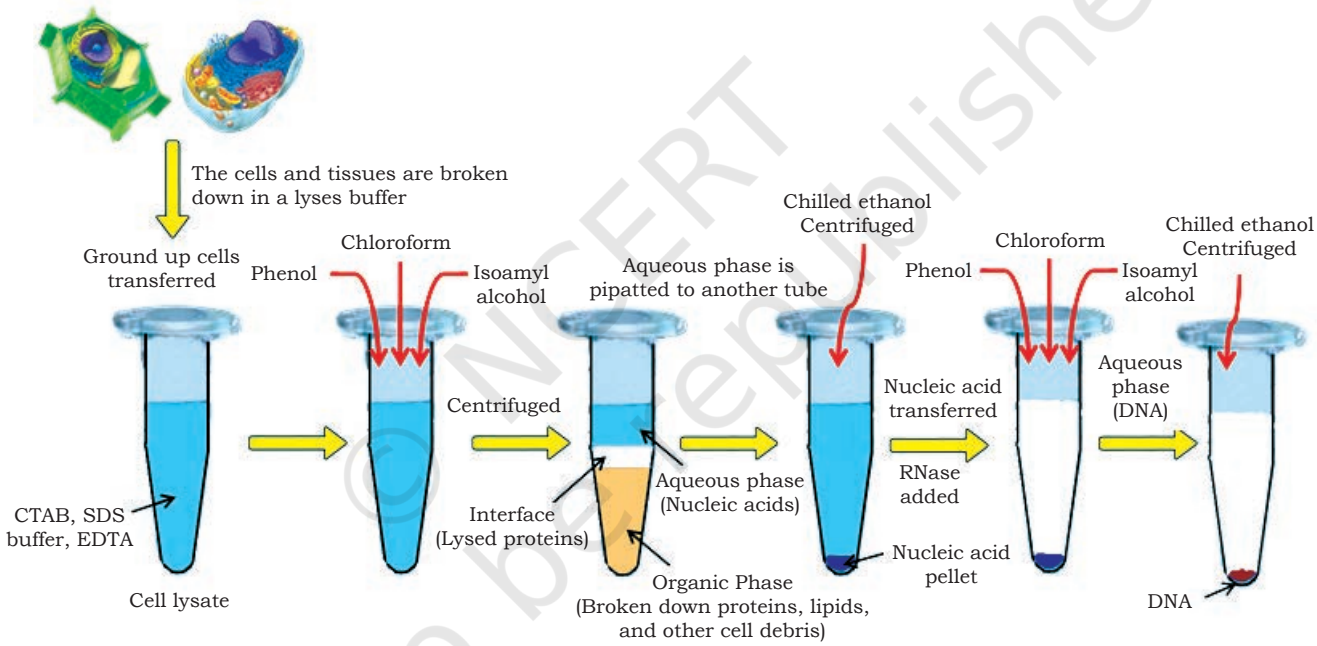


Fig. 3.1: Steps involved in the isolation of DNA

enzymes, which are released in the isolation medium with other proteins. In the third step, the nucleic acids are separated from other molecules. In the fourth and the last step, the isolated nucleic acids are precipitated and concentrated by adding ethanol or isopropanol.

Although, chemical and physical properties of nucleic acids are similar in all organisms, the outer boundary of cells differs from one organism to another. Therefore, in order to disrupt the cell boundaries for releasing nucleic acids into extraction medium, different strategies are adopted. Animal cells have plasma membrane that can be easily disrupted. On the contrary, plant cells and bacteria are protected by tough layers (e.g., cell wall), which need different approaches for their lysis. These include homogenisation, grinding, sonication or enzymatic treatment. Such mechanical or enzymatic treatment ruptures plasma membrane or cell wall so that nucleic acids get released from cells and exposes them to nuclease enzymes (deoxyribonuclease and ribonuclease), which are also released simultaneously.

Isolation of DNA

As bacterial cells have little structure beyond the cell wall and cell membrane, isolating DNA from them is much easier. An enzyme called lysozyme digest the peptidoglycan, the main component of bacterial cell wall. Detergents like sodium dodecyl sulphate (SDS) is used to lyse the cell membranes by disrupting the lipid bilayer. Plant and animal cells are ground to release the intracellular components. Plant cells are mechanically ruptured in a blender to break open the tough cell walls. For isolation of DNA from plant cells, cetyl trimethyl ammonium bromide (CTAB) is used as detergent (a cationic detergent). Plant cells have high concentration of polysaccharide and polyphenols in comparison to animal cells and pose problems during isolation of DNA. The solubility of DNA and polysaccharides to CTAB depends on ionic strength of the solution. At low ionic strength, DNA is soluble in CTAB solution while polysaccharides are insoluble; whereas at high ionic strength, polysaccharides are soluble and DNA is insoluble. In addition, being a detergent, it also

lyses cell wall. Both the molecules are separated based on their differential affinity to CTAB. Addition of polyvinyl pyrrolidone (PVP) to CTAB extraction medium neutralises phenols. Soluble DNA present in supernatant is extracted with chloroform-isoamyl solution. DNA present in aqueous phase is precipitated using ethanol or isopropanol. In case of animal cells, the cell membrane is disrupted by detergent to release the intracellular components.

The cells and tissues from which nucleic acids are to be extracted are broken down in a medium either mechanically or enzymatically. The media is usually a buffer having mild alkaline pH with minimum ionic strength (0.05 M) containing chelating agent ethylene diamine tetraacetic acid (EDTA). The mild alkaline pH facilitates the reduction of electrostatic interaction between DNA and basic proteins (histones) released during cell disruption. Chelating of divalent cations particularly Mn^{2+} and Mg^{2+} prevents the action of nucleases. Further, inhibition of their activities is achieved due to alkaline pH of the buffer. In addition, chelating of divalent cations prevents the formation of their respective salts with phosphate groups of nucleic acids.

The next step is to separate nucleic acids from its bound proteins. This is achieved by decreasing interaction between proteins and nucleic acids so that nucleic acids are free of proteins, by exposing to detergents, like SDS, an anionic detergent. Exposure to SDS makes all the protein molecules anionic. Consequently, basic proteins that are positively charged and bound to negatively charged nucleic acids become negatively charged and dissociate from the nucleic acids. In addition, SDS also prevents the activities of nucleases thereby giving additional protection to nucleic acids from nucleases. Then sodium chloride is added to the medium at high concentration. Increased salt concentration diminishes the ionic interaction between DNA and cations thus ensuring complete dissociation of DNA and protein complexes. Deproteinisation of the medium is achieved by exposing it to chloroform and isoamyl alcohol. These solvents are non-polar in nature when it is added to the medium that is polar in nature and subjected to centrifugation, it gives three distinct layers. Since, the density of organic solvent mixture is higher than water, it forms a lower layer (which contains denatured

proteins) while the upper layer is aqueous in nature and contains nucleic acids. Chloroform causes denaturation of proteins while isoamyl alcohol prevents the formation of foam and helps in stabilising the interface between lower organic and upper aqueous phase, that can be separated by pipetting. The nucleic acids from aqueous phase are precipitated by addition of ethanol to aqueous medium that reduces its polarity, which makes aqueous medium as non-polar and thus nucleic acids become insoluble that were otherwise soluble in aqueous medium. To remove RNA, the enzyme ribonuclease A is added that digests RNA into ribonucleotides. DNA is then isolated by centrifugation and stored at low temperatures (Fig. 3.1).

RNA Isolation

RNA is single stranded, while DNA is mostly double stranded. Ribonucleases (RNases), a group of enzymes that degrade RNA molecules, are abundant in the environment and it is difficult to remove or destroy RNases completely. Thus, it is often difficult to isolate intact RNA.

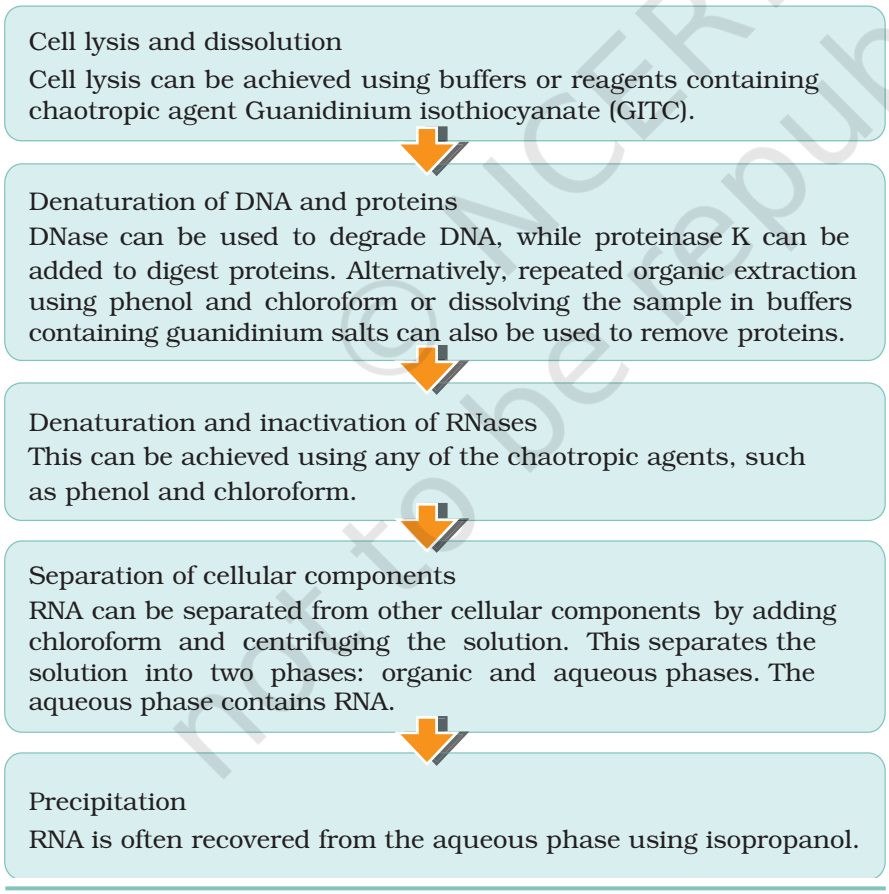


Fig. 3.2: (a) Flow chart for the isolation of RNA

Total RNA is extracted from biological samples by using a specific reagent known as guanidinium isothiocyanate (GITC)-phenol-chloroform. GITC is a chaotropic reagent and acidic in nature as it disrupts the hydrogen bond and releases energy to increase entropy (chaos) that reduces hydrophobic effect of the solution resulting in the aggregation of proteins and nucleic acids. Phenol causes denaturation of proteins

whereas chloroform solubilises lipids. Chloroform also enhances specific gravity of phenol with respect to water. When biological samples are treated with GITC solution and subjected to centrifugation, the solution gets separated into three phases: upper aqueous phase, followed by interface and organic phase. Although total RNA is extracted in the aqueous phase due to the acidic nature of the reagent whereas DNA and denatured proteins are retained in the interface or organic phase. This step also inactivates RNase enzyme that may hydrolyse RNA. Subsequently, RNA from aqueous phase is precipitated with the help of isopropanol [Fig. 3.2 (a) and b)].

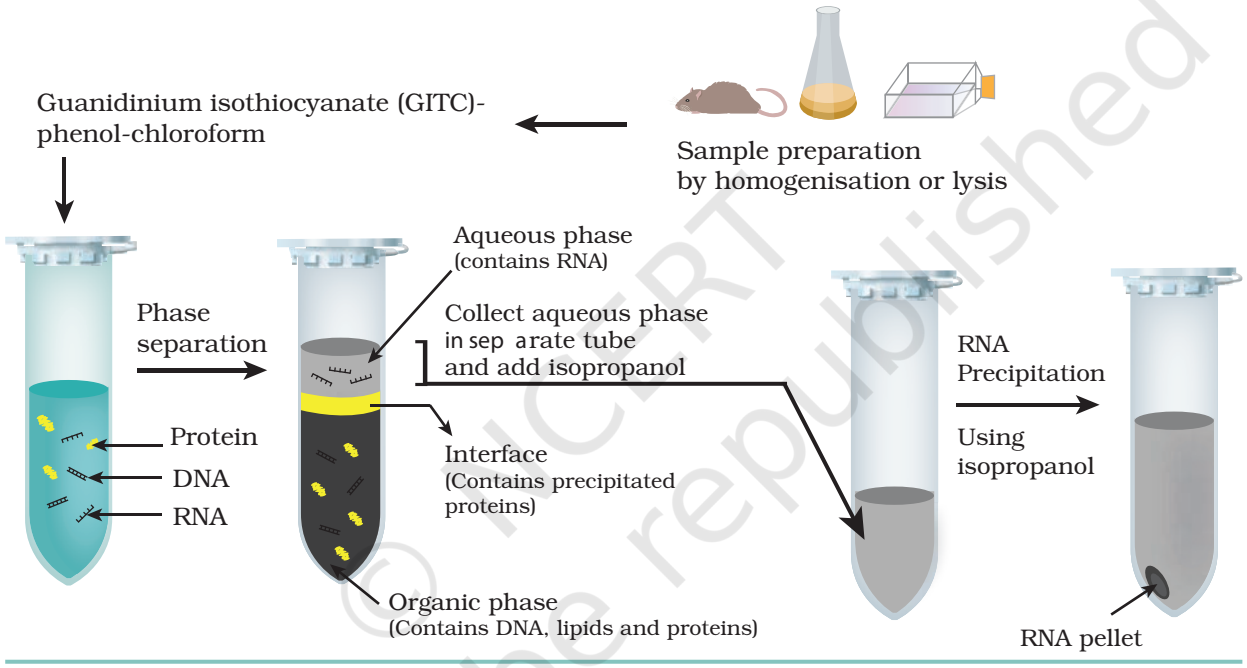


Fig. 3.2: (b) RNA extraction

Box 1: Separation of Plasmid DNA from Genomic DNA

Two types of DNA molecules are isolated in cloning experiments. One is plasmid DNA and the other genomic DNA from bacteria. Chromosomal DNA is separated from plasmid DNA by boiling bacterial lysate. The boiling of lysate causes irreversible denaturation of chromosomal DNA and denaturation of proteins including deoxyribonuclease. Boiling causes formation of a gel, which is precipitated by centrifugation. On the contrary, partially denatured plasmid DNA (due to boiling) is renatured as circular double helix and become soluble. In another method, bacterial suspension is lysed and its contents are denatured by anionic detergent SDS and NaOH solution. The broken cell wall, chromosomal DNA and denatured proteins are clumped as a large mass coated with SDS that are precipitated from solution by replacing sodium ions (Na^+) with potassium ions (K^+). The precipitate is then separated by centrifugation. The plasmid DNA is isolated from the supernatant by ethanol precipitation.

3.3 Enzymes used for Recombinant DNA Technology

Enzymes constitute an important tool in rDNA technology. The major task of the manipulation of the DNA involves cutting and ligation of the vector DNA and the gene of interest. For this, the natural abilities of different enzymes found in organisms are exploited. The major enzymes used in the rDNA technology are:

(i) Nucleases: Nucleases are the enzymes, which cleave nucleic acids by hydrolysing the phosphodiester bond that joins the sugar residues of adjacent nucleotides. Some nucleases are DNA specific called DNases and some are RNA specific called RNases. There are two major types of nuclease enzymes depending on their preference of the location of phosphodiester bonds of polynucleotide chains (DNA or RNA or synthetic polynucleotide chain) namely, exonuclease and endonuclease. Exonuclease, as the name suggests, removes the nucleotides one at a time, i.e., mononucleotides,

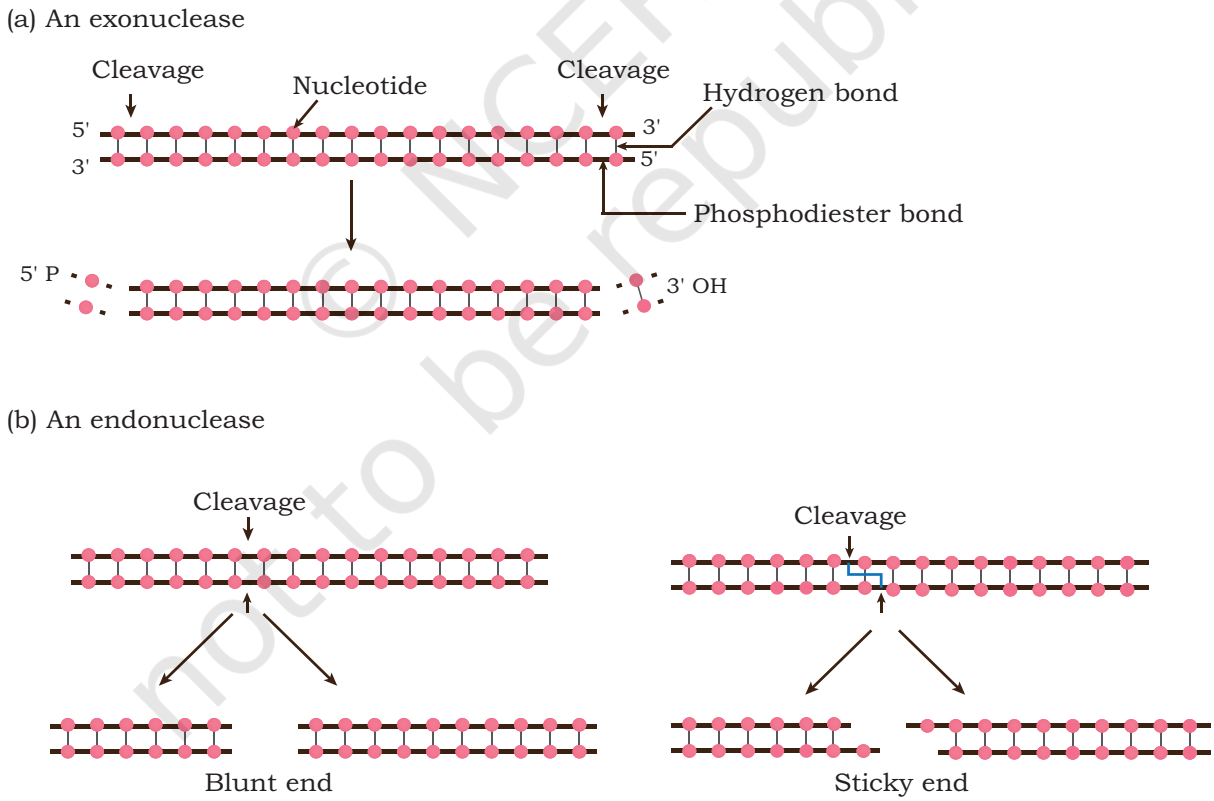


Fig. 3.3: (a) An exonuclease, which removes nucleotides from the end of DNA molecule (b) An endonuclease, which breaks internal phosphodiester bonds

either from the 3′ or 5′ ends of polynucleotide chains. Endonuclease, on the contrary, breaks internal phosphodiester bonds within a DNA or RNA molecule [Fig. 3.3 (a) and (b)].

(ii) Restriction endonuclease/enzyme (RE):

Endonuclease enzymes that cleave DNA molecules at a specific position are called restriction endonucleases or restriction enzymes. They are mostly found in bacteria and archaea that provide a defense mechanism against invading bacteriophages. RE recognises and binds to a specific DNA sequence called recognition sequence or site, often consisting of 4 to 8 bp.

Restriction enzymes are categorised mainly into three groups (Type I, II and III) based on their co-factor requirement and the position of their DNA cleavage site relative to the target sequence. Type I enzymes cleave DNA at a site that is about 1000 bp from the recognition site and require S-adenosyl methionine (SAM), Mg^{2+}, ATP and has DNA strand cleavage, methylase and ATPase activities. Type II enzymes cleave within the recognition site and require Mg^{2+} and has only DNA strand cleavage activity (Fig. 3.4). Type II REs find application in rDNA technology. Type III enzymes cleave at sites about 24 to 26 bp away from the recognition site; require S-adenosyl methionine (SAM), Mg^{2+}, ATP and has DNA strand cleavage and methylase activities (Table 3.1).

Box 2

The 1978 Nobel Prize in Physiology or Medicine was awarded jointly to Werner Arber, Daniel Nathans and Hamilton Smith for the discovery of 'restriction enzymes' and their application to the problems of molecular genetics. HindII was the first restriction enzyme to be isolated by Hamilton Smith.

Table 3.1: Types of Restriction Enzymes

	Cleavage site	Endonuclease and methylase function	Examples
Type I	Random around 1000 bp away from the recognition site	Endonuclease and methylase function on a single protein molecule	EcoKI EcoAI CfrAI
Type II	Specific within the recognition site	Endonuclease and methylase are separate entities	EcoRI BamHI HindIII
Type III	Random 24–26 bp away from the recognition site	Endonuclease and methylase function on a single protein molecule	EcoP1 HinfIII EcoP15I

The recognition sequences of widely applied Type II REs are **palindromic sequences**, meaning the sequence on the forward direction on a double stranded DNA reads same in a reverse direction on the complementary strand. These enzymes break specific phosphodiester bond in both strands of the DNA molecule within the restriction sequence recognised by the enzyme or at the site or near the sequence. It generates a 5′-phosphate group at one end of the break and a 3′-hydroxyl group at the other end of the break (Fig. 3.4). Several REs cleave at different locations on the two DNA strands to produce staggered cut having short single-stranded protruding ends called cohesive or sticky ends. Some REs produce blunt ended cut by cleaving both strands of DNA at same location (Fig. 3.4).

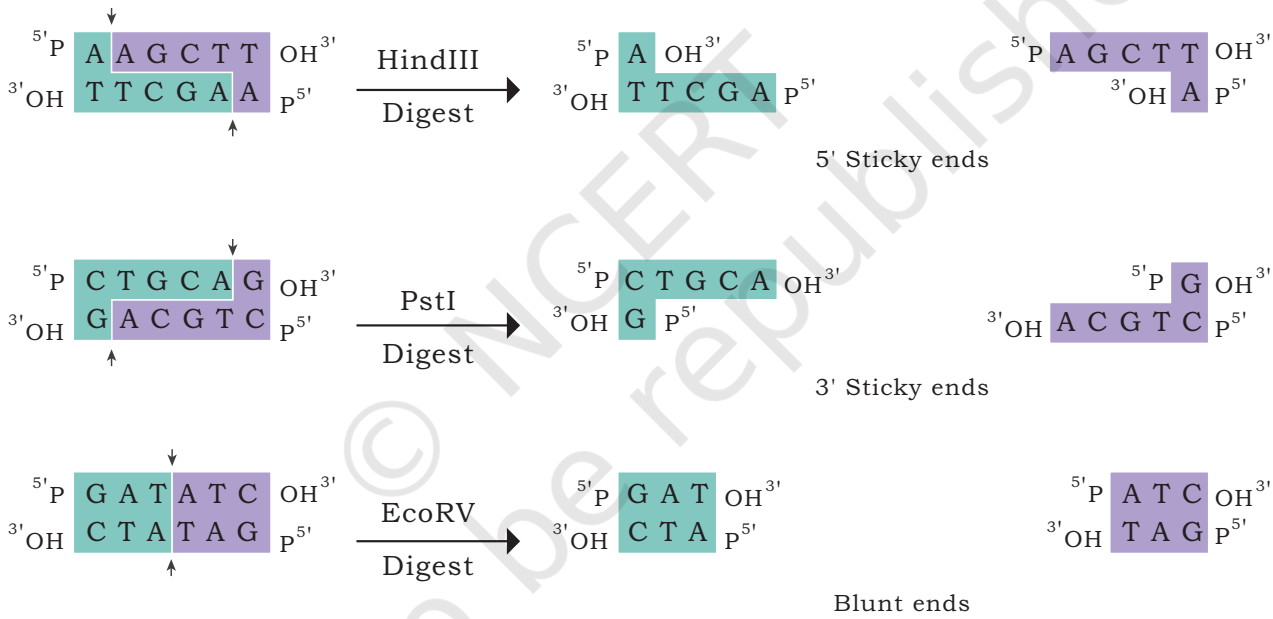


Fig. 3.4: Type II REs generating sticky or blunt ends

Let us now understand the nomenclature of restriction enzymes. The enzyme is named after the microorganism from which it is isolated. The first capital letter represents the genus, the second and third letters represent species. The fourth letter specifies the strain of the microorganism. And the last Roman number represents the number of the enzyme isolated from the species (Table 3.2).

Table 3.2: Nomenclature for restriction endonucleases

EcoRI	
Escherichia (E)	genus
coli (co)	specific epithet
strain Ry13 (R)	strain
first endonuclease (I)	order of identification
HindIII	
Haemophilus (H)	genus
influenzae (in)	specific epithet
strain Rd (d)	strain
third endonuclease (III)	order of identification

(iii) DNA ligase: Ligase enzyme facilitate the joining of DNA strands together by catalysing the formation of a phosphodiester bond in the duplex form (Fig. 3.5). Bacterial DNA ligases, from *E. coli*, use the hydrolysis of NAD as their energy source, whereas ATP is the energy source for DNA ligases from bacteriophages (e.g., T4) and eukaryotic cells. The 5′P(5′–PO_4) group of one chain makes a covalent linkage with the 3′-OH group of adjacent chain. T4 DNA ligase is used to join two DNA molecules having cohesive ends or blunt ends. *E. coli* DNA ligase is used to join cohesive ends.

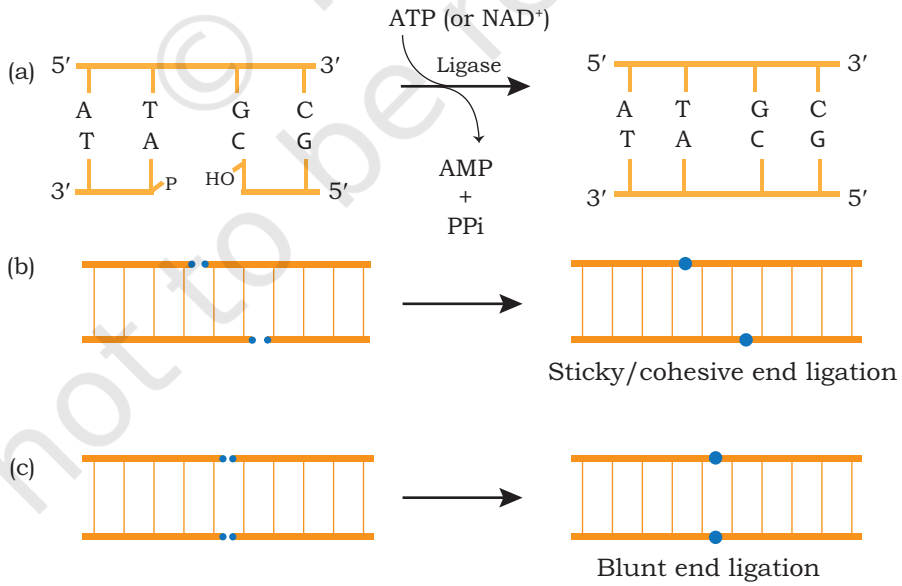


Fig. 3.5: Ligation of DNA by ligase (a) Formation of phosphodiester bond (b) Ligation of sticky end (c) Ligation of blunt end

(iv) DNA polymerases: DNA polymerases are a group of polymerases that catalyse the synthesis of new DNA strand by using mono-deoxyribonucleoside triphosphates (dNTPs) on a template strand. A DNA polymerase enzyme synthesises new DNA strand in 5′→3′ direction (Fig. 3.6). It cannot initiate synthesis of a new DNA strand. In addition to dNTPs, they require a primer (oligonucleotide) carrying a free 3′-end hydroxyl group that can be used as the starting point of chain growth. DNA polymerase I of *E. coli* exhibit several other activities, such as 5′→ 3′ exonuclease and 3′→ 5′-exonuclease.

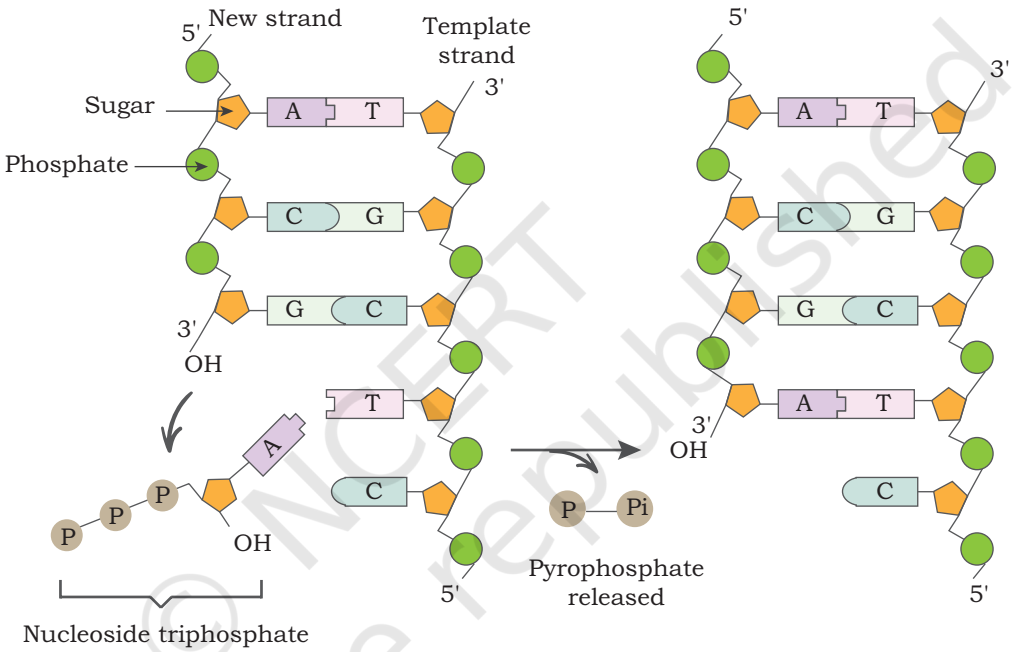


Fig. 3.6: DNA polymerase adds nucleotides at 3'OH end of the DNA molecule

(v) Alkaline phosphatase: Alkaline phosphatase is used to remove the terminal phosphate group from 5′ end of DNA strands.

(vi) Polynucleotide kinase: Using polynucleotide kinase, a phosphate group can be attached to hydroxyl (-OH) group present on 5′ end of DNA. Polynucleotide kinase has the reverse effect of alkaline phophatase, adding phosphate groups onto free 5′ termini.

(vii) Terminal deoxynucleotidyl transferase or terminal transferase: This enzyme can add similar nucleotide residues to form a homopolymer tail on 3′ end of a DNA strand. Unlike most DNA polymerases, it does not require a template.

(viii) Reverse transcriptase: It is also called RNA directed DNA polymerase and is found in many retroviruses. It is used to generate complementary DNA (cDNA) strand from a–RNA template, a process termed as reverse transcription (Fig. 3.7).

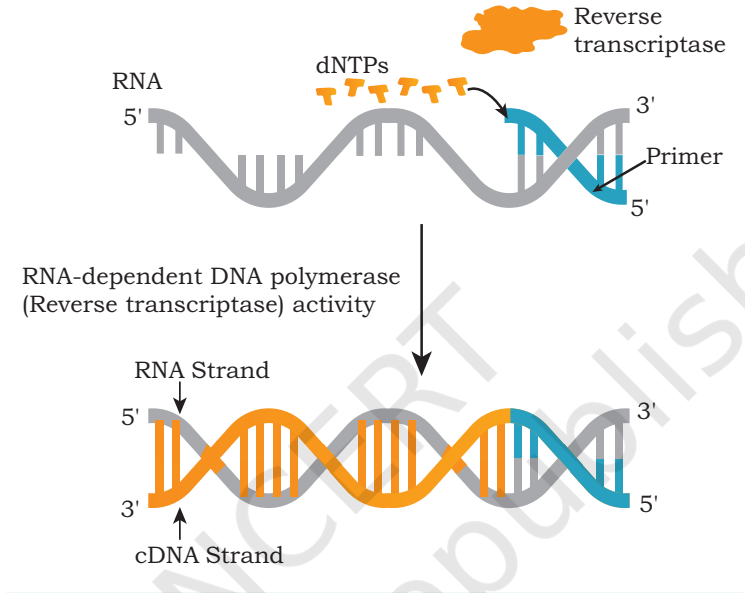


Fig. 3.7: Reverse transcription

(ix) Poly A polymerase: It incorporates adenine residues to hydroxyl group of 3′ end of RNA (Fig. 3.8).

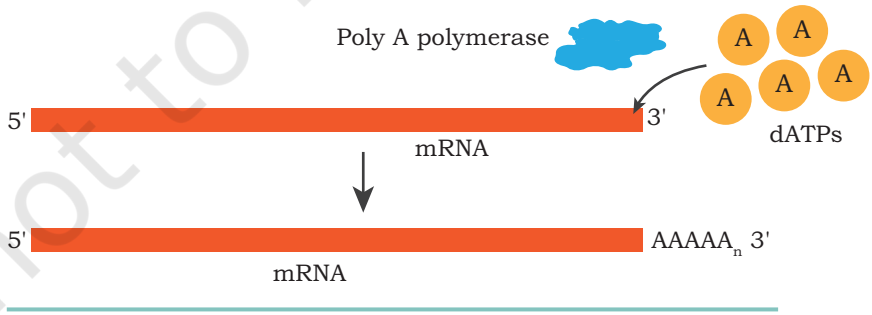


Fig. 3.8: Addition of dATPs by poly A polymerase

3.4 Modes of DNA Transfer

Transfer of a foreign DNA molecule to a host cell (prokaryotic or eukaryotic) from its surrounding environment is one of the basic steps in rDNA technology. In nature, bacteria obtain foreign DNA molecules from its surroundings in three different ways, which are: (i) transformation, (ii) transduction and (iii) conjugation.

(i) Transformation: Transformation is genetic alteration of a cell resulting from the direct uptake of exogenous DNA molecule from its surroundings through the cell membrane and gets incorporated in the recipient genetic material. Recipient cells with foreign DNA molecule are referred to as **transformants** (Fig. 3.9). Transformation occurs naturally in some species of bacteria.

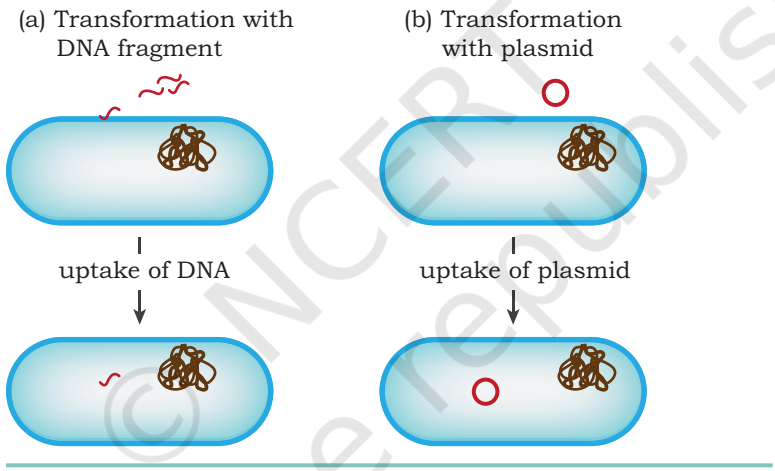


Fig. 3.9: Transformation in bacteria

(ii) Transduction: Viruses may also mediate the uptake of foreign DNA into the genome of a cell. Viruses that specifically infect bacterial cells are known as bacteriophages. Bacteriophages on infecting follow a lytic cycle or a lysogenic life cycle in the host. In **lysogenic life cycle**, the bacteriophage genome gets incorporated into bacterial DNA, and remains dormant for several generations. After a period of time when phage genome gets excised from the host DNA, they occasionally take small sequences of bacterial DNA with them. Phage genome containing bacterial DNA is then packaged into phage coat proteins to

form a complete, recombinant viral particle. When this phage infects a bacterial cell, the recombinant phage genome containing bacterial DNA is introduced into bacteria (Fig. 3.10). The recipient bacterial cell is referred to as **transductants**.

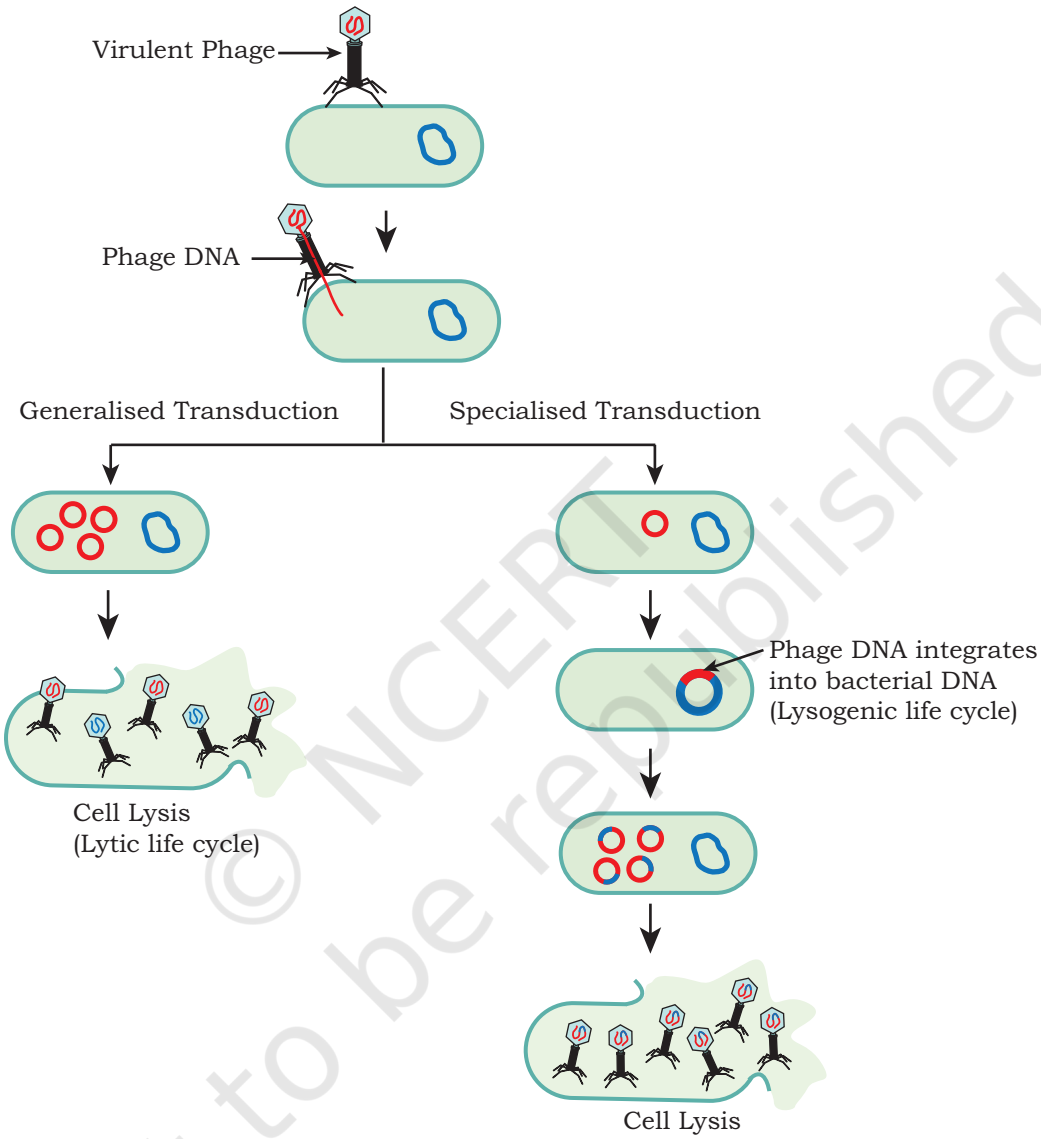


Fig. 3.10: Transduction in bacteria

(iii) Conjugation: Conjugation is referred to as transfer of genetic material (DNA) from one bacterium to another through cell-to-cell direct contact. The bacterial cell that transfers its DNA is called the donor cell and the one that receives is the recipient cell. Conjugation is usually mediated by

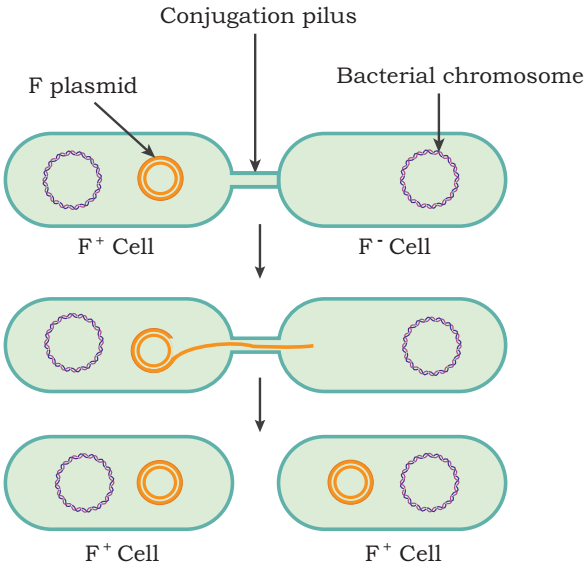


Fig. 3.11: Bacterial conjugation

F plasmids that carry a DNA sequence encoding for the fertility factor, or F–factor. The F-factor forms a thin tube-like structure called **pilus**, through which the donor cell makes contact with the recipient. A nick is made in one of the strands of double stranded F-plasmid by an enzyme relaxase in the donor cell and this strand is transferred to the recipient cell through pilus. Inside both donor and recipient cells, the single-stranded DNA undergoes replication to form double-stranded F plasmid identical to the original F plasmid (Fig. 3.11).

In rDNA technology, the rDNA is introduced (transferred) in host cells by numerous methods. Chemical (calcium chloride, lipofection, etc.) and physical (electroporation, microinjection and gene gun) methods for introducing foreign DNA molecules into host cells are commonly used. In **calcium chloride method**, the DNA to be transferred is mixed in a solution containing positively charged calcium and the negatively charged group of DNA to form a complex. The host cells take up the foreign DNA molecule by a process of heat shock. In **electroporation method**, transient micropores are created on the membrane of host cells by exposing them to mild electric current in the presence of foreign DNA molecules. The recombinant DNA molecules enter into host cells through transient micropores. **Lipofection** (or liposome transfection) is a technique used to inject genetic material into a cell by means of liposomes, which are vesicles that can easily merge with the cell membrane since they are both made of a phospholipid bilayer. Foreign DNA molecules can be introduced directly to the nucleus of host cells using specialised automated **Microinjection** apparatus. In **biolistic method**, with the help of a gene gun (particle gun), microscopic particles (gold, nickel, tungsten) coated with foreign DNA are bombarded to cells at high velocity so that foreign DNA molecule enters inside the cell (Fig. 3.12).

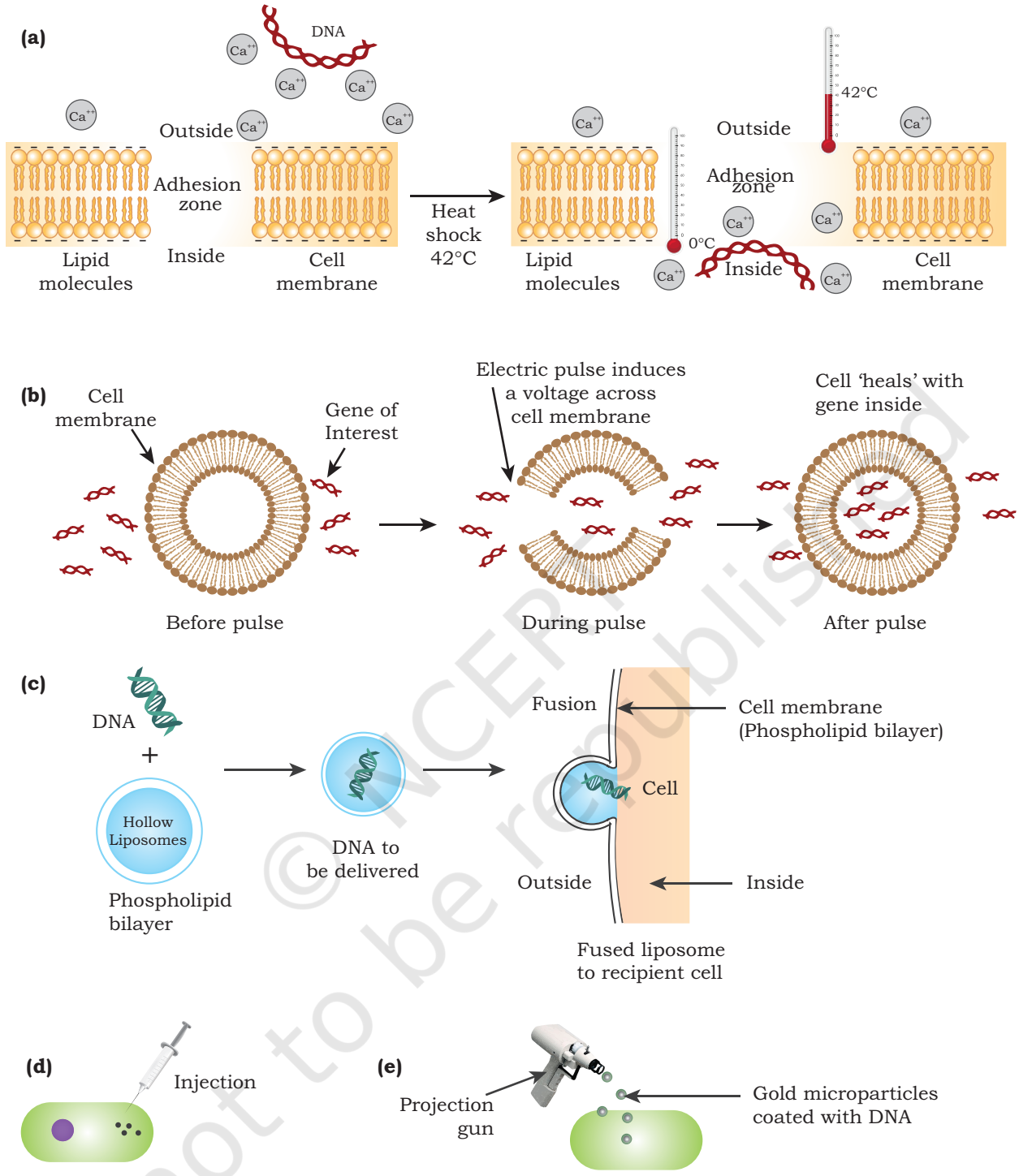


Fig. 3.12: Methods of DNA transfer (a) Chemical ($CaCl_2$), (b) Electroporation, (c) Lipofection (d) Microinjection and (e) Biolistic method

3.5 Screening and Selection

Selection of transformed bacteria with recombinant vectors is the most essential step for a successful cloning experiment. Here, the objective is to identify the transformed cells having recombinant vector from a mixture of non-transformed cells. Success rate of insertion of an insert into a plasmid and subsequent transfer of recombinant plasmids to bacteria is very low. Therefore, it is difficult to select a few bacteria containing plasmids with insert from a large number of bacterial populations without the insert.

The method of selection of recombinant cells is based on the principle of difference in biological traits present in hosts with recombinant DNA from those without recombinant DNA. Thus, the recombinant cells are distinguished from non-recombinants based on their expression or non-expression of certain traits, such as antibiotic resistance, or expression of some specific proteins, such as β-galactosidase or Green Fluorescent Protein (GFP), or dependence/independence of a nutritional requirement, such as amino acid leucine. On the basis of this principle, the selection procedure can be divided into two main types as described in the following section.

(i) **Direct selection of recombinants:** In this method of selection, transformed cells are distinguished from non-transformed cells based on the expression of certain traits. For example, bacterial cells (host) are not resistant to a particular antibiotic but when they take up the plasmids containing antibiotic resistant gene, they become resistant to that specific antibiotic. These cells will survive and grow in a media containing the antibiotic(s), whereas the host cells without plasmid will be killed when they are exposed to antibiotics.

(ii) **Selection of recombinants by insertional inactivation:** This is more efficient than the direct selection method. In this method, a vector having two markers (either two antibiotic resistant genes, or one antibiotic resistant gene and one *lacZ* gene) is used. When the gene of interest (insert DNA) is inserted into one of the selection marker genes in the vector, its expression is disrupted and hence called insertional inactivation. Let

us use a plasmid with two antibiotic resistance genes—one for ampicillin (*amp*^{R} gene) and the other for tetracycline (*tet*^{R} gene). The target DNA (insert) is inserted into *amp*^{R} gene of the plasmid making them recombinant plasmids. Now we have plasmids with insert and without insert. When this plasmid mixture is added to a culture of bacteria as described earlier, there will be three different populations of host bacterial cells: (i) host cells without plasmids (non-transformed), (ii) transformed host cells with plasmids without insert and (iii) transformed host cells with recombinant plasmids (with insert). Now it is essential to identify those cells that have received the recombinant plasmid. This process of screening is based on the property of resistance to ampicillin, which is lost in the host cell having recombinant plasmids. The insert gets cloned in amp^R gene leading to insertional inactivation of ampicillin resistance gene (amp^R) (Fig. 3.13). When these bacteria are plated on a media containing tetracycline, the non-transformed cells get eliminated as they are sensitive to it. Only transformed cells (with functional *tet*^{R}) multiply and form colonies as they are resistant to it. There will be two types of colonies (master plate)— one of transformed cells having plasmid without insert (non-recombinant) and the other of transformed cells having plasmid with insert (recombinant) (Fig. 3.13). By using nitrocellulose membrane, bacterial cells from the master plate colonies are plated on a solid media containing ampicillin. Transformed cells with vectors (without insert) will only multiply to form colonies (replica plate) while transformed cells with recombinant vectors will not grow because their *amp*^{R} gene has been inactivated. Now, if we compare the master plate with replica plate, the colonies present in master plate and absent in replica plate are the transformed cells with recombinant vector containing DNA insert of interest (Fig. 3.13).

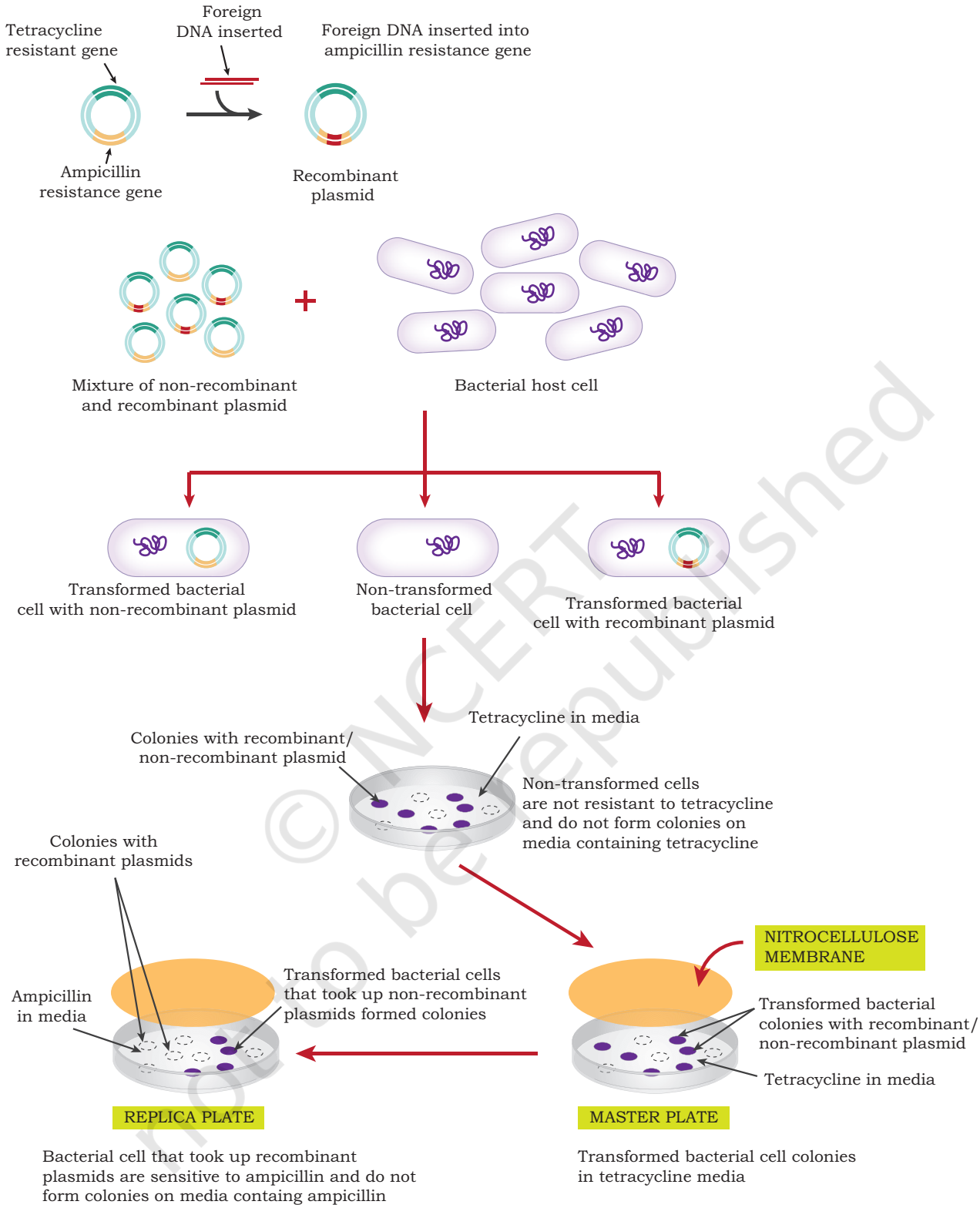


Fig. 3.13: Selection of recombinants by insertional inactivation

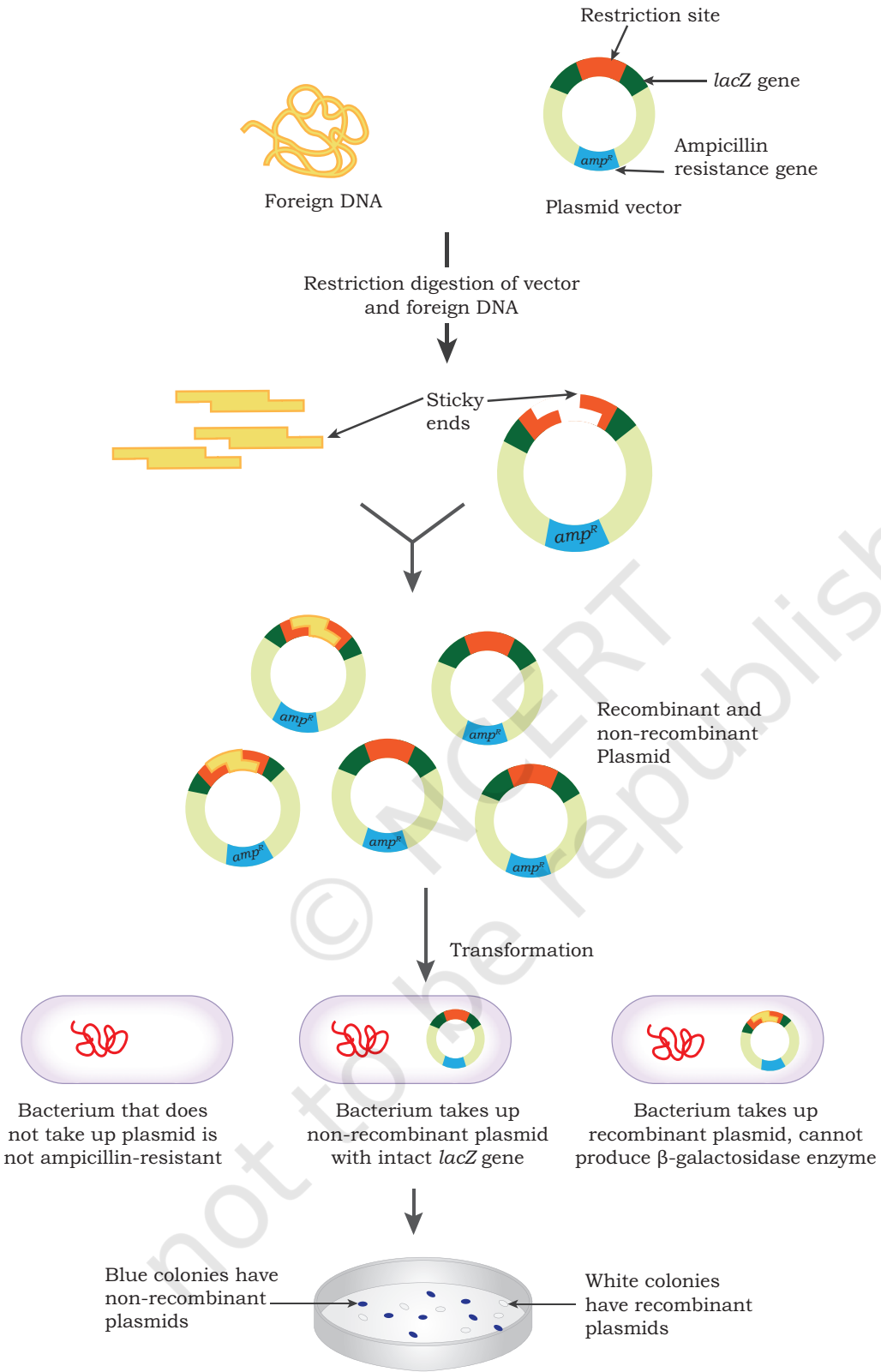


Fig. 3.14: Blue-white selection method

Blue-white selection method is another example of insertional inactivation selection method to select the recombinant transformed cells. In this method, *lacZ* gene present in plasmid vector (refer to Vector section of Chapter 2) expresses the β-galactosidase enzyme. β-galactosidase cleaves a colourless chromogenic, substrate called X-gal (5 Bromo-4-chloro-3 indolyl-beta D-galactoside), an analog of lactose to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerises to produce an insoluble blue pigment called 5,5′-dibromo-4,4′-dichloro-indigo. When *lacZ* gene in the plasmid is inactivated due to insertion of the insert DNA, then the enzyme β-galactosidase is not expressed in hosts containing recombinant plasmids (Fig. 3.14).

During transformation experiment, the bacterial cells (both transformed and non-transformed) are plated on an ampicillin and X-gal-IPTG (Isopropyl β-D-1-thiogalactopyranoside) containing solid media. The non-transformed cells get eliminated and only the transformed cells multiply and form colonies. Two types of colonies will be formed i.e., blue colour and white colour colonies. The bacterial cells in blue colonies contain a vector with an uninterrupted *lacZ*, (no insert) while cells in white colonies, where X-gal is not hydrolysed, indicate the presence of an insert in *lacZ*, which disrupts the formation of an active β-galactosidase.

Alternative methods have been developed in order to screen transformed bacteria, e.g., Green Fluorescent Protein (GFP). The concept is similar to *lacZ* in which a DNA insert can disrupt the coding sequence within a vector and thus disrupt the GFP production resulting in non-fluorescing bacteria.

3.6 Blotting Techniques

Blotting techniques are widely used by scientists to separate and identify DNA, RNA and proteins from a mixture of molecules. This technique immobilises the molecule of interest on a support, which is a nitrocellulose or nylon or polyvinylidene difluoride (PVDF) membrane. It uses hybridisation techniques for the identification of specific nucleic acids and genes. Both nitrocellulose and PVDF membranes are highly hydrophobic and chemically

resistant to a broad range of chemicals. They have high affinity for binding to proteins and nucleic acids. Once proteins or nucleic acids are transferred to membranes, they are immobilised on the membrane. A specific protein can be detected on the membrane by using its specific antibody. Similarly, by using a specific nucleic acid probe, one can detect the desired nucleic acid on the membrane by hybridisation. Detection methods used in blotting techniques are chromogenic, fluorescence, chemiluminescence or radioactive. There are mainly three types of blotting techniques used in biotechnology—southern blotting, northern blotting and western blotting.

Southern blot technique: The original blotting technique was invented by British biologist Edwin Southern as a method to detect specific sequence in DNA samples. In Southern blotting, large DNA molecules are cut into small pieces by restriction endonuclease. The DNA fragments are separated on agar gel based on their size by electrophoresis. DNA from the gel is transferred on to nitrocellulose membrane through capillary action. For this, a solid support is placed in a tray. Buffer solution is added in the tray to half the height of the solid support. A Whatman paper strip is placed on the solid support that touch the buffer on two sides. The gel having DNA is kept

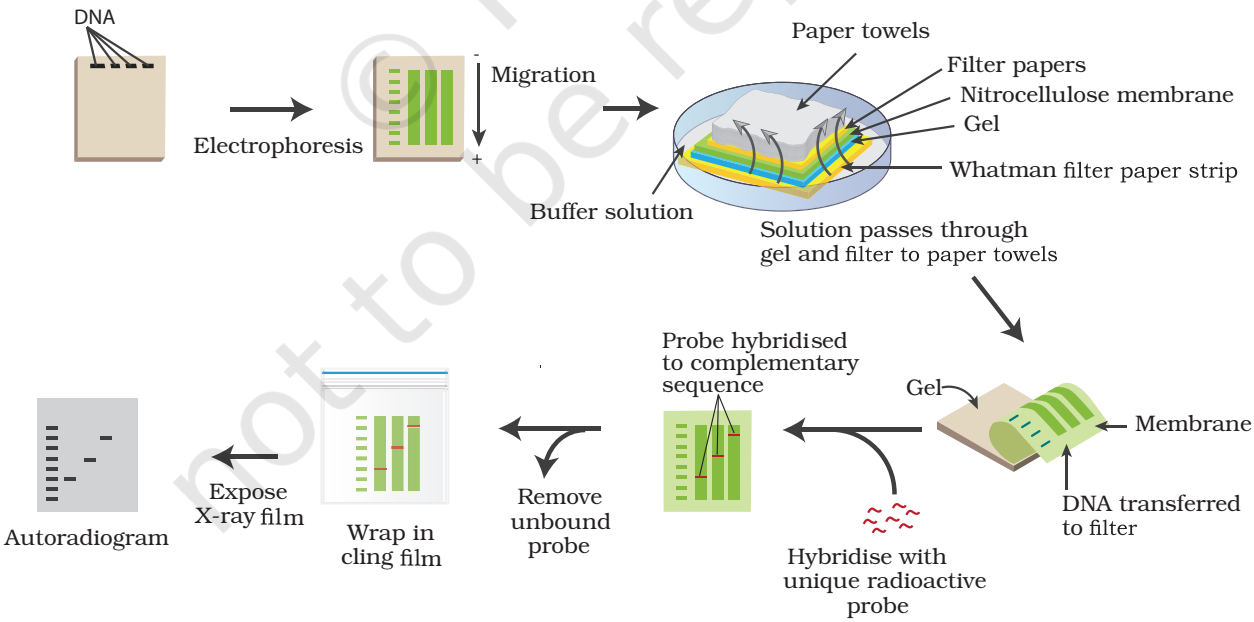


Fig. 3.15: Identification of desired DNA by Southern blotting

on this Whatman strip. A sheet of nylon or nitrocellulose membrane is placed on the top of the gel. Pressure is applied evenly on the gel by placing a stack of filter papers or paper towels and a weight on top of the membrane and gel. Buffer solution by capillary action moves through the gel and membrane onto filter papers. Along with buffer solution, DNA moves to the positively charged membrane. The membranes after transfer of nucleic acids, serve as the replica of their respective gels. The membrane is then baked to permanently attach or fix the transferred DNA to the membrane which is mixed with probes. The blot membrane is then washed to remove unhybridised probes. The desired DNA sequence on the membrane is subsequently detected using probe (Fig. 3.15). **Probe** is a single DNA strand, complementary to the sequence present in the DNA fragment to be identified. The probe is labeled with a detecting tag which may be of radioactivity, fluorescence or chemical nature. The labeled DNA probe anneals with its complementary strand in the membrane. Location of the target DNA fragment is identified by visualisation on X-ray film by autoradiography.

Northern blotting technique: It is used to detect specific RNA molecules in a mixture of RNA. It was developed by American scientists J. Alwine, David Kemp and George Stark in 1977. Like Southern blotting, it starts with the extraction of total RNA from a homogenised tissue sample or from cells. They are separated on a agarose gel based on their size by electrophoresis. Then they are transferred to a membrane where they are immobilised. A nylon membrane with a positive charge is most effective for use in northern blotting since the negatively charged nucleic acids have a high affinity for them. The transfer buffer used for the blotting usually contains formamide because it lowers the annealing temperature of the probe-RNA interaction, thus eliminating the need for high temperatures, which could cause RNA degradation. Once the RNA has been transferred to the membrane, it is immobilised through covalent linkage to the membrane by UV light or heat. It is then mixed with radioactive probes. The probes are specifically designed for the RNA of interest, so that they will hybridise with RNA sequences on the blot

corresponding to the sequence of interest. The blot is now washed to remove extra probes. The labeled probe is then detected by autoradiography, which appears as dark bands on X-ray film or by fluorescent labels (Fig. 3.16).

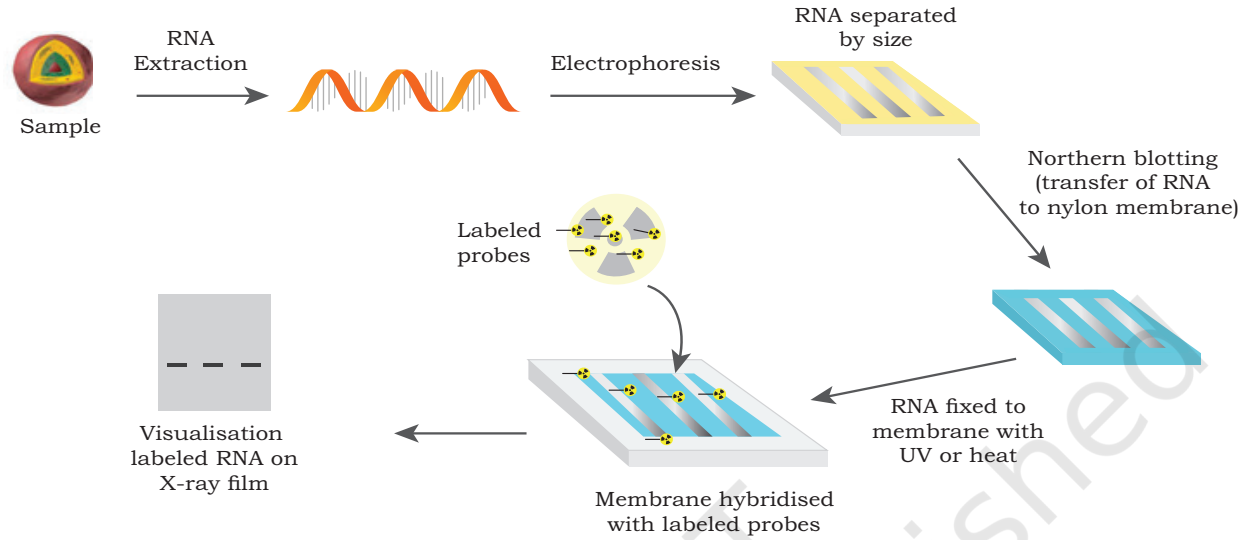


Fig. 3.16: Identification of desired RNA by Northern blotting

Western blotting technique: The name was coined by W. Neal Burnette in 1981. It is a technique used to detect specific proteins in a sample of tissue homogenate or extract. Proteins are isolated from a source. They are separated on SDS-PAGE gel based on their electrophoretic mobility, which depends on charge, molecule size and

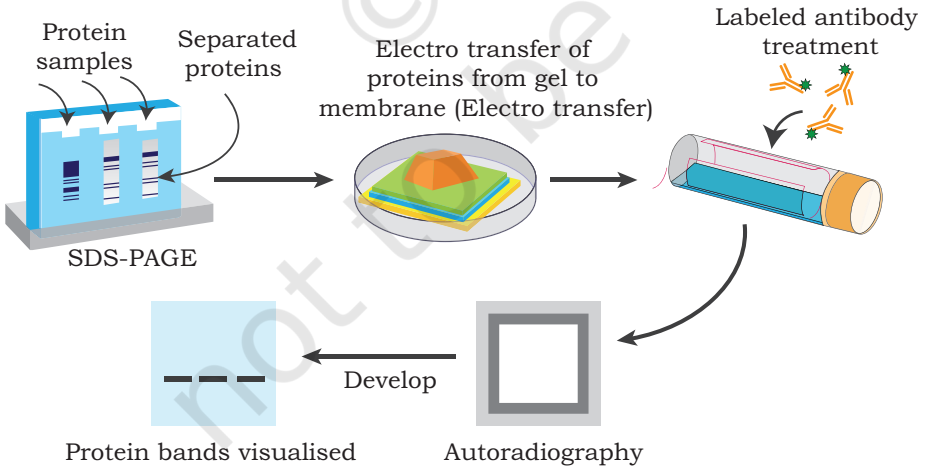


Fig. 3.17: Identification of desired protein by Western blotting

structure of the proteins. They are transferred to a nitrocellulose membrane. The desired protein is detected on membranes using an antibody specific to the protein (Fig. 3.17).

Using all the three blotting techniques, a person can identify a gene and its expression. For example, a gene in a DNA sequence can be identified by Southern blotting, and its transcripts (RNA) can be identified by northern blotting, and finally the expression of a protein from mRNA (by translation) by western blotting.

Box 3: Eastern Blot

Eastern blot is used for the detection of specific post-translational modification of proteins. Proteins are separated by gel electrophoresis before being transferred to a blotting matrix where upon post-translational modifications are detected by specific substrates (cholera toxin, concanavalin, phosphomolybdate, etc.) or antibodies

3.7 Polymerase Chain Reaction (PCR)

Several molecular and genetic experiments require significant amount of DNA. In order to generate multiple copies of DNA from a few copies, a technique was developed by Kary B. Mullis, which is known as 'Polymerase Chain Reaction (PCR)'. In this technique, a very small amount of DNA can be exponentially amplified to generate thousands to millions of copies. PCR, sometimes called 'molecular photocopying', is often heralded as one of the most important scientific advances in molecular biology that revolutionised the study of DNA to such an extent that its inventor, Kary B. Mullis was awarded the Nobel Prize for Chemistry in 1993.

PCR technique is based on the principle that cells use to replicate its DNA. As the name implies, it is a chain reaction carried out in repeated cycles, which involves the process of heating and cooling called thermal cycling carried out by a machine called thermocycler. It requires a heat stable DNA polymerase enzyme that can make new strands of DNA on template strands at a high temperature of about 72 to 78°C (a temperature at which a human or *E. coli* DNA polymerase would be non-functional). DNA polymerase typically used in PCR is called ***Taq* polymerase**, an enzyme isolated from the

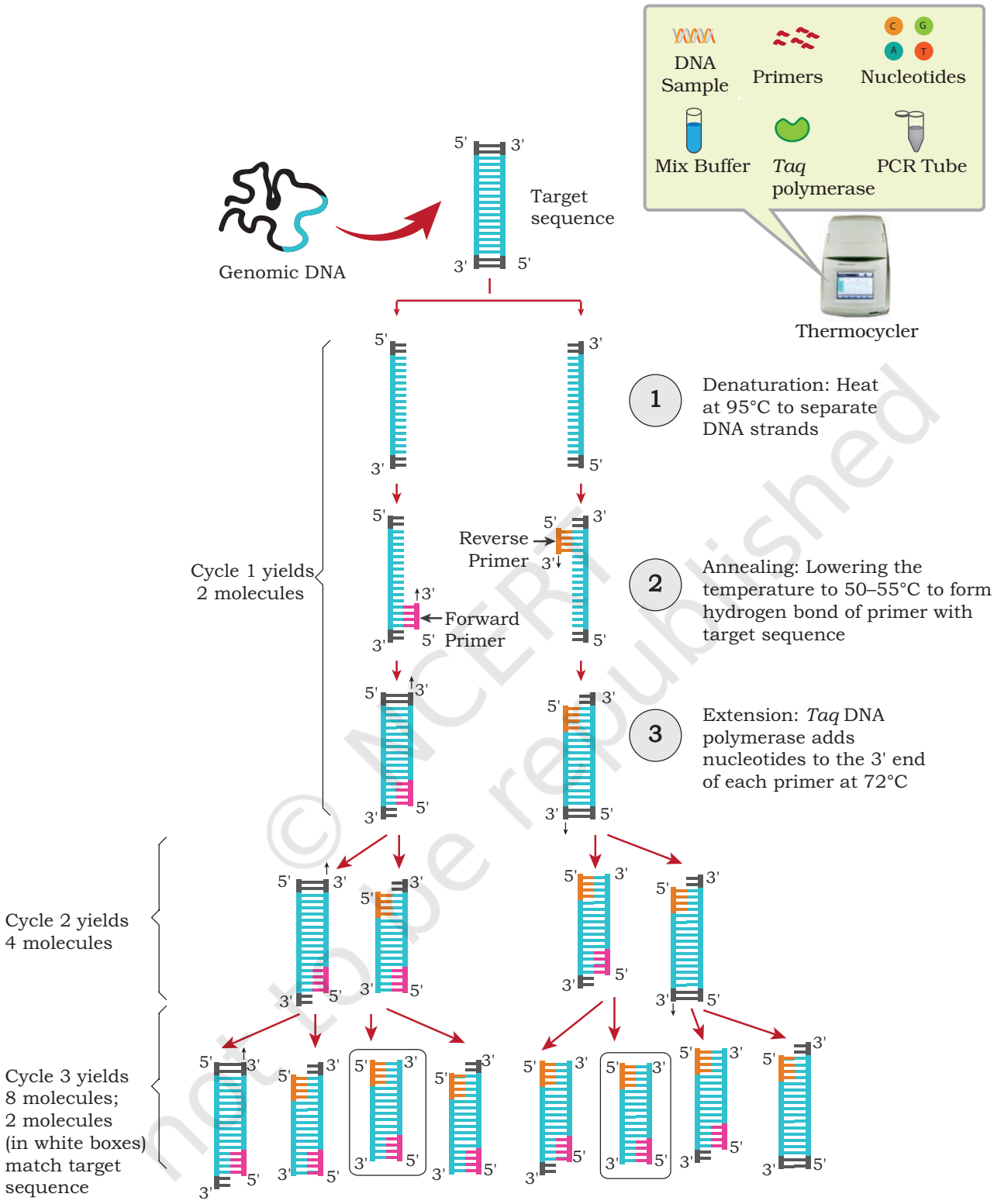


Fig. 3.18: Steps of polymerase chain reaction

thermophilic bacterium, *Thermus aquaticus*, which inhabits hot springs. Another enzyme *Pfu* polymerase isolated from *Pyrococcus furiosus* is used widely because of its higher fidelity when copying DNA. Like other DNA polymerases, *Taq* polymerase also requires a primer, a short sequence of nucleotides that provides 3′-OH end to start DNA synthesis. Two types of single stranded synthetic deoxyoligonucleotide primers (called forward and reverse primers) are used in each PCR reaction that is complementary to the DNA sequences in the template strands flanking the target region (region that should be copied). They are designed from the pre-existing knowledge of the sequence of DNA template to be amplified.

PCR involves three steps — denaturation, annealing and extension (Fig. 3.18). The first step, i.e., denaturation is accomplished by heating the double stranded DNA to be amplified to a temperature of about 94–95°C. At high temperature, hydrogen bonds that hold two complementary strands of DNA molecule break down and each strand serves as template for the synthesis of its new complementary strands. The second step is annealing during which the temperature is lowered to around 50–55°C so that the specific primers can anneal to their respective template strands at their complementary sites and serve as the starting point for copying. Lowering of temperature depends upon the length of the primer and sequence of the primer. In the third step, i.e., extension, the temperature is raised to about 72°C, and the heat stable DNA polymerase begins adding deoxyribonucleotides (dNTPs – dATP, dTTP, dCTP and dGTP) onto the 3′-OH ends of the annealed primers. Thus, a new chain of DNA grows from 5′ to 3′ direction on each template. Copies of DNA strands formed by PCR are known as amplicons. At the end of the cycle, again the temperature is raised and the process is repeated. The number of DNA copies doubles after each cycle hence the number of copies at the end of each cycle would be 2^n (where 'n' is the cycle number). Usually, 25 to 30 cycles are carried out in a typical PCR reaction.

In PCR, the amplified product is analysed by gel electrophoresis at the end of reaction (end point analysis). The amount of DNA in the band of gel plate is then estimated by measuring the intensity of the band by computer

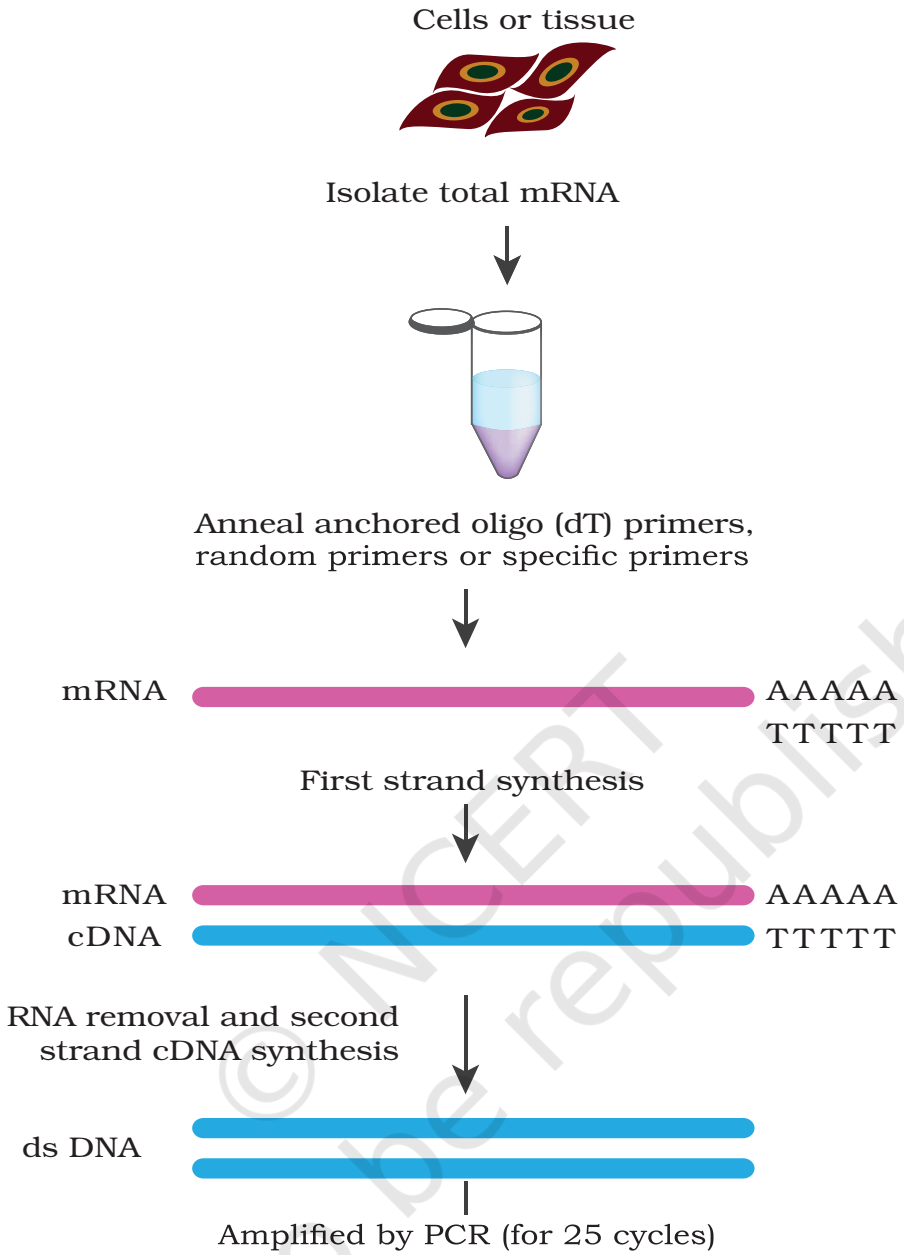


Fig. 3.19: Steps of RT-PCR.

programs and transferred into a quantitative data. This is called **semi-quantitative** PCR. If DNA material is formed from mRNA by reverse transcriptase and used in PCR for amplification (Fig. 3.19), the method is known as **reverse transcription PCR** (RT-PCR).

The latest advancement in PCR technology is **real-time quantitative PCR** (real-time qPCR). In this method,

fluorescent markers are used, which have specific binding affinity to double stranded DNA. When bound to dsDNA, they exhibit fluorescence behaviour. Fluorescence emission is detected and quantitated by a detector. The amount of fluorencence emitted is directly proportional to the amount of double stranded PCR product. Since, the amount of PCR product formed can be measured after each PCR cycle, and hence, it is called real-time quantitative PCR. One of the fluorescent dyes used in real time PCR is SYBR green. The dye only binds to double stranded DNA.

The machine in which PCR reaction is conducted is known as thermocycler. These are automated machines having control points where one can set three gradients of temperature (for denaturation, annealing and extension) for different time periods for each step. For real-time qPCR thermocycler it has a detector to measure emitted fluorescence. In real-time PCR, gel electrophoresis is not needed as in case of conventional PCR.

PCR has several applications in molecular biology and rDNA technology. One of the applications of PCR is to quantify mRNA to assess the expression of a gene. It is also used to amplify minute DNA samples collected from crime scenes and fossils for further investigation.

Box 4

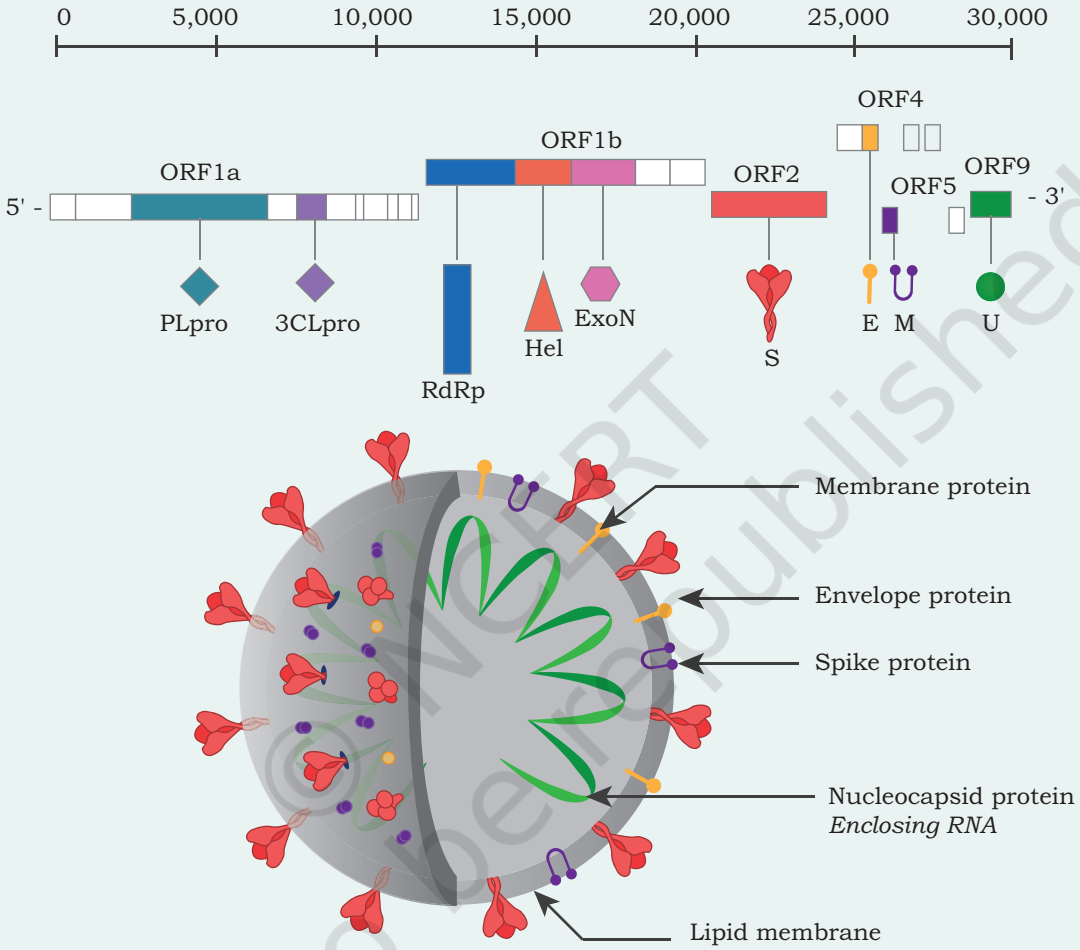
The Novel Coronavirus (nCoV) Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) and COVID-19 Disease (Corona Pandemic)

The International Committee on Taxonomy of Viruses on 12 February, 2020, officially named 2019-nCoV virus as SARS-CoV-2, and on the same day, World Health Organization (WHO) announced it to be responsible for the pandemic Coronavirus disease 2019 (COVID-19). SARS-CoV-2 is an enveloped virus, which contains crown-like spikes on its outer surface.

The genome of SARS-CoV-2 is a single-stranded positive (sense) RNA of 30 kb with G + C content of 38%. Two-thirds of viral RNA encode a number of non-structural proteins (NSPs), which include papain-like protease (PLpro), 3-chymotrypsin-like protease (3CLpro), RNA-dependent RNA polymerase (RdRp), helicase (Hel) and exonuclease (ExoN) as major proteins while the rest

are accessory proteins that are involved in the transcription and replication of the virus. The rest part of the virus genome encodes four essential structural proteins, including spike (S) glycoprotein, small envelope (E) protein, membrane (M) protein, and N phosphoprotein (N) protein, and also several accessory proteins that interfere with the host immune response.

On the basis of the structure, the RT–PCR tests have been efficiently optimised, and mRNA vaccines have been designed and being administered (Chapter 4).

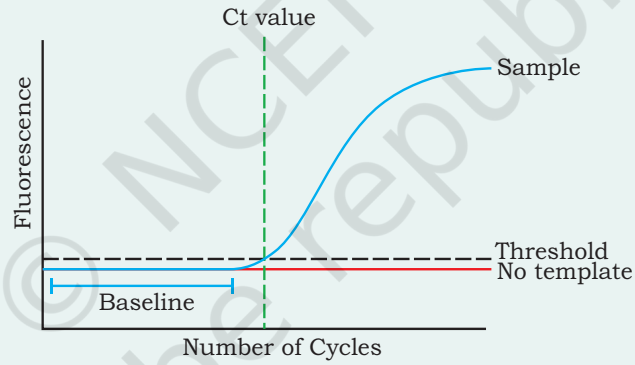


Schematic diagram of the SARS-CoV-2 genome organisation and a virion. The genome contains a 5'-untranslated region (5''- UTR), open-reading frames (ORFs) 1a and 1b encodes non-structural proteins, 3-chymotrypsin-like protease (3CLpro), papain-like protease (PLpro), helicase (Hel), and RNA-dependent RNA polymerase (RdRp) besides accessory proteins, The other ORFs code for structural S protein (S), E protein (E), M protein (M), and N phosphoprotein (N).

Box 5: Application of RT-PCR and COVID-19 detection test

RT-PCR plays an important role in the COVID-19 detection test. It is based on the principle of real-time reverse transcription polymerase chain reaction (rRT-PCR) test that qualitatively detects the nucleic acid from SARS-CoV-2 in the lower and upper respiratory tract specimens [sputum, broncho-alveolar lavage (BAL)] collected by health care staff, from individuals that are suspected of COVID-19.

Principle of the RT-PCR test is same as described in the chapter. For testing, primer and probes are selected from Open Reading Frame gene region (ORF1a/b) and viral nucleocapsid region (N), or the spike protein (S) of SARS-CoV-2 genome. The kit contains primer/probe specific for N gene, ORF1a/b gene and the human RNase P. RNA is separated and purified from the upper and lower respiratory tract specimens is firstly converted to cDNA by reverse transcription and then amplified in real-time PCR thermal cycler. Probes consist of a reporter dye at 5′ and quenching dye at 3′. The fluorescent signals emitted from reporter dye are absorbed by the quencher, so it doesn't emit signals. During amplification, probes are allowed to bind to templates and are cut off by Taq enzyme (5′-3′ exonuclease activity), separating reporter dye from the quencher, and generating fluorescent signals. The PCR instrument can then inevitably draw a real-time amplification curve that is based on the change in signal, and finally realising the qualitative detection of SARS-CoV-2 novel coronavirus at the nucleic acid level. **Amplification plots** shown in the figure signify the accumulation of the product over the duration of the **real-time PCR** experiment. The fluorescent signal from individual sample is plotted against the cycle number.



Threshold level and Ct value on a RT-PCR amplification curve

The threshold cycle or Ct value is the cycle number at which the fluorescence generated within a reaction crosses the fluorescence threshold — a fluorescent signal significantly above the background fluorescence. Ct refers to the **number of cycles** needed to amplify the viral RNA to a detectable level. At the threshold cycle, a detectable amount of amplicon product has been generated during the early exponential phase of the reaction. The Ct value is **inversely proportional** to the amount of the gene of interest in the sample.

3.8 DNA Libraries

DNA molecules present in a genome of an organism are very large in terms of the number of base pairs it contains. The size of a DNA molecule present in any diploid cell from any organ of your body has around 3×10^9 bp. In a genome, gene sequences are arranged randomly and selecting or isolating a gene of interest is a big task, especially when the genomic sequences are not known. Also, a small portion of genome is transcribed to give mRNA, whereas a major portion of the genome remains untranscribed. It will be very difficult to isolate a gene of interest or a sequence of genome whose location and sequence is not known. Hence, DNA libraries are constructed by collecting DNA fragments that have been cloned into vectors so that the specific DNA fragments of interest can be identified and isolated for further study. There are basically two types of DNA libraries (genomic and cDNA libraries), which are described in the following section.

(i) Genomic DNA Library

A genomic library is a collection of clones of small fragments of DNA that together represents the complete genome of an organism. A population of identical vectors store DNA inserts, each containing a different insert. In general, construction of genomic library is done as shown in Fig. 3.20. First, genomic DNA is isolated from the source, which is too large to be incorporated into a vector and needs to be broken down into desirable fragment sizes. Therefore, the genomic DNA is digested with a restriction enzyme to cut the DNA into fragments of a specific size. DNA fragments are then inserted into vectors using DNA ligase to form recombinant vectors. This generates a pool of recombinant DNA molecules. The recombinant DNA molecules are now taken up by host bacterial cells by transformation and then allowed to multiply in a nutrient medium to form colonies. All host cells containing recombinant vectors represent a genomic library. The library created contains representative copies of all the DNA fragments present within the genome of an organism.

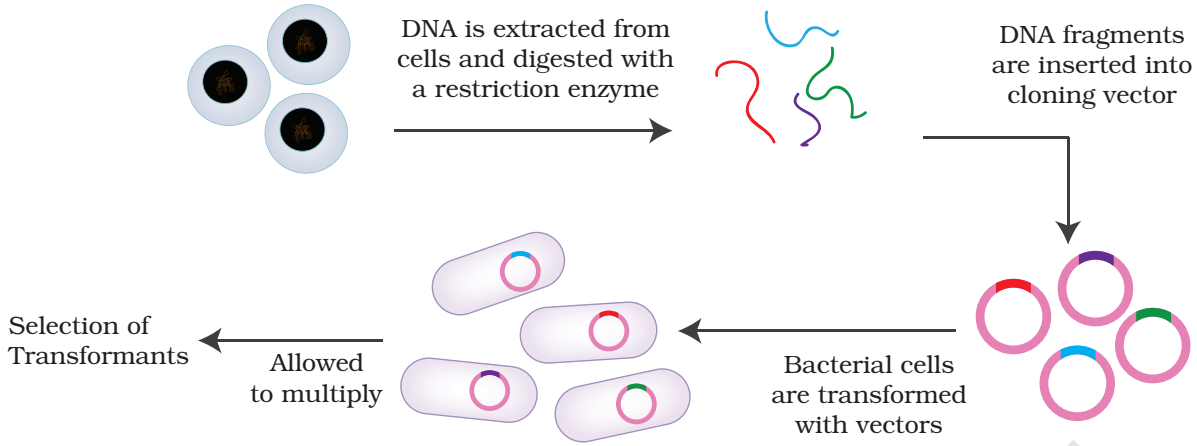


Fig. 3.20: Construction of genomic library

Genomic library has several applications in biotechnology. Genomic library of a species may be helpful for complete sequencing of its genome. Also, one can search for many genes that are not expressed in the genome of an organism. It is also helpful in understanding the evolution of species. Genomic library can be used to compare the sequences of healthy and diseased tissues of the same organism to identify genetic aberrations.

(ii) cDNA Library

Gene expression in higher eukaryotes is tissue-specific. In specific cells, certain genes undergo moderate to high expression. For example, the genes encoding insulin proteins are expressed only in beta cells of pancreas while albumin encoding genes are expressed in liver cells. Using this information, a target gene can be cloned by isolating the mRNA from a specific tissue. The specific DNA sequences are synthesised as copies from mRNAs of a particular cell type called cDNA (complementary DNA). Clones of such DNA copies of mRNAs are called cDNA clones. The cDNA clones of all the genes expressed in a specific cell type or tissue of an organism represent cDNA library.

Construction of cDNA library involves the isolation of total mRNA from a cell type or tissue of interest. mRNA being single-stranded cannot be cloned as such and is not a substrate for DNA ligase. It is first converted into cDNA before insertion into a suitable vector, which can be

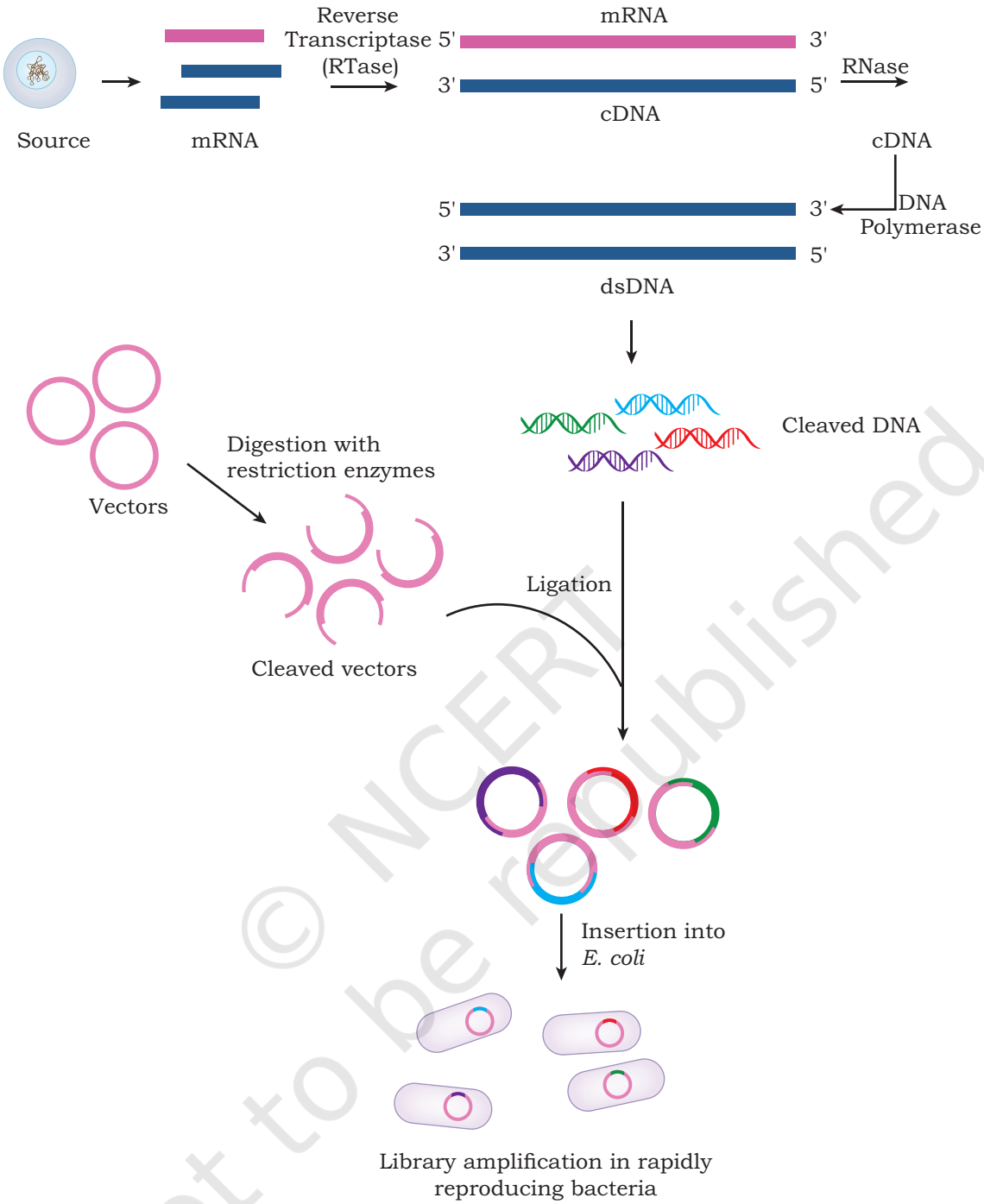


Fig. 3.21: Construction of cDNA library

achieved using reverse transcriptase (RNA-dependent DNA polymerase or RTase). RTase synthesise a complementary DNA strand on mRNA by using mRNA as a template. mRNA is then removed by RNase and the single stranded

cDNA is converted into double-stranded cDNA by DNA polymerase. cDNA molecules are cloned in appropriate host-vector system (Fig. 3.21). The total clones of cDNA are the representative of cDNA library of the source. Since the expressions of genes are different in different organs or cells of an organism at different physiological states, cDNA libraries prepared from different sources of an organism may vary from each other.

The cDNA library has a great significance in the applications of biotechnology. The most important application of cDNA library is to know which genes are active in particular tissues under a particular physiological state. It also helps us to isolate a specific gene. Using cDNA as probes, we can screen genomic libraries for a particular gene.

SUMMARY

- Isolation of nucleic acids from different organisms is the most essential requirement for any molecular biology experiment. There are four steps in the process of extraction of nucleic acids, i.e., disruption of biological samples, protection of nucleic acids from its degrading enzymes, separation of nucleic acids from other molecules and assessment of purity and quality of the isolated nucleic acids.
- Various enzymes play an important role in recombinant DNA (rDNA) technology. These are nucleases, DNA ligase, alkaline phosphatase, polynucleotide kinase, poly A polymerase, etc.
- The major task of the manipulation of DNA involves cutting and ligation of the gene of interest into the vector DNA.
- Nucleases are the enzymes that cleave nucleic acids by hydrolysing the phosphodiester bond that joins the sugar residues of adjacent nucleotides. Two major types of nuclease enzymes depending on its action on the phosphodiester bonds of polynucleotide chains have been identified, which are exonuclease and endonuclease.
- Exonuclease enzymes can remove mononucleotide either from the 3′ or 5′ end of the DNA molecule.

- Endonuclease enzymes cleave DNA molecules at a specific sequence, hence called restriction endonucleases or restriction enzymes (REs). REs are mainly categorised into three groups (i.e., Types I, II and III) based on their cofactor requirement and the position of their DNA cleavage site relative to the target sequence. Type II REs find application in rDNA technology.
- DNA ligase can join two DNA strands together by catalysing the formation of a phosphodiester bond in the duplex form.
- DNA polymerases are a group of enzymes that catalyse the synthesis of new DNA strand by using dNTPs on a template strand.
- Alkaline phosphatase is used to remove the terminal phosphate group from 5′ end of DNA strands.
- Reverse transcriptase is used to generate complementary DNA (cDNA) strand from an RNA template, a process called reverse transcription.
- In rDNA technology, the recombinant DNA is introduced (transferred) in host cells by a number of methods, such as chemical based transfection (calcium chloride, lipofection etc.) and physical transfection (electroporation, microinjection and biolistic) methods.
- Selection of transformed bacteria is the most essential step for a successful cloning experiment, i.e., to identify the transformed cells having recombinant vector (with gene of interest) from a mixture of transformed and non-transformed cells. These selection methods may be direct or through insertional inactivation.
- In direct selection, the transformed cells are distinguished from non-transformed cells based on expression of certain traits, such as resistance to antibiotics.
- In insertional inactivation method, a vector is used having two markers (either two antibiotic resistant genes or one antibiotic resistant gene and *lacZ* gene).
- Blue–white selection method is another example of insertional inactivation to select recombinant transformed cells in which the expression of *lacZ* gene can directly be observed in bacterial colonies.
- Blotting techniques are widely used to separate and identify DNA, RNA and proteins from a mixture of molecules.
- Southern blotting technique is used to detect specific sequence of DNA in DNA samples.

- Northern blotting technique is used to detect specific RNA molecules in a mixture of RNA.
- Western blotting is used to detect specific proteins in a sample of tissue homogenate or extract.
- Polymerase Chain Reaction (PCR) is used to amplify a small amount of DNA into thousands to millions of copies, which involves three steps, i.e., denaturation, annealing and extension. The amplified product of PCR can be analysed by gel electrophoresis at the end of reaction (end point analysis).
- The latest advancement in PCR technology is real-time quantitative PCR (qPCR), in which the fluorescent markers are used that have specific binding affinity to double stranded DNA. In qPCR, gel electrophoresis is not needed as in the case of conventional PCR.
- DNA libraries are constructed by collecting DNA fragments that have been cloned into vectors so that specific DNA fragments of interest can be identified and isolated. There are basically two types of DNA libraries — genomic and cDNA library.
- A genomic library is a collection of clones of small fragments of DNA that together represent complete genome of an organism.
- The cDNA library constitutes cDNA clones of all the genes expressed in a specific cell type or tissue of an organism.

EXERCISES

1. Describe the methods used for isolation of DNA.
2. What is the role of biological detergent in the process of isolation of nucleic acid?
3. How does DNA isolation from plant tissue differ from that of bacterial cell?
4. How many types of restriction enzymes (REs) are there? Can all REs be used in rDNA technology? Give justification.
5. What are the challenges faced during the process of nucleic acid extraction?
6. Write the role of alkaline phosphatase, DNA ligase, terminal transferase in rDNA technology.
7. Describe the role of chelating agent in the process of DNA extraction.

8. Briefly describe the modes of DNA transfer into the host.
9. Identify the correct statement for blue–white selection method.
   (a) A specific dye is used to stain bacterial colony.
   (b) It is based on the expression of *lacZ* gene.
   (c) The recombinant bacterial colony remains blue.
   (d) *lacZ* gene is inserted in an antibiotic resistant gene.
10. Identify the correctly matched pair from the following options.
   (a) Northern blot: Detect specific sequence of DNA
   (b) Southern blot: Detect specific sequence of RNA
   (c) Western blot: Detect specific proteins
   (d) Eastern blot: Detect transcriptional modifications in RNA
11. Identify the incorrect matched pair from the following options.
   (a) Taq polymerase: *Thermus aquaticus*
   (b) Pfu polymerase: *Pyrococcus furiosus*
   (c) HindIII: *Haemophilus influenzae*
   (d) PstI: *Pyrococcus stuartii*
12. How are recombinants screened? Describe the methods in detail.
13. Differentiate between the Southern, Northern and Western blotting.
14. What is PCR? Describe in detail.
15. Write a comparative account of the genomic and cDNA libraries.
16. Diploid human genome contains:
   (a) 3.2×10^9 base pairs
   (b) 6.4×10^8 base pairs
   (c) 3.2×10^8 base pairs
   (d) 6.4×10^9 base pairs
17. Select the incorrectly matched pair from the following.
   (a) Nucleases : Hydrolyse phosphodiester bond
   (b) Restriction enzymes: Cleave DNA at specific sequence
   (c) Palindromic sequence: Read same backwards and forward
   (d) EcoRI: Type I Restriction Enzyme

18. **Assertion:** PCR can be used to amplify very small amount of DNA using DNA modifying enzymes.

    Reason: PCR uses Taq Polymerase.

    (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.

    (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.

    (c) Assertion is true but reason is false.

    (d) Both assertion and reason are false.

19. **Assertion:** Foreign gene can be introduced into host bacterium by transformation techniques like electroporation.

    Reason: Bacteria have cell wall/membrane.

    (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.

    (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.

    (c) Assertion is true but reason is false.

    (d) Both assertion and reason are false.

Chapter 4

Applications of Recombinant DNA Technology

Recombinant DNA (rDNA) technology has revolutionised our life in various ways. In recent years breakthrough discoveries have provided solutions to many of the problems. These include establishing the identity of an individual, introduction of foreign genes into other organisms, diagnosis of many diseases and their treatment, production of therapeutic agents/molecules and so on. In this chapter, students will be acquainted with a few applications of rDNA technology, like DNA fingerprinting, developing transgenic organisms, gene therapy, recombinant vaccines and production of therapeutic agents/molecules.

4.1 DNA Fingerprinting

As you are aware, chemical structure of every individual's DNA is identical and is made up of four nitrogenous bases: A (Adenine), G (Guanine), C (Cytosine) and T (Thymine). The arrangement of these bases along the DNA strands is specific to an individual. The human genome consists of 3.2×10^9 bp. However, there is very little uniqueness in the genetic makeup of humans. About 99.9% of the genome among humans is same and the remaining 0.1% of the genome consists of sites of inherited variations. It is these variations in DNA, which make each of us unique. Identifying these differences is helpful in determining the

relatedness between two individuals. One way of achieving this is by DNA sequencing. However, sequencing and comparing DNA of individuals every time would not be feasible. Therefore, to study and compare the inherited variations in human DNA without sequencing, a new technique known as 'DNA fingerprinting' was developed by a British geneticist Sir Alec Jeffreys in 1984 at the University of Leicester.

More than 90% of the human genome consists of DNA which does not code for protein. Within the non-coding regions of an individual's genome, there exists short sequences of nucleotides which are repeated a number of times in tandem called STR (short tandem repeats) at a locus which are called **VNTR** (variable number tandem repeat). VNTRs are commonly subdivided into two principal families: microsatellites (repeated sequences of 1 to 9 bp) and minisatellites (repeated sequences of 10 to 100 bp) as shown in Fig. 4.1.

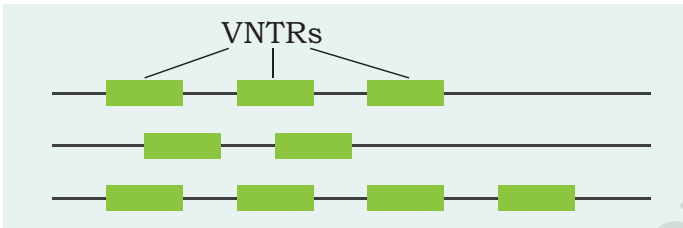


Fig. 4.1: Schematic representation of VNTRs in 3 alleles

These sequences show a high degree of polymorphism (or variations) and form the basis of DNA fingerprinting. Furthermore, as the polymorphisms are inheritable from parent to offspring, DNA fingerprinting is the basis of paternity testing in case of disputes.

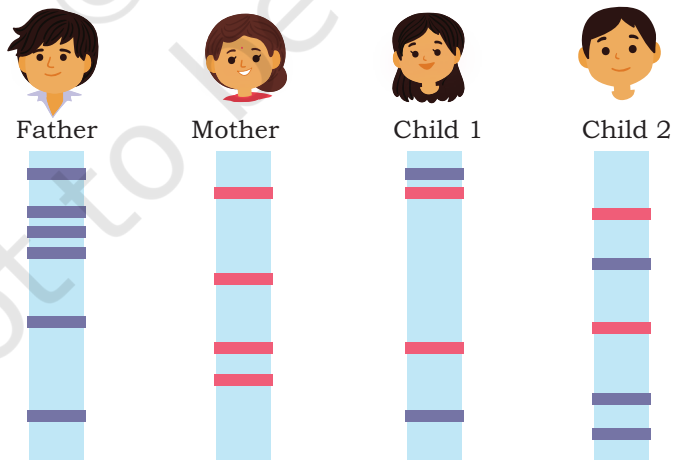


Fig. 4.2: DNA profiling to determine the child of a couple. In the above figure, the DNA profile of a child is compared with father and mother to confirm paternity. Here, father and mother are parents of child 1 but not of child 2.

We carry two different copies of every VNTR locus because we inherit one chromosome from mother and one from father. In simplest way, DNA fingerprinting can be performed using restriction digestion of DNA. This technique is referred to as Restriction Fragment Length Polymorphism (RFLP). In RFLP, after restriction enzyme digestion of individual's DNA in specific regions, unique patterns are generated that are used for genetic analysis and identification (Fig. 4.2).

Conceptually, the DNA fingerprinting shown in Fig. 4.2 is correct but in reality, the identification of individual bands on gel is difficult and therefore, hybridisation (Southern Hybridisation) using a VNTR probe is used. Hybridisation with VNTR probes produces a pattern of bands which are characteristic to every individual (Fig. 4.3). The steps involved in this technique are as follows:

1. DNA is isolated from different samples like blood, hair, skin, semen, buccal swab, etc.
2. The collected DNA sample is cut into several fragments of different sizes using one or more restriction enzymes.

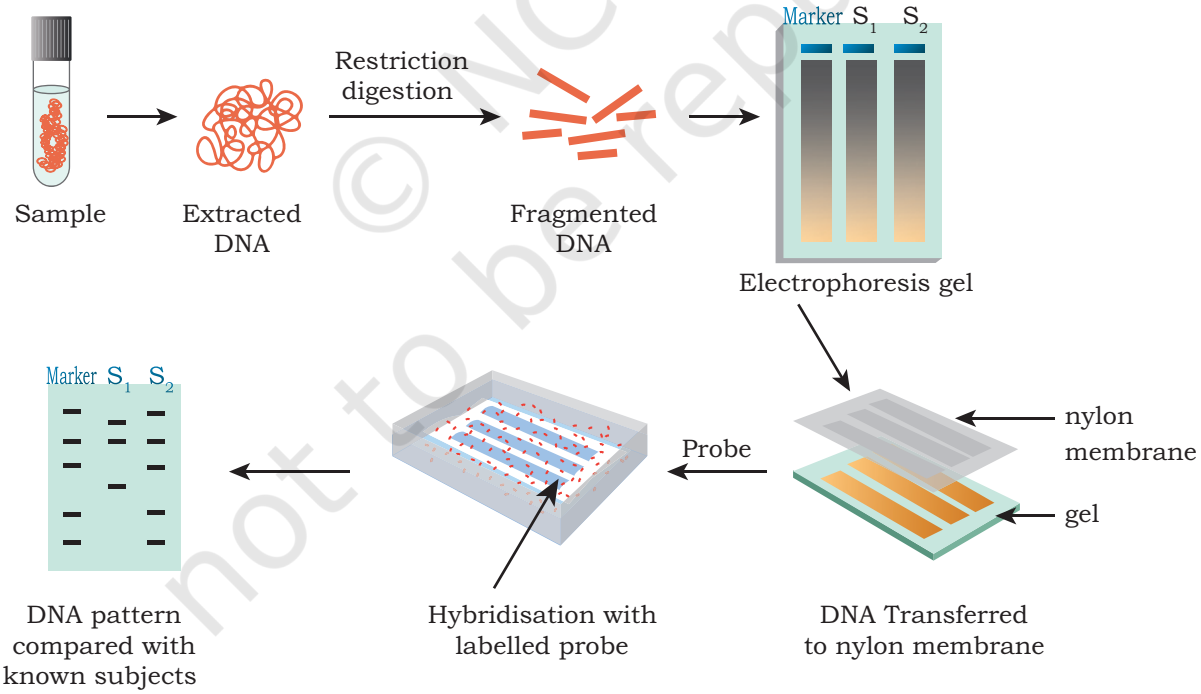


Fig. 4.3: Steps involved in DNA Fingerprinting

3. The DNA fragments are then separated by agarose gel electrophoresis. The different sized DNA pieces are separated based on size.
4. The separated DNA on the gel is thus transferred to a nitrocellulose/nylon membrane. The nylon membrane is then exposed to UV radiation on UV transilluminator for three minutes or baked at 80°C for two hours to permanently attach DNA to the membrane.
5. Now, Southern hybridisation is performed using VNTR Probes (the labeled stretches of single-stranded DNA used to detect the presence of complementary target sequences).
6. Finally, the hybridised DNA fragments are detected.
7. The patterns of DNA bands are highly specific for each individual and can be used in forensics and paternity disputes.

Note: Polymerase Chain Reaction (PCR) is often used to increase the sensitivity of the technique as it amplifies the DNA, irrespective of the amount of DNA.

Applications of DNA Fingerprinting

1. VNTR patterns are used to ascertain the paternity and maternity of a person given the fact that they are inherited from their parents. Since, these patterns are very specific, even a parental VNTR pattern can be reconstructed from their known offspring(s) VNTR patterns. Therefore, VNTR patterns of parent–child can be used to solve paternity and maternity cases.
2. DNA isolated from tissues like blood, hair, skin, semen, etc., from the scene of a crime is used for VNTR patterns analysis as evidence, where such a pattern of DNA isolate is compared with VNTR patterns of a criminal or suspect for establishing guilt or innocence. Hence, DNA fingerprinting helps in criminal identification and forensic studies.

3. DNA fingerprinting is also used to compare DNA extracts from fossils to modern day counter parts and therefore, finds great significance in evolutionary biology studies.
4. DNA profile of people suffering from some particular disorder, or comparing it to a large number of people with and without the disorder helps to identify the DNA patterns in studying inherited disorders.
5. In addition to social security numbers, picture ID and other more routine methods, even the DNA profile (VNTR patterns) of an individual are also being proposed to be used as a sort of genetic barcode for personal identification.

4.2 Transgenic Organism

You must have heard about 'Bt Cotton' or 'Rosie, the cow', but have you ever wondered what these are? Are these naturally found in the environment? If not, then how have these been created? Or why these have been created at all? Both the examples mentioned above are a transgenic plant and animal, respectively. These have been produced by the introduction of new gene segments through the process of transgenesis and these are not naturally found in the environment. These have been created for the benefit of human beings.

The process of insertion of a foreign gene (transgene) into the genome of an organism and its transmission and expression in the organism's progeny is termed as transgenesis. The organisms carrying the transgene, are known as **transgenic organisms** or **genetically modified organisms (GMOs)**.

4.2.1 Historical background

The first genetically modified organism was a bacterium made by Herbert Boyer and Stanley Cohen in 1973. In the subsequent year, it was followed by the engineering of first transgenic animal (transgenic mice) by Rudolf Jaenisch and Beatrice Mintz in 1974. In 1994, Flavr Savr tomato was released as the first genetically modified (GM) food crop approved by the US Food and Drug Administration (USFDA).

Box 1: History of GMO Technology

7,800 BCE

Oldest evidence of artificially selected crops

1974

Jaenisch & Mintz create the first GE animal

1980

U.S. Supreme court allows patenting of GE organism

1994

Flavr Savr Tomato becomes the first GE food crop approved by U.S. Department of Agriculture

1996

Herbicide-resistant crops first introduced

2009

The drug ATryn, the first biological product produced by GE animal approved by US FDA

30,333 BCE

Artificial selection of wolves began

1973

Boyer & Cohen develop Genetic engineering (GE)

1975

Asilomar conference sets guidelines for future GE experiments

1982

FDA approves Humulin, the first pharmaceutical manufactured using GE technology

1995

US EPA approves the first insecticide producing crop

2000

Golden Rice engineered to mitigate Vitamin A deficiency

Timeline for 'Advancements in the concept of transgenic organisms'
Ref: Rangel, G. (2015). From Corgis to Corn: a Brief Look at the Long History of GMO Technology. Science in the News.

4.2.2 Production of transgenic organisms

Transgenic Plants

Transgenic or genetically modified plants are those plants whose genome is modified (by the introduction of one or more genes from another species) through genetic engineering. Basic requirement for genetic transformation is construction of genetic vehicle (vector), which carries the genes of interest flanked by necessary regulating sequences, like promoter or terminator. Most commonly used techniques for gene transfer are of two types.

- Vector-mediated or indirect gene transfer
- Vector-less or direct gene transfer

A. Vector-mediated or indirect gene transfer

- ***Bacteria-mediated transfer:*** *Agrobacterium tumefaciens* is a rod shaped Gram-negative soil bacterium that is capable of naturally transferring DNA into the plant genome, and causes crown gall disease (a type of plant tumor). *Agrobacterium* has the natural ability to transfer its own DNA to plant genome randomly, where it can produce copies of itself, and therefore, is also known as 'Natural Genetic Engineer'. It contains a large size tumor inducing (Ti) plasmid. A portion of the plasmid carrying tumor inducing genes is called **T-DNA**, which is transferred to plant genome upon infection. These genes encode for phytohormones, like auxin and cytokinins, which induce rapid cell division in the host plant cell and cause the formation of tumor called crown gall. This natural ability of *A. tumefaciens* to transfer T-DNA has been exploited to transfer the genes of interest to plants and produce transgenic plants. The tumor inducing genes of T-DNA are replaced with genes of interest like *Bt* gene for insect resistance. Ti plasmid without tumor inducing genes is a disarmed plasmid and this process is called disarming. Antibiotic marker genes can be introduced along with genes of interest, to differentiate among the transformed and non-transformed cells. Vectors containing modified T-DNA (recombinant disarmed T-DNA) are transferred to *Agrobacterium*

cells, which are further used for plant transformation. *Agrobacterium* infection causes the T-DNA to get transferred into plant cells and subsequent integration of T-DNA carrying the target gene into plant nuclear DNA. These are further cultured on regeneration media supplemented with the bacteriostatic agent to stop further growth of *Agrobacterium* and lethal concentrations of marker antibiotic, which selectively eliminates the non-transformed plant cells. In a few weeks, the transformed tissue forms shoots, which are transferred onto the rooting media, where the transformed shoots make roots within two to three weeks. Plantlets are then transferred to soil. A schematic representation of *Agrobacterium* mediated plant transformation is given in Fig. 4.4.

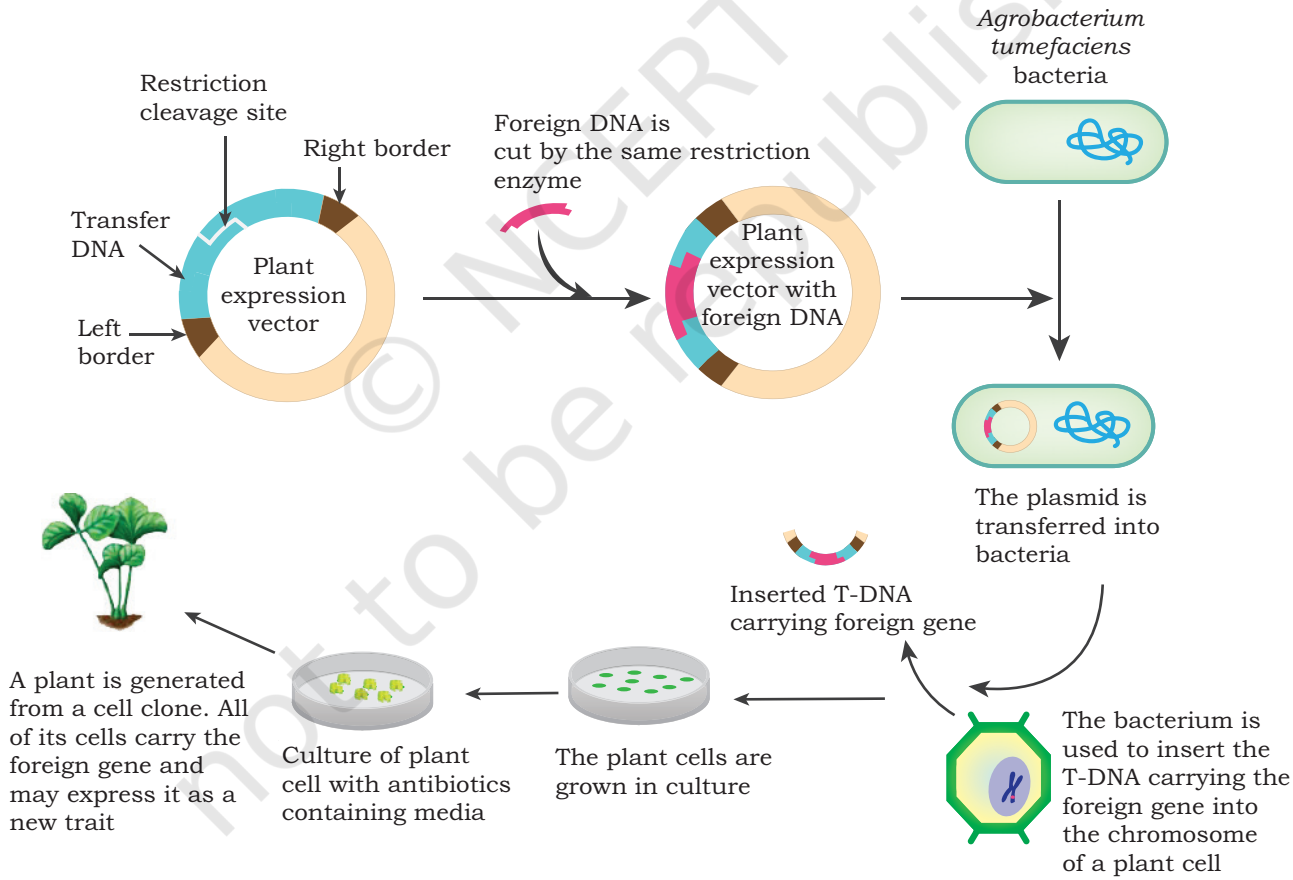


Fig. 4.4: Stepwise procedure for Agrobacterium mediated transformation in plants

- ***Plant virus-mediated transfer:*** Plant viruses, such as *caulimoviruses* have the ability to enter intact plant cells and introduce their own DNA into the plant's DNA. Use of plant viruses constitutes another method to transfer gene of interest into plant cells. However, this is not a common method of plant transformation as compared to *Agrobacterium*.

B. Vector - less or direct gene transfer

Many physical or mechanical methods have been developed to help direct entry of DNA in plant cells.

- ***Particle Bombardment gene transfer:*** It is a popular direct gene transfer method and is capable of delivering foreign DNA in both monocot and dicot plants. It is also called **biolistics**. The DNA having genes of interest and antibiotic resistance marker gene is coated on to the surface of gold or tungsten particles (1 μm – 3 μm in size). These particles are accelerated with high velocity and bombarded onto the target tissue or cells using a powerful gun, which is called a **particle gun** or **gene gun**. Tissues bombarded are then cultured on the nutrient media having an appropriate antibiotic. Transformed cells grow to make callus and then transgenic plants are regenerated. This method is very useful for those plant species, where *Agrobacterium*-mediated transformation may not be very successful, for example, cereals. Particle bombardment technique is also utilised for the transformation of chloroplast. As a plant cell contains many chloroplasts, this method allows much higher transgene expression.
- ***Protoplast Transformation and Electroporation:*** Protoplasts are spherical naked plant cells, which are produced by the removal of cell walls. These naked cells are capable of taking up DNA from their surroundings, which get integrated into the genome of transfected cells. Gene transfer process can be accelerated with the application of polyethylene glycol (PEG) and dextran sulphate. Alternatively, electric current can also be used for gene transfer in protoplast and the method is known as electroporation. Transfected protoplasts are allowed to regenerate cell walls and then the cell

division begins. Transformed cells are selected on the selective nutrient medium and transgenic plants are regenerated.

Transgenic Animals

In transgenic animals, genetic makeup has been altered by the use of various genetic engineering techniques. Most commonly used techniques for developing transgenic animals are:

1. DNA pronuclear microinjection
2. Embryonic stem cell-mediated gene transfer
3. Retrovirus-mediated gene transfer

1. DNA Pronuclear Microinjection

This is one of the earliest strategies used for creating transgenic mice. Just after fertilisation, the egg contains a small female pronucleus and a large male pronucleus. In pronuclear microinjection technique, transgene is directly injected into the larger male pronucleus. The embryos with injected transgene are cultured *in vitro* and then implanted into the uterus of foster mothers. The transgene may get integrated into the DNA of zygote, resulting in animals that are transgenic.

The technique is simple and reliable, but the rate of success is low as the transgene may or may not get integrated into the host DNA. Also, the site of integration of transgene is random which may lead to unpredictable effects.

2. Embryonic stem cell-mediated gene transfer

Embryonic stem (ES) cells have the potential to differentiate into any other cell type of the body including germ cells. ES cells are collected from the inner cell mass region of the blastocyst stage. The r-DNA is transfered into these ES cells by electroporation. Few ES cells take up the transgene and get transformed. These transformed ES cells are then transferred back to the inner cell mass of blastocyst through micro-injection. The blastocyst is implanted into the uterus of a foster mother. The offspring are then monitored for expression of the transgene.

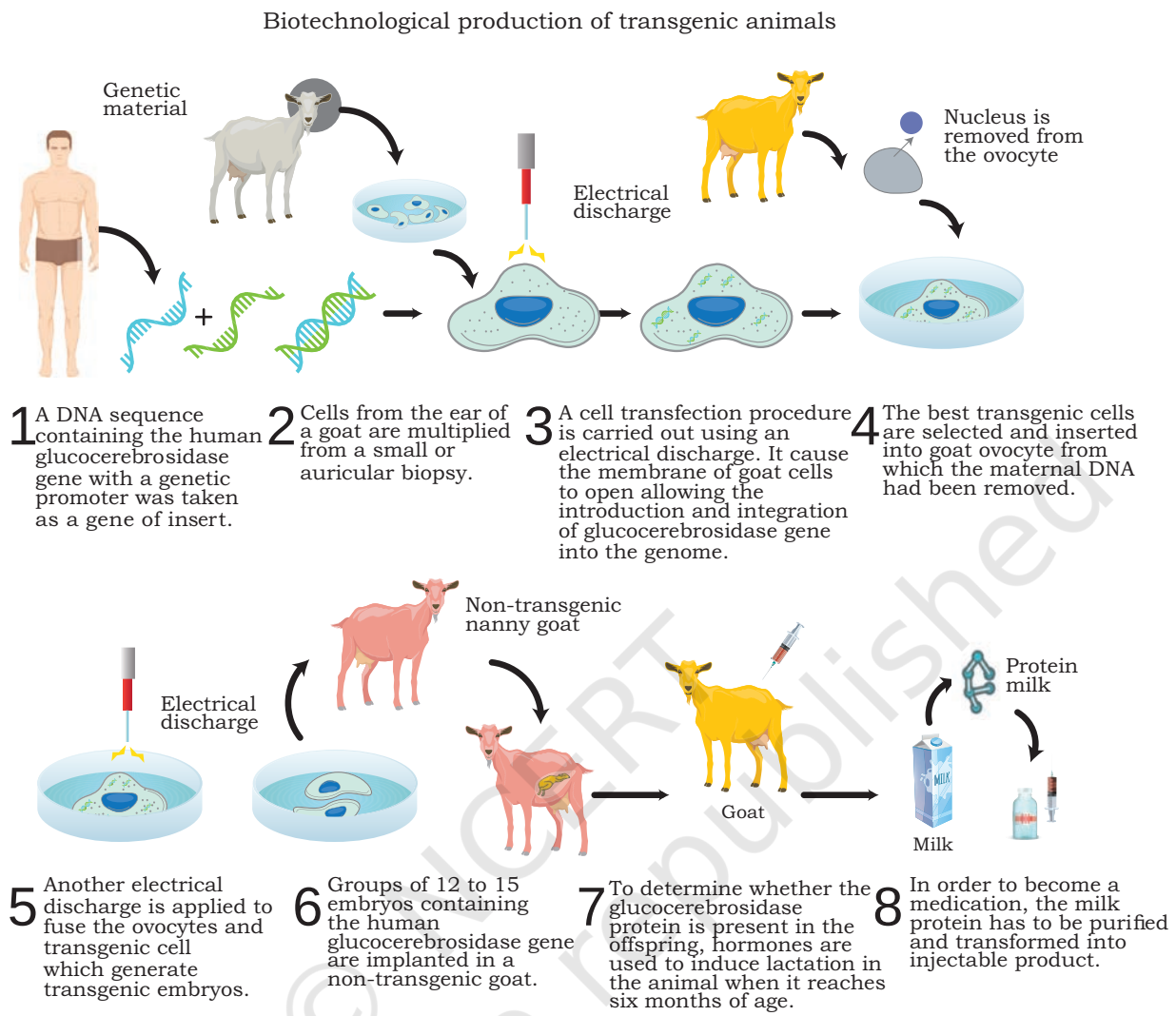


Fig. 4.5: Stepwise procedure for production of transgenic animals

Box 2: Chimeric Mouse

Chimeras are organisms composed of at least two genetically distinct cell lineages originating from different zygotes. A chimeric mouse contains both normal cells and genetically manipulated 'knockout' cells. Coat colour can reflect this with a spotted pattern. It is a patchwork of normal cell and genetically manipulated 'knockout' cells. Embryonic chimeras of the mouse have become a tool to investigate the critical developmental processes, including cell specification, differentiation, cell lineage, potential patterning and the function of specific genes. In addition, chimeras can also be generated to address biological processes in the adult, including the mechanisms underlying diseases or tissue repair and regeneration.

3. Retrovirus mediated gene transfer

The genetic material of retroviruses is RNA. Retroviral vectors are used to stably introduce transgene into early embryos or ES cells. The advantage of this method is that a single copy of retrovirus carrying the transgene integrates at a particular location in the host genome. An example of such retroviruses is the lentivirus.

4.2.3 Applications of Transgenic organisms

Applications of Transgenic Plants

In Agriculture

One major goal of plant transgenics has been to increase the yield of crops and also to improve the nutritional quality of crop products to cater to the needs of ever increasing human population. Biotic stress due to pathogens, like bacteria, fungi, viruses or insects, and abiotic stress factors, like drought, salinity, extreme temperatures causes huge loss to crop productivity in terms of yield. Biotechnological strategies have been used effectively to generate newer and effective cultivars with increased resistance to pathogens or perform better in harsh environmental conditions. Abiotic stress response reaction involves the production of stress related osmolytes, like sugar (e.g., trehalose and fructans), amino acids (e.g., betaine, proline) and certain proteins have been

produced, which over express one of the above-mentioned compounds. These show better tolerance to environmental stresses. A common example of a plant offering resistance to insect is *Bt* cotton. This insect resistant transgenic crop has been developed by transferring and expressing *Bt* gene (also called *cry* gene) from bacterium *Bacillus thuringiensis* into a cotton plant. *Bt* gene encodes a toxin (*Bt* toxin), which is naturally produced by *B. thuringiensis* as an insecticidal protein to kill insects. There are several classes of *cry* genes and products of each class are harmful only to a specific group of insects and do not affect other useful insects, like butterflies and silkworms. For example, *cryIAc* and *cryII Ab* codes for a toxin that control cotton bollworms whereas *cryIAb* encodes for a toxin that controls corn borers. Some of the other crops for which transgenic plants have been developed by introducing *Bt* genes are brinjal, maize, rice, tomato, etc. All these transgenic crops have been proven to be effective in controlling the insect pests. Many of these transgenic crops are currently grown commercially and have resulted in significant increase in the total yields. Most notable example in India is *Bt* cotton (offering resistance to the insect pest pink bollworm) which is grown commercially since 2002.

Similarly, transgenic crops have been generated for several other traits, which results in significant enhancement in the crop productivity. These traits include tolerance to herbicides; resistance to bacteria, pests, viruses or nematodes; tolerance to environmental stresses; nutrient quality and delayed fruit ripening, etc.

Strategies for increasing crop yield also include modifying the photosynthetic machinery of the plant, manipulating the enzyme activity, increasing sugar to starch conversion, improving the nutritional quality; for example increasing the expression of vitamins, such as vitamin A in plants, e.g., Golden Rice. Many traits have been already commercialised and several others are being tested for their performance in the field conditions.

Box 3: List of some transgenic crops and their engineered traits

Crop	Source of Inserted Trait	Trait
Corn	Bacteria, and other species of corn	Resistance to insects
		Tolerance to herbicides
		Male corn sterility
		Alpha-amylase expression
		Increased lysine level for use in animal feed
		Reduction of yield-loss under water-limited conditions
Cotton	Bacteria	Tolerance to herbicides
		Resistance to insects
Soybean	Bacteria, corn, oats, and other species of soybean	Tolerance to herbicides
		High oleic acid soybean oil
		Resistance to insects
	Mustard greens	Resistance to insects
Canola	Bacteria	Tolerance to herbicides
		Fertility restoration
		Male canola sterility
	Fungus	Degradation of phytate in animal food
Potato	Bacteria	Resistance to insects
	Potato virus	Resistance to potato viruses
	Other species of potato	Lower level of reducing sugars
		Lower level of free asparagine
		Reduced black spot bruising
Tomato	Bacteria, potato	Delayed softening
	Bacteria	Resistance to insects
Radicchio	Bacteria	Tolerance to herbicides
		Male radicchio sterility
Alfalfa	Bacteria	Tolerance to herbicides
Sugar beet	Bacteria	Tolerance to herbicides
Rice	Bacteria	Tolerance to herbicides
Apple	Other species of apple	Reduced browning and bruising
Cantaloupe	Bacteria	Delayed ripening
Squash	Viruses	Resistance to viruses
Papaya	Viruses	Resistance to viruses
Flax	Mustard green	Tolerance to herbicides
Plum	Virus	Resistance to viruses
Creeping Bentgrass	Bacteria	Tolerance to herbicides

Antisense technology

The antisense technology is used to inhibit/silence the gene expression. In this technique, the inhibition of expression of a specific gene is achieved by preventing the translation of its mRNA. There are two approaches, the first one is known as antisense oligonucleotide (ASO) technique. In this technique, a synthetic oligonucleotide (a short RNA or DNA molecule), which is complementary to the mRNA of the target gene, is introduced into a cell. The ASO can mediate its effect in two ways: (i) by binding of ASO to the target mRNA followed by RNase H mediated degradation (ii) by blocking of its translation by hindering its binding to ribosomes. The second approach is known as RNA interference (RNAi) technique. In this technique, the expression of target gene is inhibited by inactivating its mRNA through RNA induced silencing complex (RISC). A double stranded RNA of exogenous (viral origin) or endogenous origin (cellular origin) is cleaved in the cytoplasm into shorter fragments by the endoribonuclease named DICER. The shorter double stranded fragments are separated by a multiprotein complex RISC into guide strand and passenger strand. The passenger strand is degraded while the guide strand attached to RISC binds to the target mRNA and cleaves it, thereby the target gene is silenced (Fig. 4.5).

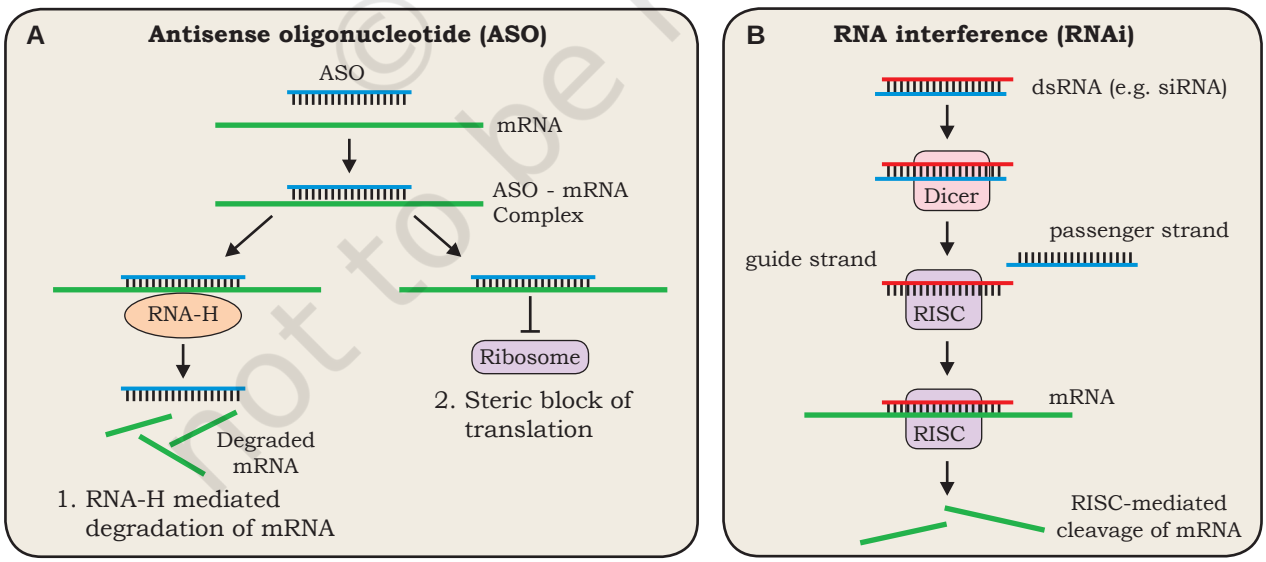


Fig. 4.5: (A) Mechanism of antisense oligonucleotide-mediated gene silencing, (B) Mechanism of RNA interference (RNAi).

Using a combination of *Agrobacterium*-mediated transformation and antisense technology, pest-resistant plants have been created. An example is provided by the tobacco plant, which is infected by nematode *Meloidogyne incognita*. Nematode-specific genes were introduced into the plant using *Agrobacterium* as a vector. The genes, when transcribed, produced antisense RNA, which binds to the sense RNA of the nematode, being complementary to each other, and thus prevents its translation into proteins that are necessary for nematode survival. This makes transgenic plants resistant to the parasitic attack of the nematodes.

For Industrial Production

Plants are the backbone of all life forms on earth as they convert the energy of sun to chemical energy that feed the world. Today thousands of plants are being used for food, feed and medicines to cure acute and chronic health problems. Advances in our understanding of plant genetics and biotechnological tools has led to the development of relatively new bioscience '**Molecular Farming**'. It involves the genetic modification of the host plant through purposeful addition of a gene or group of genes leading to the production of new desired biomolecules. Foreign genes can be inserted either to nuclear genome or chloroplast genome. As chloroplast has several copies of the genome, the insertion of gene of interest in chloroplast results in higher accumulation of target biomolecules. In this way, a large number of products have been produced in plants which include vaccine antigens, therapeutic proteins, diagnostic reagents, nutritional products, bioplastics, or industrial enzymes, etc. In 2012, U.S. FDA approved the first plant-made human biologics 'Elelyso'. It is an enzyme that is used to treat Gaucher's disease and has been produced in carrot cell cultures. Table 4.1 lists the examples of a few therapeutic molecules that have been produced in plants through genetic engineering.

Table 4.1: List of industrial or therapeutic products produced in plants through genetic engineering.

Transgenic Plant	Industrial/ Therapeutic Product(s)	Disease/ Other Usage
Lettuce	Hepatitis B antigen	Hepatitis B
Tobacco	Cancer vaccine	Non-Hodgkin's Lymphoma
Carrot	Human glucocerebrosidase	Gaucher's Disease
Safflower	Insulin	Diabetes
Rice	Human lysozyme	Anti-infection, anti-inflammatory

For Environmental benefits

• **Biodegradable plastics:** Plastics are non-biodegradable and are harmful to the environment. Transgenic plants can be exploited to produce biopolymers that can replace plastics. Polyhydroxyalkanoates (PHAs) are an attractive source of non-polluting and biodegradable plastic. Production of PHAs using transgenic plants provides an economically viable alternative. An example is the production of bacterial polyester polyhydroxybutyrate (PHB) in transgenic sugarcane.

• **Phytoremediation:** Phytoremediation refers to the use of plants for the removal of pollutants from the environment, especially soil. Metal detoxifying genes from bacteria and other organisms are introduced into plants, and the transgenic plants then uptake and convert the toxic elements into less toxic forms.

Mercury is a hazardous heavy metal, largely found in the aquatic ecosystems. Transgenic plants have been designed to incorporate the mercuric reductase gene, which helps in the detoxification of harmful mercury into less toxic forms. Such transgenic plants act as 'Mercury-breathing plants', and can be used for environmental remediation.

Applications of Transgenic Animals

For Industrial production

Transgenic animals are used as bioreactors for production of normal and recombinant protein products in large quantities (**Molecular Pharming**). An early example of success in 1990s was the transgenic ewe, Tracy, which

produced high levels of human protein α1-antitrypsin in her milk. Deficiency of α1-antitrypsin in humans causes lung diseases. The protein produced in the milk of Tracy was thought to cure the symptoms of this deficiency but clinical trials for this recombinant protein revealed the side effects in patients.

In 1997, the first transgenic cow, Rosie was created that could produce human α-lactalbumin-enriched milk. Human α-lactalbumin-enriched milk was found to be a more balanced product for human infants compared to natural cow milk. Table 4.2 lists the examples of a few human recombinant proteins that have been produced in transgenic animals through genetic engineering.

Table 4.2: List of human recombinant proteins produced in the milk of transgenic animals through genetic engineering

Name	Transgenic Animal
Albumin	Cow
α-fetoprotein	Goat
Growth hormone	Goat
Tissue plasminogen activator	Goat
Coagulation factor IX	Mouse
Coagulation factor VIII	Rabbit

For Research

Transgenic animals are also used as model organisms by scientists for studying the normal human physiology, development and diseases.

The transgenic animals are genetically manipulated to develop symptoms of diseases, such as cancer, Alzheimer's, etc. This also allows the researchers to understand the function of genes involved in various diseases.

Transgenic animals are also used for toxicity testing of vaccines, drugs and chemicals before they can be used on humans.

4.2.4 Concern over GMOs

The major concern over GMOs is the potential risk to human health and the environment. Transgenic crops may produce proteins that may cause allergic reactions in

humans. Concerns arise over the use of viral vectors and promoters in GMOs, as these can lead to viral infections in humans. The improved characteristics of GMOs may make them invasive for the native or wild type species. Variable insertion of transgene can lead to unpredictable or non-target effects.

For ethical issues related to the use of transgenic animals, India has established biosafety regulations for the production and use of GMOs. The Ministry of Environment, Forest and Climate Change has established the Genetic Engineering Appraisal Committee (GEAC) which regulates manufacture, use, import, export and storage of genetically modified organisms and cells/ hazardous microorganisms and decides upon the validity and safety of introducing GMOs.

4.3 Gene Therapy

Many diseases in humans are known to be caused either due to the absence of normal gene, or the presence of defective and disease-causing gene. Can such disorders be corrected? The answer lies in the approach known

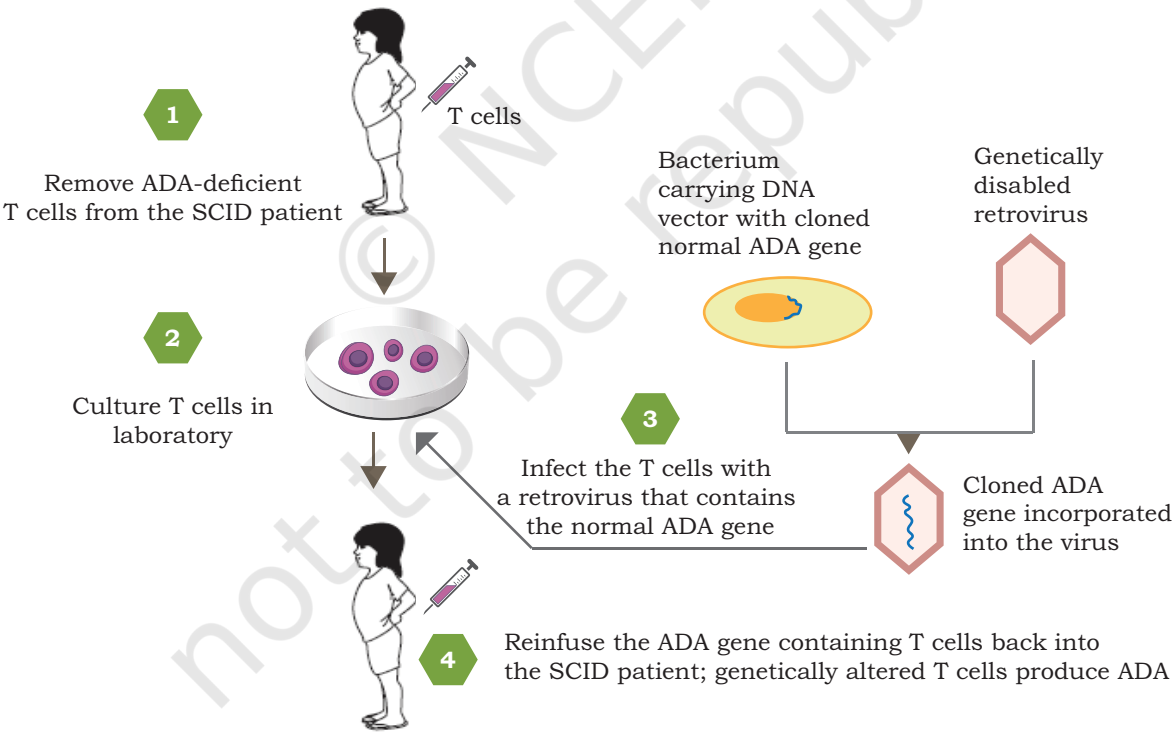


Fig. 4.6: Steps involved in gene therapy for SCID by replacement of a functional adenosine deaminase (ADA)

as 'Gene therapy'. Gene therapy is a technique designed to repair faulty genes in humans by introducing correct genetic material inside the cells. The obvious candidate diseases for gene therapy include immune disorder called **Severe Combined Immunodeficiency (SCID)** and inherited genetic disorders, such as cystic fibrosis, hemophilia, muscular dystrophy, etc. The first gene therapy was performed in 1990 in a four-year-old girl suffering from SCID caused due to the defect in adenosine deaminase (ADA) coding gene. Deficiency of ADA led to the inability to produce sufficient immune cells, which subsequently made the girl suffer from frequent life-threatening infections. A functional copy of ADA gene was inserted into a viral vector, which was then introduced into lymphocytes collected from the patient and these lymphocytes were then reintroduced into the patient's bloodstream (Fig. 4.6). The immune system functioning of the patient improved but the cure was not permanent.

4.3.1 Approaches for Gene Therapy

There are three main approaches for gene therapy—**(i) gene replacement or gene addition, (ii) gene inhibition** and **(iii) gene repair or gene editing therapies**.

In gene replacement or gene addition therapy, a functional copy of the gene is delivered into the genome to replace the non-functional defective gene. The new gene carries the instructions to synthesise the protein that was not available in the cell due to defective gene. For example, cystic fibrosis is an inheritable disease caused due to a defective membrane protein known as cystic fibrosis transmembrane conductance regulator (CFTR) protein. The defective protein is produced due to a mutation in the CFTR gene. Due to the defective protein, the movement of salt and water are hampered in body cell causing damage to respiratory and digestive tract (Fig. 4.7). If the mutated CFTR gene is replaced by normal CFTR gene, the disease can be cured. Another example is p53 gene that normally prevents tumor growth. Several types of cancers have been linked to defective forms of p53.

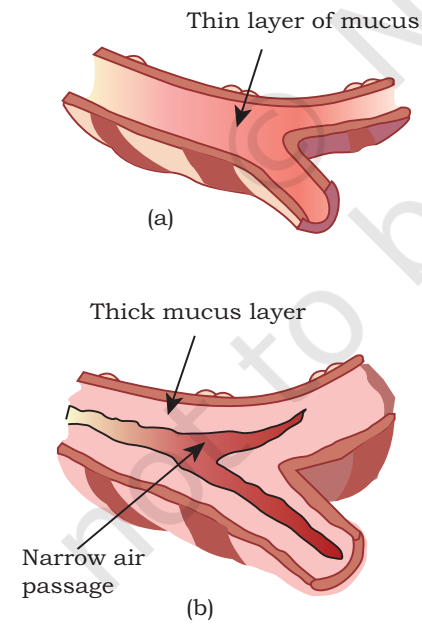


Fig. 4.7: Cross section of air passage (a) normal and (b) in cystic fibrosis

Replacement of the defective form of p53 in cancer cells may cause inhibition or death of cancer cells thereby, curing cancer.

In case of gene inhibition, the function of a gene, which causes a disease in cell is inactivated. Therefore, gene inhibition approach is applicable in case of infectious diseases such as Tay-Sachs disease, phenylketonuria and colour-blindness. In gene inhibition, a gene is introduced in a cell whose product either blocks the expression of the faulty gene or disarms the products of the faulty gene.

Finally, in case of gene repair or gene editing, the DNA is inserted, deleted, modified or replaced in the defective gene in living cells. This is achieved by a recent technology known as CRISPR/Cas9 system. (Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR associated protein 9). The CRISPR/Cas9 system was adopted from the natural defense system of bacteria or archea against viruses that attack them. With CRISPR system, bacteria recognise the invading virus and with the help of Cas9 enzyme, they cut the viral DNA into small pieces. The technique offers an easy way to snip out mutated DNA and replace it with correct sequence. This technique shows the possibility of treating several genetic disorders in future for details (refer section 5.4.2, Chapter 5).

4.3.2 Types of Gene Therapies

On the basis of the strategy used for gene delivery, gene therapy can be classified into *ex vivo* and *in vivo* (Fig. 4.9). In *ex vivo* (*ex* means 'out of' and *vivo* means 'something alive') gene therapy, cells are taken out from the diseased person, grown in culture, normal genes are then introduced into these cells, and finally these transformed cells are reintroduced into the patient. This is also known as **cell-based delivery**. *Ex vivo* approach is much simpler as compared to *in vivo* approach, because it becomes easier to manipulate the cells externally (Fig. 4.8).

In case of *in vivo* gene therapy, normal functional genes are directly introduced into the target cells and tissues of the person with the diseased condition. This is also known as direct delivery method.

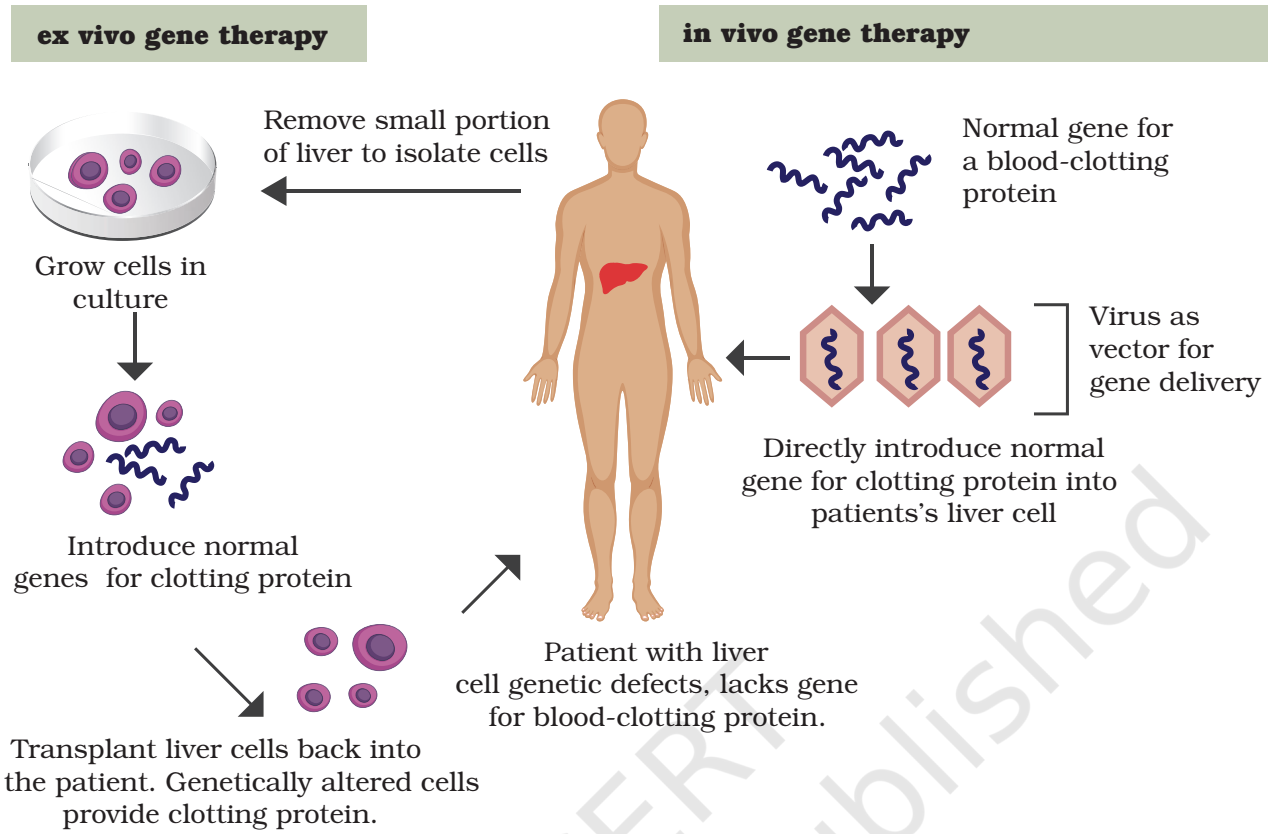


Fig. 4.8: ex vivo and in vivo gene therapies

On the basis of the nature of target cells or tissues, gene therapy can be classified into: *somatic* and *germ-line* gene therapy. In somatic gene therapy, functional copies of the target gene are introduced into the somatic cells of the patient. In germ-line gene therapy, functional genes are introduced into the germ cells (egg or sperm). The functional gene then gets integrated into the genome of the germ cells. Changes made to the germ-line are heritable.

Thus, based on the functional strategy used, gene therapy can be classified into:

- **Gene augmentation therapy**: Addition of functional gene to the genome with the aim of replacing the missing gene product.

- **Gene inhibition therapy**: Using antisense RNA or other inhibition techniques to block the expression of dominant acting mutated genes.
- **Gene targeting therapy**: Replacing the non-functional gene with a normal gene using homologous recombination.

Attempts to correct the function of a defective gene as described above could become possible only after advancements in rDNA technology, gene transfer and gene editing mechanism. You have already studied the mechanism of the delivery of target gene (desired DNA sequence) either through viral (direct gene transfer) or non-viral (indirect gene transfer) method earlier in this chapter and also in Chapter 3.

4.3.3 Merits and Demerits of Gene Therapy

Gene therapy provides a potential cure for the treatment of the devastating inherited diseases for which conventional treatment strategies provide little hope. Advancements in human genomics have shown that cancer is caused due to somatic aberrations in the human genome. This has led to enthusiasm among cancer researchers to use gene therapy approach for genetic manipulation of cancer cells and to find a possible cure to the disease.

However, uncontrolled expression of the therapeutic gene needs to be resolved for efficient gene therapy mechanism. Introduction of therapeutic gene in non-target cells or its random integration at a wrong place in the host genome is another concern. For example, if the DNA gets integrated into a tumor-suppressor gene, this can result in tumor. There may be a requirement of frequent administration of the therapeutic gene as gene therapy is short-lived in nature. Host rejection of the therapeutic gene or the viral vector employed in the process can stimulate the host's immune system. Besides being a costly process, gene therapy is effective for single-gene defects but not for multigene disorders.

4.3.4 Ethical Issues

Bioethics refers to the assessment of risks associated with the procedures and moral implications of new techniques. While genetic modification of somatic cells is approved by a large part of scientific community, genetic therapy in germ line cells often forms a subject for heated discussion in the field of science. Since, gene therapy includes making changes to the body's basic set of genes, it raises unique ethical considerations. Some of the ethical concerns associated with gene therapy include:

1. Use of gene therapy for gene enhancement in another concern.There is a need for strict regulations so that it is not used, where the outcomes are unpredictable
2. Should gene therapy be allowed to enhance the basic human trait like athletic abilities intelligence etc?
3. Germ line alternation are controversial. Though it can cure a genetic disorder in future generations, it may result in long term side effects.

Gene therapy as an approach is often criticised for promising too much but delivering very little. However, with constant efforts it is expected to become a common practice for the treatment of genetic diseases with single gene defects.

4.4 Recombinant Vaccines

A preparation of killed or weakened pathogen or their components given to elicit an immune response that subsequently recognises the infectious agent and fights off the disease is known as **vaccine**. The word vaccine was coined by Edward Jenner (Latin word *vacca* means cow), who injected cow pox virus in the skin of a person to confer successful protection against small pox. The process of injection and administering vaccines is known as 'vaccination'. The term vaccination was coined by Louis Pasteur in 1881. During vaccination, the pathogen (live or inactivated) or a component of pathogen known as antigen is injected into an individual with a purpose to induce immunity. Consequently, on the subsequent exposure

to the same pathogen, a quick and enhanced immune response is elicited involving antibodies and memory immune cells. The most common vaccines given to infants and young children are DPT (diphtheria, pertussis, tetanus) and MMR (measles, mumps, rubella), etc. Initially, vaccines were developed mostly by attenuation or inactivation of pathogens. To avoid several potential concerns raised by conventional vaccines, like chances of infection in case of live attenuated vaccine, reversal of the toxoids to their toxigenic forms, or co-purification of undesirable components and to overcome the complexity involved in obtaining sufficient quantities of purified antigenic components, recombinant vaccines were developed using the various tools of rDNA technology. There are three main types of recombinant vaccine:

1. Live genetically modified vaccines

The live pathogenic organism (bacteria or virus) is modified into a risk free and safe non-pathogenic organism by either the deletion or inactivation of one or more gene. These deletion or gene-inactivated vaccines are primarily developed to weaken or attenuate the disease agent.

Vector-based vaccines that carry a foreign gene from another disease agent also falls under this category of live genetically modified organisms (GMD). These when injected into the host, induces an immune response. Examples of such vaccines include *Salmonella* vaccine (for sheep and poultry) and a *Pseudorabies* virus vaccine (for pigs). Foreign genes must be inserted into the genome of the vaccine vector in such a way that the vaccine remains viable. Example of vector-based vaccines includes vaccinia virus, an enveloped virus belonging to the pox family. It has a large linear double stranded DNA genome with around 200 genes. The genome of this virus can accommodate stretches of foreign DNA, which can be expressed alongside with its own genes.

With the help of rDNA technology, vaccinia virus carrying antigenic genes of several different pathogens have been constructed which are capable of simultaneously providing

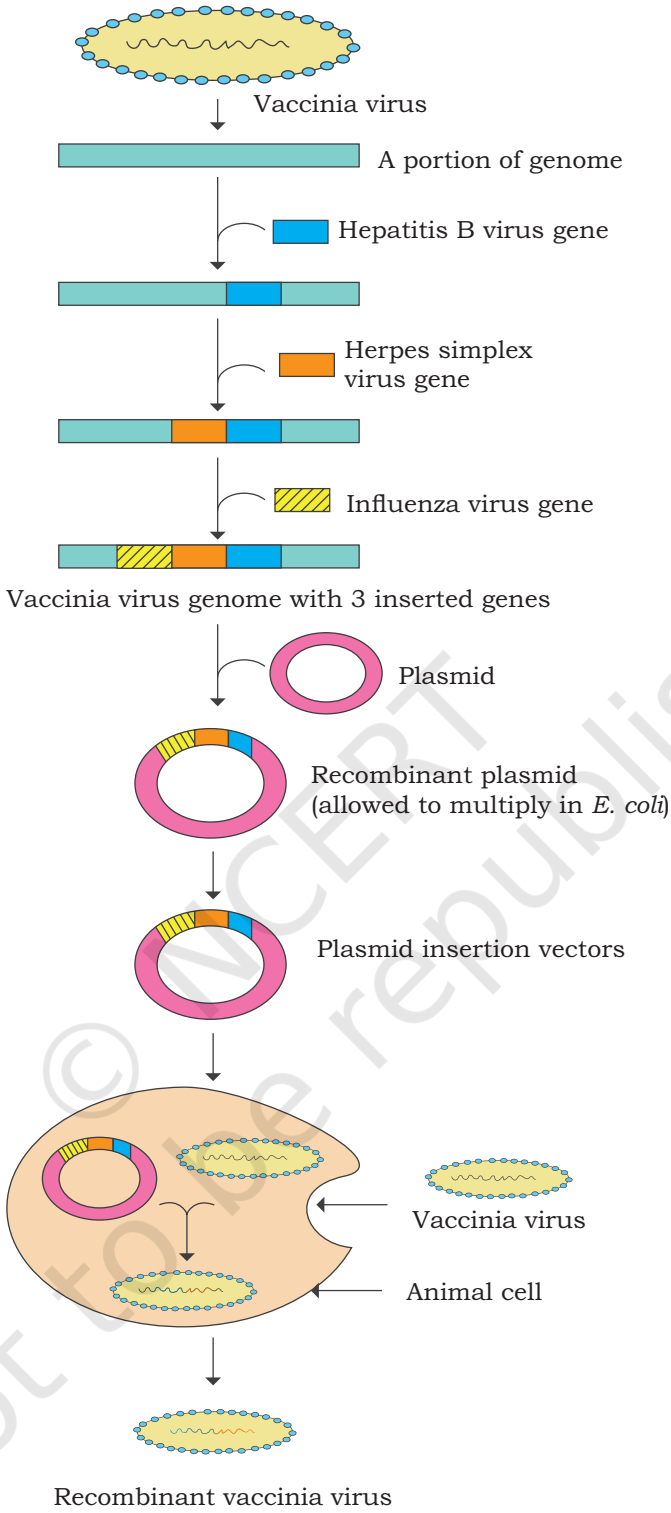


Fig. 4.10: Production of vector based vaccine using virus

protection against several different diseases. Such vaccines are known as **polyvalent** or **multivalent** vaccines based on their antigenic sites (Fig. 4.9).

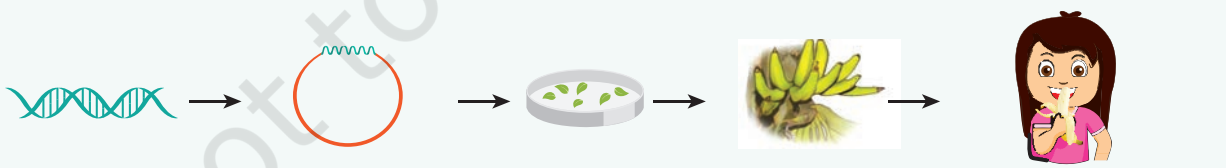
Thus, for the production of vaccinia vector to be used as vaccine, a small DNA portion of vaccinia virus is cloned in a plasmid vector (now known as insertion vector). This part of vaccinia DNA is used as recombination site in the later stage, for homologous recombination.

After this, the antigen gene of hepatitis virus, herpes simplex virus and influenza virus are inserted into this insertion vector and a recombinant plasmid is formed, which is the normal vaccinia virus host cells along with the normal vaccinia virus. During viral DNA replication, recombinant plasmid DNA recombines with viral DNA and antigen coding genes get incorporated into normal vaccinia virus genome. Thereby, generating recombinant vaccinia virus. Consequently, vaccinia virus offers elevated levels of protection against pathogens.

Box 4: Edible Plant Vaccines

Edible vaccines are vaccines manufactured in plants that can be administered easily by consuming the plant material containing the vaccine e.g. fruits, leaves and seeds, etc. The significant antigenic genes have been inserted into different plants, like tobacco, banana, potatoes, tomatoes and rice, etc. using various vectors. These recombinant vectors are introduced within the plants by using either a bacterial transformation system, like *Agrobacterium tumefaciens* or by microprojectile bombardment method. The ingestion of the edible vaccine stimulates the mucosal immune system and elicits an immune response.

Example: Edible vaccine for Hepatitis B banana has been successfully made by inserting the HBsAg gene in banana genome. Banana is the best plant for oral vaccine production as it is easy to digest, requires no cooking, palatable, inexpensive and available throughout the year.



Gene from a human pathogen is inserted into a vector

Vector introduced into plant cells

Leaf segments sprout into whole plants carrying the gene from human pathogen

Eating banana triggers immune response against pathogen

Reprint 2025-26

2. Recombinant subunit vaccines

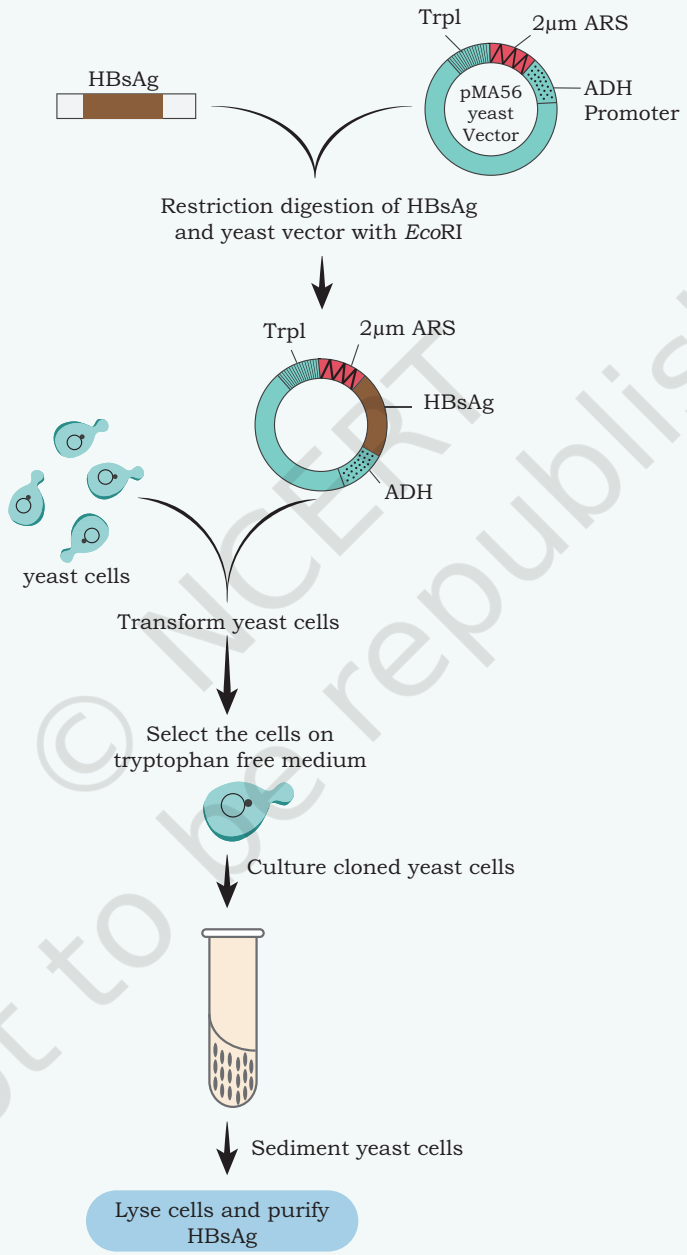
Subunit vaccines contain only a part of the whole pathogenic organism. These can be either synthetic peptide or an expressed whole protein extracted from the pathogenic organism or expressed from cloned genes in the laboratory. Prokaryotic systems, such as *Escherichia coli* or eukaryotic systems, such as yeast can be used to express recombinant proteins. The advantages of these vaccines include purity, stability and safe use. One such example of a subunit vaccine developed against Hepatitis B, a widespread disease that mainly affects liver leading to cirrhosis, chronic hepatitis and cancer. Hepatitis B virus consists of a core containing a viral genome (DNA) surrounded by a phospholipid envelope carrying surface antigens HBsAg, the key element for the hepatitis B vaccine. Culturing Hepatitis B virus and producing these surface antigens is very complicated. Therefore, with the advent of rDNA technology, the gene coding HBsAg surface protein was identified and inserted in pMA56, a yeast plasmid vector and expressed in *Saccharomyces cerevisiae* yeast cells. (Box 5)

3. DNA vaccines

In recent years, DNA vaccines are one of the promising advancements in vaccine technology. For DNA vaccines, genes of interest (antigens) are identified and cloned in plasmids. DNA vaccine carrying the plasmid harbours a promoter site, cloning site, origin of replication, a selectable marker sequence and a terminator sequence, like a poly-A tail. During vaccination, DNA is directly injected into the muscles of the animal model system like mice, generally using a 'gene gun' that is based on compressed gas, to inject the DNA into the muscle. DNA vaccines can also be administered by nasal spray. The antigenic DNA is taken up by a few muscle cells, which after protein expression, induce the immune system. DNA vaccines are capable of inducing both humoral and cellular immune responses.

Box 5

The HBsAg gene is cleaved and ligated to a strong alcohol dehydrogenase promoter (ADH) of a pMA56 yeast vector promoter. These plasmids are then transferred and cultured. pMA56 plasmid also carries a termination sequence from yeast gene and the origin of replication from bacteria. For selection of recombinants, an antibiotic resistance marker and a gene that permits growth only in the absence of amino acid tryptophan were used in pMA56. The protein is separated, purified and used for immunisation. This was the first recombinant subunit vaccine for public use, licensed in 1987 and marketed as Recombivax® and Engerix-B®.



Production of HBsAg in yeast cells (ADH: alcohol dehydrogenase TrpI: Tryptophan biosynthesis gene I; 2μm ARS (Autonomously replicating sequence of yeast)

4. RNA Vaccines

RNA vaccines contain mRNA. Once injected for vaccination, the mRNA is directly taken up by antigen presenting cells (APCs) and other target cells, where the mRNA is expressed as properly folded and glycosylated protein that acts as an antigen (Fig. 4.10). These vaccines elicit both humoral and cellular immune response against the encoded protein.

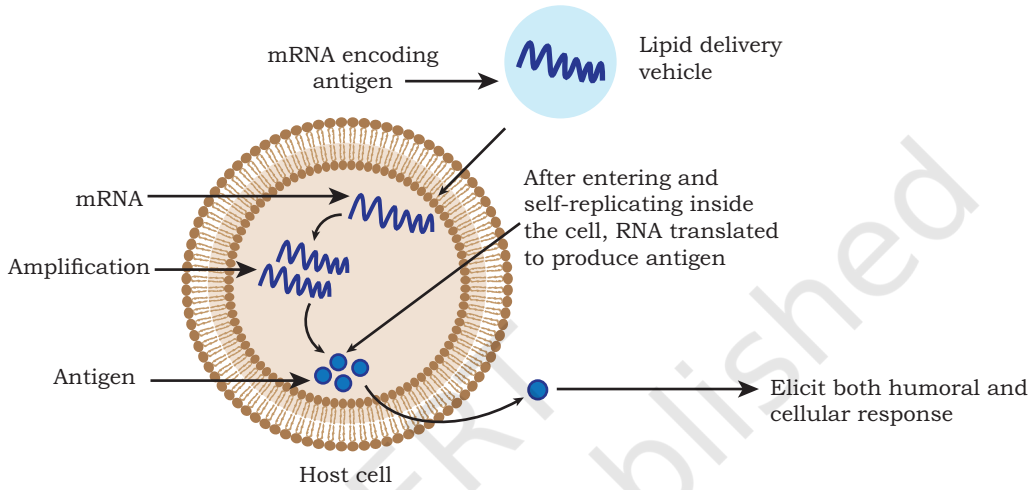


Fig. 4.10: RNA vaccine

RNA being labile, the stability of injected mRNA is prevented by modifying it in a variety of ways, e.g., the addition of a 5' cap; the length and structure of a 3' PolyA tail, etc. Therefore, RNA vaccines are formulated with specific delivery systems such as lipid nanoparticles (LNPs), which protect RNA from degradation and increase target cellular uptake.

Thermostability of a vaccine is vital to reduce the need for cold conditions during vaccine storage and use. Since RNA is unstable, it needs to be frozen when stored to ensure long-term stability. Efforts are underway to improve the thermostability of RNA vaccines. Lyophilisation or freeze-drying has been proposed as a way to improve their thermostability. In this direction, formulation of RNA with thermostable lipid nanoparticles, as mentioned above, has

also been suggested for improving the thermostability of RNA vaccines.

RNA vaccines are comparatively simple and have high yield production. Their virus-free production process enables modest and scalable production facilities. As a result of cell-free production, there is less concern with respect to contaminating agents. Another important advantage of mRNA vaccine technology is its ability to respond rapidly to emerging viral variants. This means making modified mRNA vaccines for mutants with changed sequences, is quick and simple.

Thus, RNA vaccines offer several advantages over other categories of vaccines including rapid and low-cost development, reduced need for optimisation and regulatory testing (due to already existing optimised and licensed formulation and delivery method). Furthermore, mRNA vaccines require less amount of vaccine dose in each shot and may not even require two doses. Also, it can be produced more quickly and efficiently as compared to other vaccines in case of new emerging variants of the pathogens. RNA vaccines are comparatively safe. There is no risk of pathogen reactivation and RNA is degraded *in vivo* with no risk of antigen persistence or integration into the genome.

mRNA Vaccine Development against COVID-19

The COVID-19 pandemic demonstrated the threat of pandemics caused by novel, previously unrecognised, or mutated Severe Acute Respiratory Syndrome Corona Virus-2 (SARS-CoV-2) strain with high transmissibility. Vaccines namely, Covaxin (inactivated whole virus) and Covishield (Adenovirus based DNA vaccines) are used in India for protection against COVID-19. However, for effective control of pandemics, RNA-based vaccines have also being developed. A member of mRNA-based vaccine candidates for COVID-19 are in different phases of preclinical and clinical trials.

4.5 Therapeutic Agents/Molecules: Monoclonal Antibodies, Insulin and Growth Hormone

Recombinant DNA technology has revolutionised health care by facilitating the large-scale biological production of a variety of safe, pure and efficient human proteins. Compounds used for treating a disease and for improving the health of human beings are called **therapeutic agents**. With the advent of rDNA technology, not only the newer therapeutic agents are produced but also more efficient forms of conventionally produced therapeutics are being manufactured. Since these recombinant therapeutics produced are identical to the natural human proteins unlike those isolated from animals (like cow and pig), hence, they do not induce immune responses and are free from threat of any allergic responses. Presently, numerous products, like antibiotics, monoclonal antibodies, blood clotting factors, hormones (insulin and growth hormone), cytokines and vaccines are being produced for human welfare.

Antibodies are the protein molecules produced by B cells of the immune system and protect the body against the invading foreign substances called **antigens**. In human serum, five different types of antibodies are present: IgA, IgD, IgE, IgG and IgM. B cells, in response to a foreign particle, produce many different types of antibodies specific to different regions of antigen called **epitopes** present on the surface of antigen and hence, designated **polyclonal antibodies**. This polyclonal antibody-mediated response leads to the antigen processing and degradation and therefore, has obvious advantage for the organism, *in vivo*. However, due to their heterogenic nature, the efficacy for *in vitro* use is restricted. On the contrary, antibodies, specific to a particular region or epitope of an antigen, known as **monoclonal antibodies** (MAbs) can be very well exploited for *in vitro* purpose, like in therapeutic and diagnostic applications.

Earlier, following a brief exposure of an animal to an antigen, B cells were isolated and cultured for producing monoclonal antibodies. However, B cell culturing is very

tricky and complicated, and also the monoclonal antibodies produced were short lived. An invention made by George Kohler and Cesar Milstein (1975) provided a way out, through which the large-scale production of monoclonal antibodies using **hybridoma technology**, where the short-lived antibody secreting normal plasma cell is fused with immortal myeloma cells with the help of Polyethylene Glycol (PEG) leading to the formation of hybrid cells called **hybridoma**. Hybridoma cells acquire immortal growth, a typical attribute of a myeloma cell, besides the antibodies production property bestowed by B plasma cell. Myeloma cells are genetically engineered such that they cannot use hypoxanthine, aminopterin and thymidine (HAT medium) as a source for nucleic acid biosynthesis and will die in culture. On the contrary, B cells are able to survive in HAT medium but have a definite life span and die after that. Only B cells that have fused with these engineered myeloma cells will survive in culture when grown in HAT medium (Fig. 4.11). With the advances in rDNA technology, it is possible to develop mouse antibodies carrying a few human segments known as **chimeric** or **humanised antibodies** possessing higher effectiveness and activity.

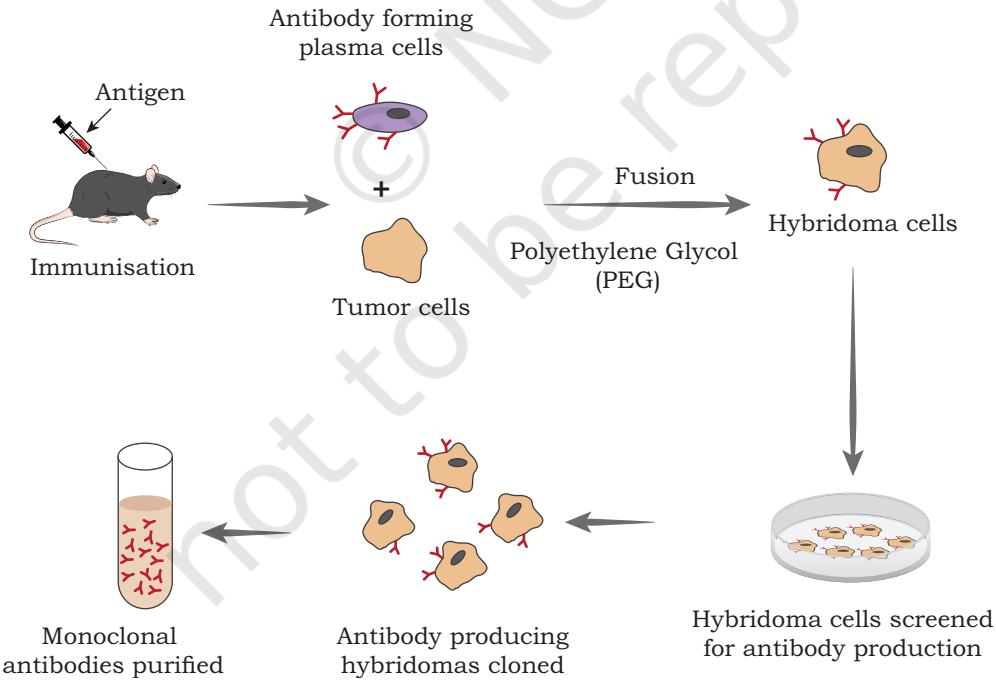


Fig. 4.11: Production of monoclonal antibodies using hybridoma technology

Monoclonal antibodies have revolutionised the diagnosis of various diseases and are used in a number of commercially available diagnostic kits, like pregnancy detection kits and use of monoclonal antibodies in the diagnostic imaging of cancer, wherein the tumor specific monoclonal antibodies can help in differentiating cancerous and non-cancerous cells. In recent years, monoclonal antibodies are employed as a very specific targeted therapeutic agents and are coupled or attached with toxins or drugs and carried to target tissues for efficient action.

Box 6

In 1921, a series of classic experiments were performed by Frederick Banting and Howard Best, which lead to the isolation of insulin. Insulin is distributed to all cells of the body through the bloodstream. The primary structure of insulin was studied by Sanger (1955) for which he was awarded Nobel Prize in 1958.

Insulin, a hormone secreted by beta cells of pancreas acts on different types of cells resulting in the uptake of glucose from blood. Insulin also stimulates the conversion of glucose to glycogen (glycogenesis) in the target cells. A disease caused by the deficiency of insulin hormone and characterised by the presence of sugar in urine is **Diabetes mellitus**.

Structurally, insulin consists of two chains: an A chain of 21 amino acids and a B chain of 30 amino acids interconnected with disulphide bonds (Fig. 4.12). After Banting and Best experiments, insulin was purified from the pancreas of pigs and cows. Human insulin, porcine insulin (from pig) and bovine insulin (from cow) are very similar except one or three amino acids. But these minor differences in the chemical structure can lead to an allergic response in some diabetic patients. As a result, the use of human insulin for treatment is more suitable as compared to the animal insulin. Physiological insulin first gets translated into a primary protein called **preproinsulin** composed of 110 amino

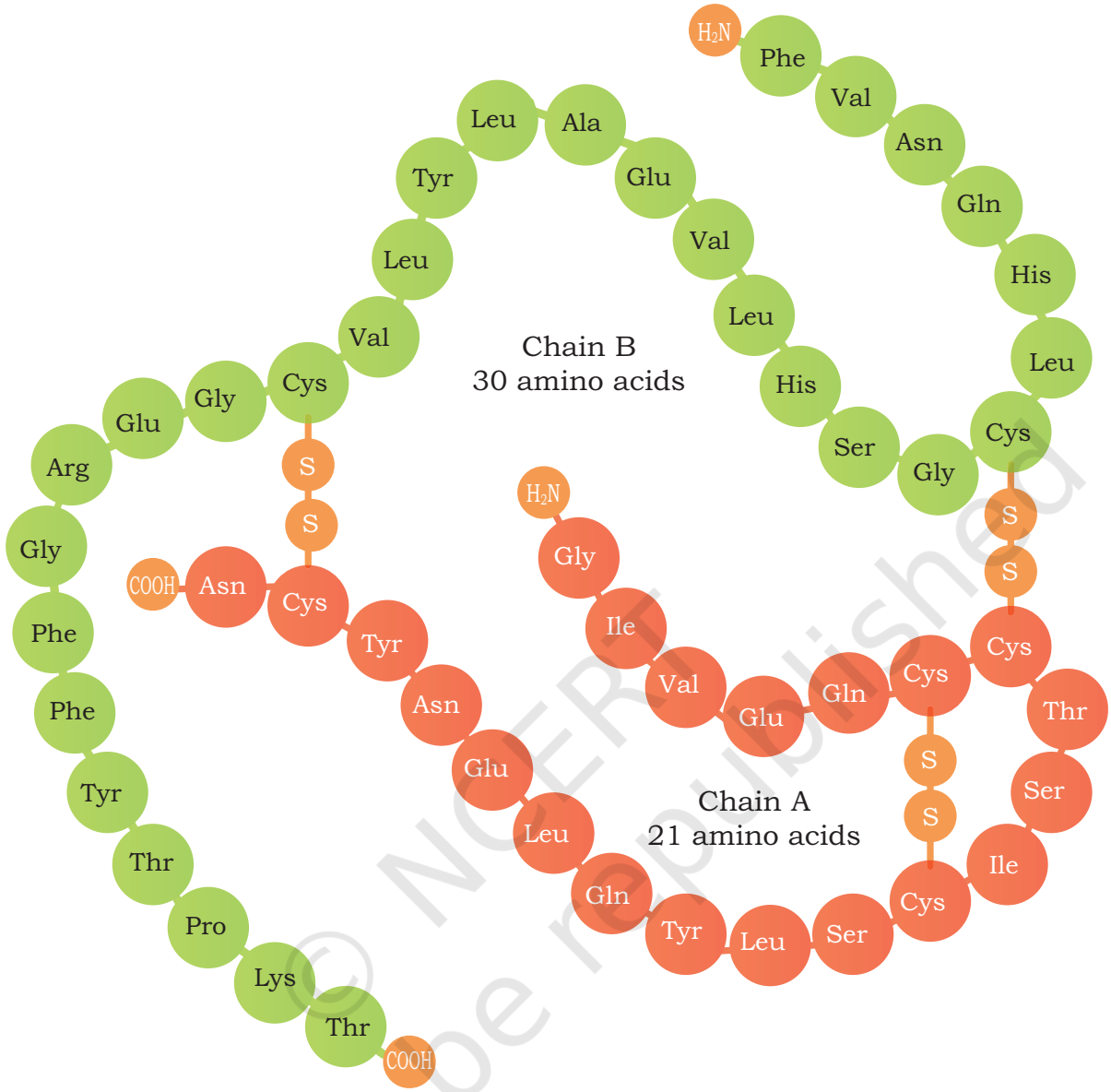


Fig. 4.12: Insulin Structure: Primary structure of human insulin. A-chain is shown in red and the B-chain in green

acids. In endoplasmic reticulum, first the signal peptide fragment is cleaved off from preproinsulin, thus turning preproinsulin to **proinsulin** (composed of 86 amino acids). Proinsulin carry three distinct peptide chains: A-chain, B-chain, and C-chain (composed of 31 amino acids). In the next step inside endoplasmic reticulum, the C-chain and additional four connecting amino acids are removed to produce biologically active insulin of 51 amino acids residues (Fig. 4.13).

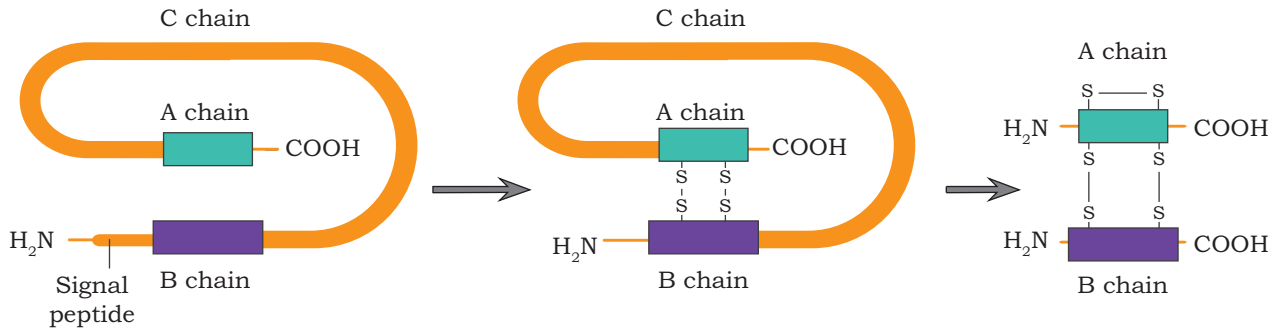


Fig. 4.13: Conversion of Preproinsulin to Insulin

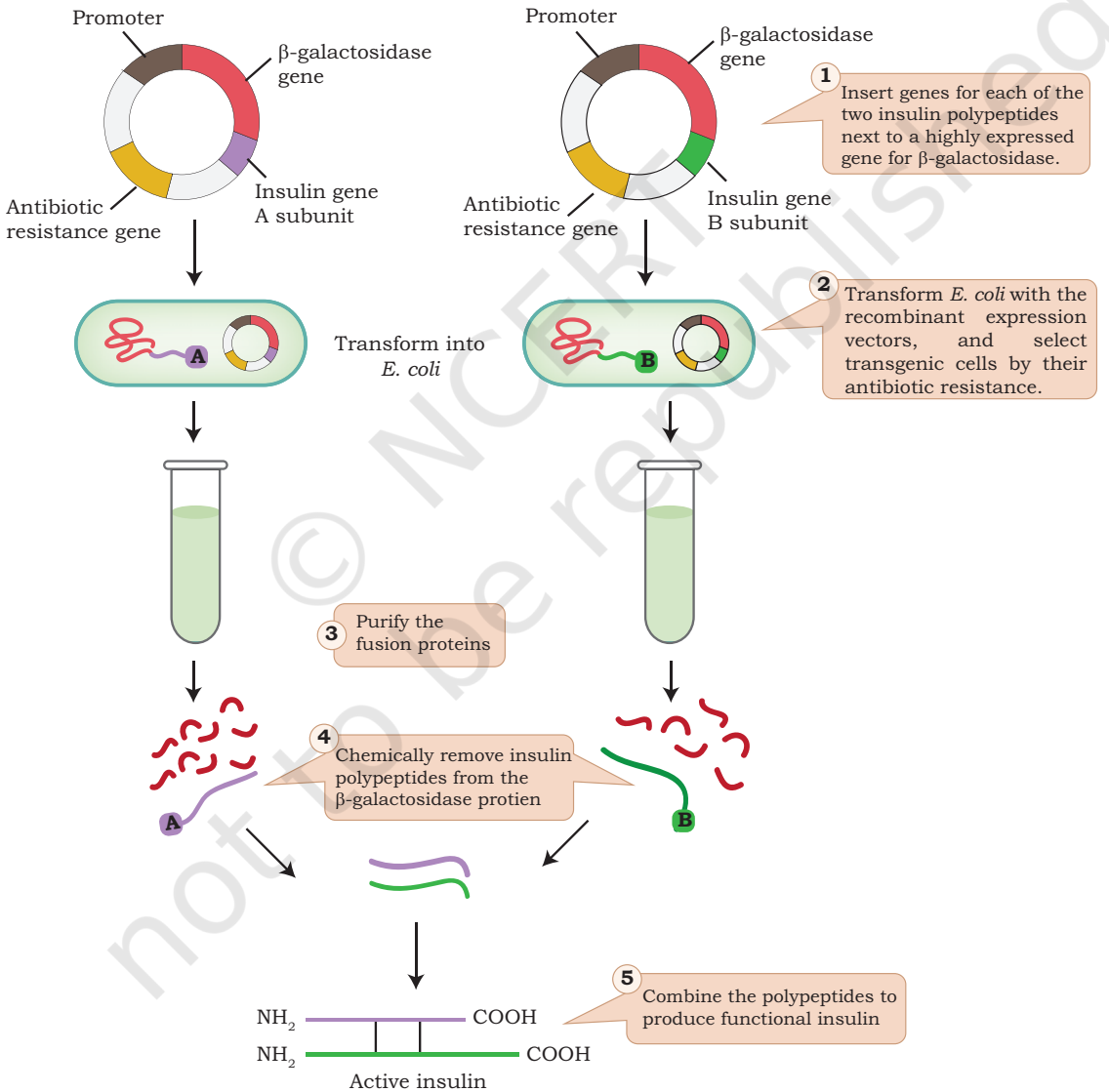


Fig. 4.14: Steps involved in the production of genetically engineered insulin

In late 1970s, biochemists exploited various tools of rDNA technology for the production of insulin. Isolated insulin gene from a gene library was inserted in a bacterial plasmid of *E. coli*. A promoter site derived from the lac operon was inserted next to the insulin gene. The gene expression produced a fusion protein, i.e., insulin molecule fused with the enzyme β-galactosidase (β-gal), which was later cleaved to produce the human insulin. Because the processing of **preproinsulin** was not feasible in bacteria, scientists sidestepped this difficulty by cloning separately the genes for A and B insulin chains in bacteria. The fusion protein, β-gal-insulin was extracted and treated with cyanogen bromide (CNBr) to separate β-gal fragment from A and B chains. Subsequently, the disulfide bonds were again reestablished between A and B chain to reconstruct the final product, insulin (Fig. 4.14).

The first genetically engineered human insulin marketed as **Humulin**® (Fig 4.15) was manufactured in 1982 which was more effective as compared to the pig and cow-derived insulin and the correct sequence was completely free from any allergic response.

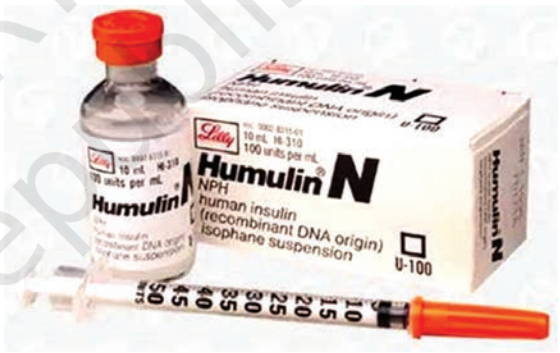


Fig. 4.15: Eli Lilly Company has been marketing E.coli expressed insulin as Humulin

Another important hormone secreted by the pituitary gland, ***human growth hormone*** (HGH), was produced using rDNA technology. HGH containing 191 amino acids is peptide hormone and promotes body growth by increasing the amino acids, lipids and carbohydrate metabolism during childhood as well as the adulthood. Childhood deficiency of HGH leads to ***Dwarfism.*** Dwarfism is characterised by retarded body growth, chubby face, added fat deposition at waist and short height of about four feet, while the deficiency in adult leads to an average intelligence but with an abnormal body proportion (Fig. 4.16). Since the bone growth is not fully complete at an early stage, dwarfism can be treated at this stage by providing HGH shots. To obtain sufficient HGH quantity for one year using conventional treatment, the pituitary glands from more than 80 cadavers were required. Not only, this method was expensive but also carried a risk of developing disease from infected brain tissue of cadavers.

Box 7

In 1978, the use of cadaver tissue was restricted in the United States and Great Britain because of the possibility of transferring Creutzfeldt-Jacob (CJ) Syndrome. This disease is believed to be caused by a virus-like agent 'prion' and it is accompanied by tremors, convulsions, dementia and wasting of the muscles. Indeed, by 1993, 24 cases of CJ syndrome had been identified in French recipients of HGH from cadaver tissue.

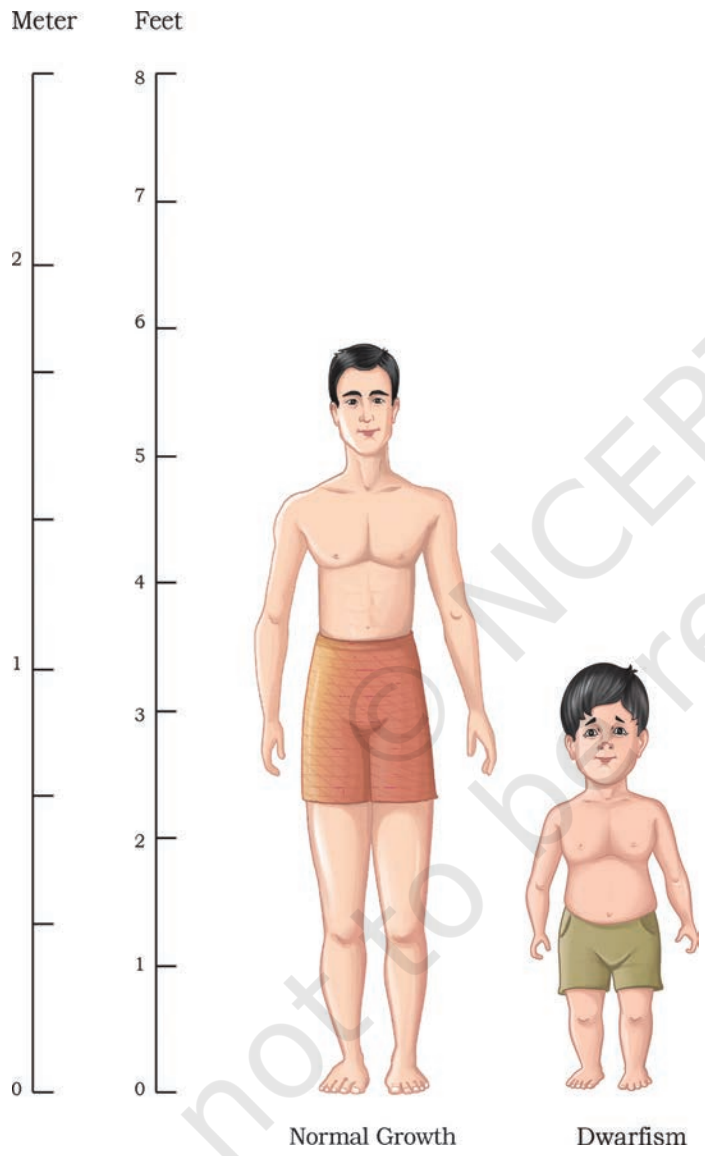


Fig. 4.16: Deficiency of human growth hormone results in retarded growth and unusual body proportions

rDNA technology has enabled the production of the entire HGH. The production process for HGH is similar to that for human insulin production.

Though the natural HGH molecule consists of 191 amino acids but during its production in the body, an intermediate molecule having an extra 26 amino acids is encoded as a signal peptide. This signal peptide chain is finally cut free during secretion. Consequently, to synthesise the gene for HGH, the HGH encoding mRNA was used as a template, and a complementary DNA (cDNA) molecule was synthesised. However, the bacterium fails to remove the signal peptide from the intermediate HGH molecule.

Nucleotide sequence coding for signal peptide was removed from the HGH gene. Since, no known restriction enzyme can make this cut, restriction enzyme EcoRI was used to remove the base sequences for the signal peptide (26 amino acids) plus an additional 24 amino acids (a total of 50 amino acids) from the cDNA molecule. The extra 24 amino acids base sequence removed were chemically synthesised and put back on the cDNA molecule to generate the complete

human growth hormone gene (Fig. 4.17). In the absence of signal peptide sequence, the human growth hormone gene was inserted in bacterial cells for hormone production. In 1985, genetically engineered human growth hormone was produced and marketed as **Humatrope**® and **Protropin**®.

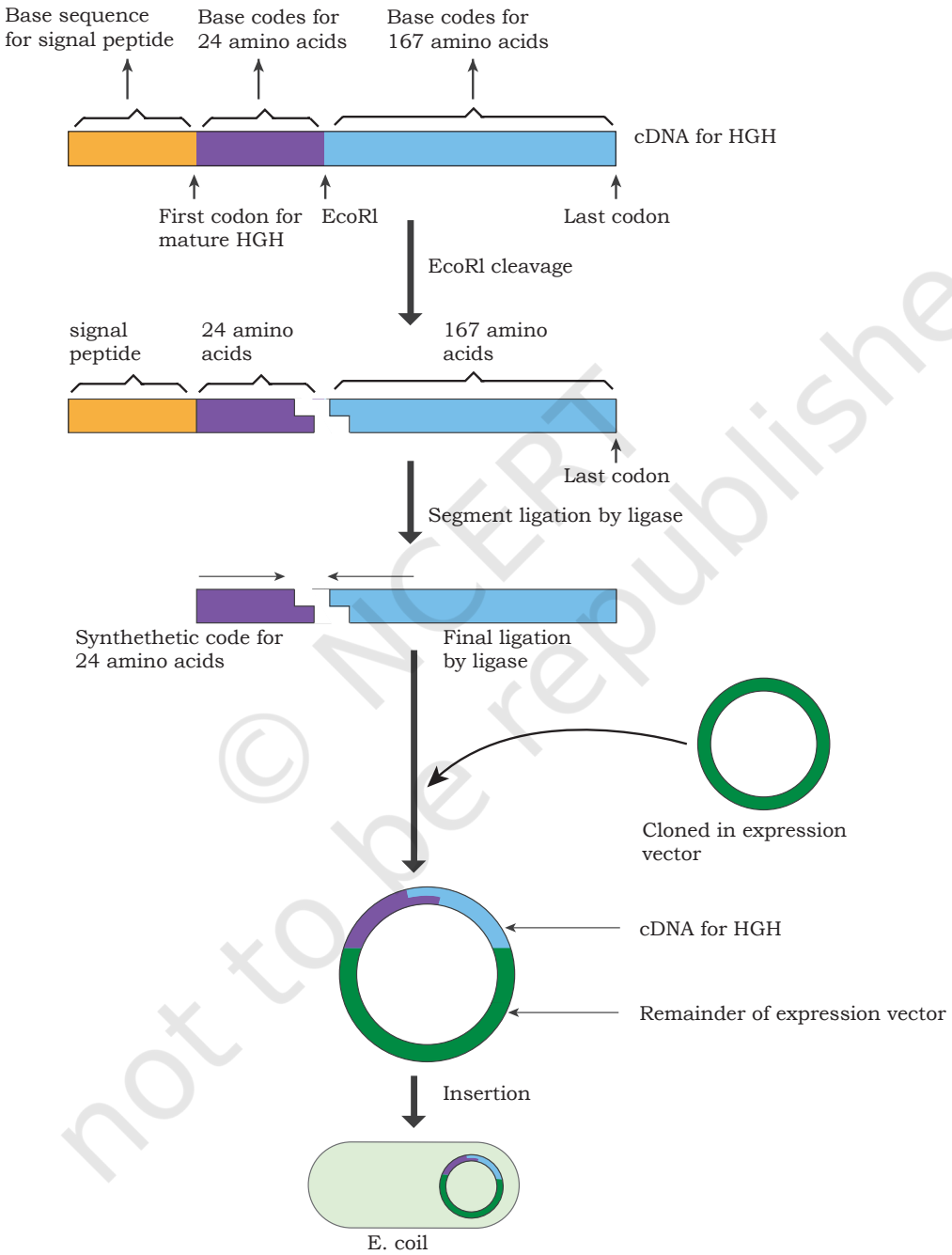


Fig. 4.17: Production of genetically engineered human growth hormone

SUMMARY

- To study and compare the inherited variations in human DNA without sequencing, a new technique, known as 'DNA fingerprinting' was developed by Sir Alec Jeffreys in 1984 at the University of Leicester.
- In DNA fingerprinting, a stretch of mini-satellite DNA known as Variable Number Tandem Repeats (VNTR) tandemly arranged are exploited using the technique, referred to as Restriction Fragment Length Polymorphism (RFLP).
- The process of insertion of a foreign gene (transgene) into the genome of an organism and its transmission and expression in the organism's progeny is termed as transgenesis. The organisms carrying the transgene are known as transgenic organisms.
- Transgenic plants are also called genetically modified plants, whose genome is modified, like introduction of one or more genes from another species through genetic engineering techniques.
- Basic requirement for genetic transformation is construction of genetic vehicle, which carries the genes of interest flanked by necessary regulating sequences, like promoter or terminator. The most commonly used techniques for gene transfer are of two types: vector-mediated or indirect gene transfer and vector-less or direct gene transfer.
- Vector-mediated or indirect gene transfer includes transformation using *Agrobacterium tumefaciens*, *in* planta transformation, plant virus-mediated transfer while vectorless or direct gene transfer includes particle bombardment, protoplast transformation and microinjection.
- Transgenic animals are animals whose genetic makeup has been transformed by the use of various genetic engineering techniques, such as DNA pronuclear microinjection, embryonic stem cell-mediated gene transfer and retrovirus-mediated gene transfer.
- Transgenic plants have been developed with improved agronomic traits in crop plants and products, for example, resistance to biotic and abiotic stresses nutrient quality and delayed fruit ripening, etc.
- Transgenic plants are used in Molecular Farming for large scale production of industrial and therapeutic products.
- Transgenic animals have also been used in molecular pharming for large scale production of proteins, such as α1-antitrypsin, human α-lactalbumin, etc.
- There are a number of concerns related to use of GMOs on human health and environment. The Genetic

Engineering Approval Committe (GEAC) established by Ministry of Environment, Forest and Climate Change regulates the manufature, use, import, export of hazardous microorganisms.

- Gene therapy is a technique designed to repair faulty genes in humans by introducing correct genetic material inside cells. There are three main approaches for gene therapy, they are: (i) Gene replacement / Gene addition, (ii) Gene inhibition and (iii) Gene repair/ Gene editing.
- Since, gene therapy includes making changes to the body's basic set of genes, it raises unique ethical considerations.
- A preparation of killed or weakened pathogen or their components given to elicit an immune response that subsequently recognises the infectious agent and confers protection against disease is known as 'Vaccine'.
- To avoid several potential concerns raised by conventional vaccines like reversal of the toxoids to their toxigenic forms, recombinant vaccines were developed using the various tools of rDNA technologies.
- There are three main types of recombinant vaccines: Live genetically modified vaccines, recombinant subunit vaccines and RNA/DNA vaccines
- rDNA technology enables health care by facilitating large scale biological production of a variety of safe, pure and efficient therapeutic agents, such as a Drugs: Monoclonal antibodies, human proteins, e.g., Insulin, HGH.
- With the advances in rDNA technology, it is possible to develop mouse antibodies carrying a few human segments known as chimeric or humanised antibodies possessing higher efficacy and activity.
- In late 1970s, biochemists exploited various tools of rDNA technology for the production of insulin. They isolated the insulin gene from a gene library and then inserted this gene in a bacterial plasmid of *E. coli*.
- The first, genetically engineered human insulin marketed as Humulin® was manufactured in 1982. Successful production of human insulin proved without any ambiguity, the possibility of genetically engineering diverse biological organisms to produce human proteins for medicinal and therapeutic use.
- DNA technologists can now produce Human Growth Hormone entirely using rDNA technology. In 1985, genetically engineered human growth hormone was produced and marketed as Humatrope® and Protropin®.

EXERCISES

1. What do you mean by DNA fingerprinting? Explain it through RFLP.
2. What are GMOs? Describe the method of development of transgenic plants.
3. Differentiate between direct and indirect method of gene transfer. Name one indirect method suitable for gene transfer in dicot plants.
4. What is molecular pharming? Give applications of transgenic animals in molecular pharming.
5. Differentiate between gene gun and gene therapy.
6. Give the procedure of development of recombinant subunit vaccines.
7. Write a short note on DNA vaccines.
8. Describe the advantages of monoclonal antibodies developed by rDNA technology over that developed by Hybridoma technology.
9. Briefly describe the development of Humulin through rDNA technology.
10. Write a short note on humatrope and Protropin.
11. Briefly describe the applications of rDNA technology in crop improvement.
12. List the ethical issues related to the use of transgenic animals?
13. What is the role of vaccinia virus in the development of recombinant vaccine?
14. Write a short note on recombinant therapeutic agents.
15. Write a short note on humanised antibodies.
16. **Assertion:** In hybridoma technology, B cells are fused with myeloma cells.

    Reason: Myeloma cells are immortal.

    (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.

    (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.

    (c) Assertion is true but reason is false.

    (d) Both assertion and reason are false.
17. **Assertion:** In Humulin, polypeptide A and polypeptide B are linked with disulfide bridges.

Reason: C peptide is removed from proinsulin to form biologically active insulin.

(a) Both assertion and reason are true and the reason is the correct explanation of the assertion.
(b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.
(c) Assertion is true but reason is false.
(d) Both assertion and reason are false.

18. DNA fingerprinting depends on identifying specific:
(a) Coding sequences
(b) Non-coding sequences
(c) mRNA
(d) Promoter

19. Short stretch of DNA used to identify complementary sequences in a sample is called:
(a) Probe
(b) Marker
(c) VNTR
(d) Minisatellite

20. Variable number tandem repeat (VNTR) are:
(a) Repetitive coding short DNA sequences
(b) Non-repetitive non-coding short DNA sequences
(c) Repetitive non-coding short DNA sequences
(d) Non-repetitive coding short DNA sequences

21. Cry genes or Bt genes are obtained from:
(a) Cotton pest
(b) Tobacco plant
(c) *Bacillus thuringiensis*
(d) *E. coli*

22. When gene therapy is done in somatic cells, it is _____.
(a) non-heritable
(b) heritable
(c) rarely heritable
(d) not related to heritability

23. In gene augmentation therapy, genetic material is _____.
(a) modified
(b) replaced
(c) suppressed
(d) removed

24. Germ cell therapy is used for _____.
    (a) RBC
    (b) Stomach cells
    (c) Egg cells
    (d) Bone marrow cells
25. For the first time, from which animal material was isolated for vaccination?
    (a) Cat
    (b) Cow
    (c) Goat
    (d) Horse
26. Vaccination was invented by:
    (a) Jenner
    (b) Pasteur
    (c) Watson
    (d) Crick
27. For the production of insulin by rDNA technology, which bacterium was used?
    (a) *Saccharomyces*
    (b) *Rhizobium*
    (c) *Escherichia*
    (d) *Mycobacterium*
28. Genetically engineered insulin is called _____.
    (a) Humulin
    (b) Promulin
    (c) Bovulin
    (d) Proculin
29. Monoclonal antibodies are produced by_____.
    (a) Mutations
    (b) Transfection
    (c) Hybridoma technology
    (d) RNA interference

UNIT II

Genome Engineering

Chapter 5: Genome Technology and Engineering

Stanley Norman Cohen
17 February 1935—present

Stanley Cohen was born in Perth Amboy, New Jersey. After graduating from Rutgers College in 1956, Cohen went to the University of Pennsylvania School of Medicine. He finished his medical degree in 1960. He accepted a position at Stanford University's Medical School in 1968 and began experimenting with plasmids. Cohen is a Professor of Genetics at Stanford University. He is a member of the National Academy of Sciences, and in 1980, won the Albert Lasker Basic Medical Research Award. He was awarded the National Medal of Science in 1988. Stanley Cohen is well-known for his innovation with Herbert Boyer of recombinant DNA technology, which they published in 1973. Stanley Cohen and Herbert Boyer were the first two scientists to transplant genes from one living organism to another, a fundamental discovery for genetic engineering.

Chapter 5 Genome Technology and Engineering

Gradual developments in the area of biotechnology and molecular biology, especially in genome mapping and its implication, has led to the opening of new frontiers for research as well as its applications in many fields. Advancement in genome research has opened up scope for understanding of transcriptome and proteome of any organism on one hand, and application of genomics and related engineering in prediction and treatment of diseases on the other. Applications in crop improvement, gene therapy and remediation of environmental hazards are yet another frontier in which genome technology has tremendous potential and scope. In this chapter, concepts related to genome, its sequencing, analysis and its impact on understanding the biology of organisms have been detailed. In Chapter 7 of Class XI, students have already studied as to how genetic information is encoded in DNA and the processes, like transcription and translation.

5.1 Mapping of Genome: Genetic and Physical

Genome of an organism is the complete genetic information present in the cell of an organism. For prokaryotes, it includes

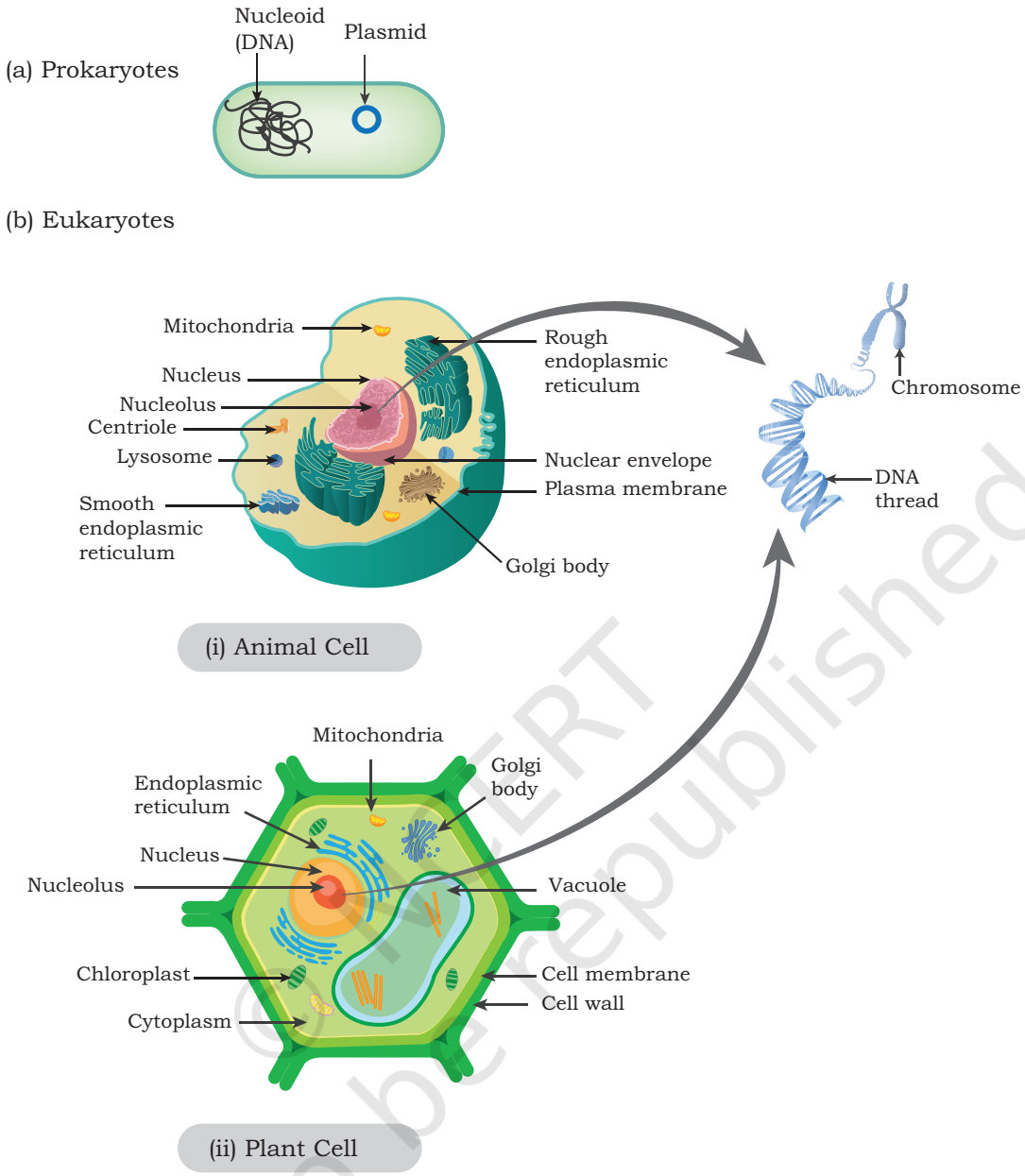


Fig. 5.1: Genome represents DNA content present in Prokaryotes or Eukaryotes. (a) For prokaryotes, it includes the DNA present in the nucleoid and plasmid (b) For eukaryotes, it includes the DNA present in the chromosomes of the nucleus as well as in organelles, like mitochondria and chloroplast

the DNA present in the nucleoid region and plasmid (as it is present in bacteria); and for eukaryotes, it includes the DNA present in the chromosome of the nucleus as well as in organelles, like mitochondria and the plastids, e.g. Protists and all multicellular organisms (Fig. 5.1).

One of the important tasks for many biologists is to compare genomes or DNA of two organisms for various purposes including the understanding of conserved genes or DNA sequences and relationships, etc. In simple words, from multiple samples of a biological organism, a scientist wants to know whether DNA samples from two or more sources are similar or different. If DNA from two sources are different, then how are these different from each other. Further, if an interesting phenotype is noticed, the scientist might also be interested to know the genetic basis (genes and mutations in it) of a particular phenotype. Therefore, a set of techniques is required to answer such questions. Ideally, if we know the complete DNA sequence of each sample, we can compare them and answer all such questions.

In genome research, it is extremely important and crucial to create a map of the genome of an organism from the point of view of identification and relative position of genes or loci. Maps of genome can be created by two different approaches, i.e., the genetic approach and the physical approach, giving rise to genetic and physical mapping techniques for genome mapping in biological organisms (Fig. 5.2).

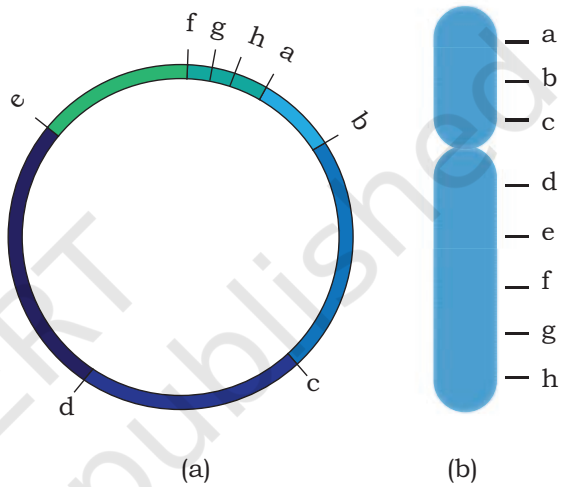


Fig. 5.2: Genome map (a) Map of a prokaryotic/ organelle genome. (b) Map of a eukaryotic genome, where a, b, c, d, e, f, g, h are genetic loci or physical sites like SSLP, STS arranged based on genetic distances

5.1.1 Genetic Mapping

Genetic mapping experiments give us an estimate of distances between two or more genetic loci responsible for a set of well-known phenotypes. A genetic map, as shown in Fig. 5.2, is created by carrying out crossover analysis. You have already studied about the phenomenon of crossing over in Class XI (refer section 6.2, Chapter 6). Rate of crossing over (also referred to as recombination frequency) between two or more genes or loci has been successfully used to measure relative genetic distance between them. One map unit is equal to one per cent of observed

cross-over (recombinants). The unit centimorgan (cM) is used to denote genetic distance between genes based on offspring phenotype frequency. Chances of segregation of two loci by recombination are directly proportional to the distances between them. Therefore, decreased recombination frequency is an indication that distance between said loci is less. When genetic loci are arranged based on the genetic distances between them, it creates a genetic map of the organism. The main drawback of this approach is the availability of limited number of genetic loci responsible for a set of well-known phenotypes. This makes genetic maps sparsely populated or coarsely grained and therefore, it limits on the utility of such maps for fine mapping of new phenotypes to the genome.

5.1.2 Physical Mapping

An alternative to genetic mapping is physical mapping of the genome in which specific locations on the genome are identified using DNA based map features. The presence of restriction enzyme sites (restriction mapping), Simple Sequence Length Polymorphism (SSLP), and Sequence Tagged Sites (STS) have been used as map features (Fig. 5.2). These features are helpful in creating a high resolution physical map of genome. Let us briefly understand these genome map features.

Restriction Fragment Length Polymorphism (RFLP): RFLP is a mapping technique that uses a DNA endonuclease enzyme called restriction enzymes (refer Section 3.3, Chapter 3) that cuts DNA at a specific base sequence resulting in the fragmentation of DNA. The fragmentation pattern of the digested DNA can be visualised when the fragments are separated based on their sizes by agarose gel electrophoresis. Typically, a single cut in a circular DNA yields one fragment of DNA, while the dual cut leads to two fragments. For linear DNA, a single cut results in two fragments of DNA while the dual cut results in three fragments. The DNA is digested with some of the enzymes such as a EcoRI, BamHI, etc., either singly or sequentially

to create a map of restriction sites based on the size of fragments they produce. This exercise results in a high-resolution physical map of restriction sites of the genome. Such a map can easily be compared with others noticing the gain or loss of certain restriction sites resulting in observed RFLP from DNA samples by the changes in length of DNA fragments (Fig. 5.3). For example, in a family with a history of certain disease, comparison of these maps among the diseased and disease-free members may help to identify specific RFLP locus associated with the disease. Association mapping can also help to identify the genetic divergence between populations. However, it is also important here that a mammalian genome may have several sites for different restriction enzymes and it is quite possible that a restriction enzyme like EcoRI may cut a mammalian genome in lakhs of fragments and these cannot be distinguished based on the electrophoresis. This problem is addressed by the technique of Southern hybridisation in which specific DNA probe between different RFLP sites are used. (Fig. 5.4).

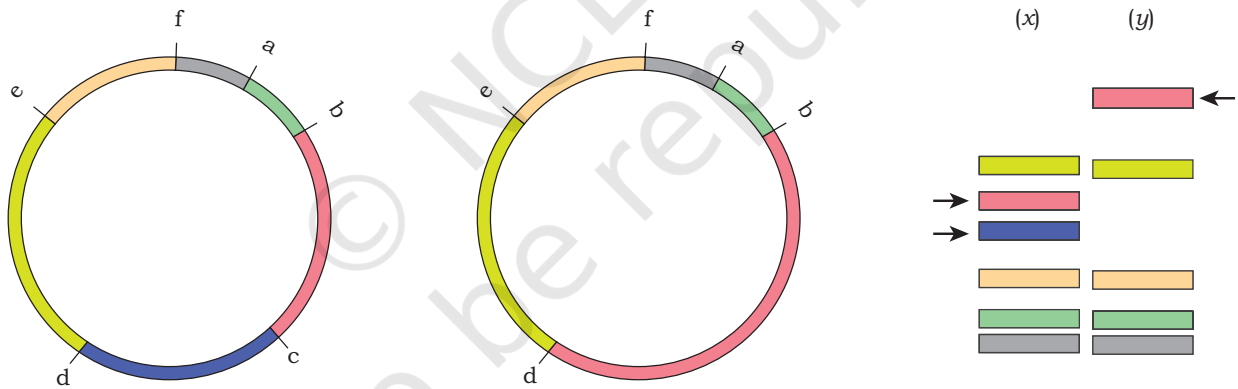


Fig. 5.3: Comparing physical genome maps. The two maps differ at site c. The restriction site c is absent or lost in genome (2) with respect to genome (1) or the restriction site c is present or gained in genome (1) with respect to genome (2). Physical map of a prokaryotic/organelle genome (1) and genome (2), where a, b, c, d, e, f are physical sites (such as restriction enzyme sites) arranged based on physical distances, (3) RFLP pattern of genome (1) and genome (2) obtained by restriction digestion of both the genomes and separating on agarose gel electrophoresis. Presence of restriction site c in genome (1) results in two smaller unique bands in (x) whereas absence of restriction site c in genome (2) gives one larger unique band in (y).

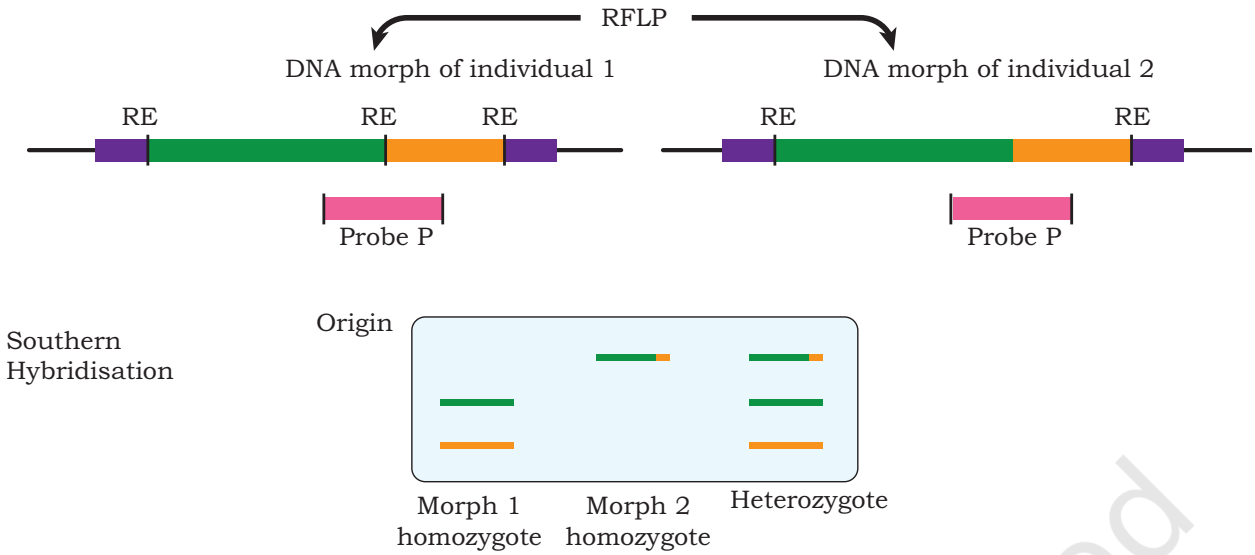


Fig. 5.4: Physical mapping using restriction fragment length polymorphism—A probe P detects two DNA morphs when the DNA is cut by a certain restriction enzyme (RE).

Simple Sequence Length Polymorphism (SSLP) map: A large genome contains arrays of different repeat sequences, which may be of varying length. Microsatellites and minisatellites are such repeat sequences that can also be used for physical mapping of genome based on its length variant (refer Section 4.1 in Chapter 4 of this book).

Physical Mapping using Sequence Tagged Sites (STS): Sequence tagged sites are a useful addition to physical mapping. STS are unique DNA sequence (200–500 bp) that occur once in a genome and whose unique location in the genome is known. The presence or absence of STS site in a given fragment of DNA can be easily demonstrated using PCR. Hence, the presence of these sites serve as a useful location landmark in the creation of physical map of a genome. They are often combined with the restriction maps to improve the resolution and effectiveness of association mapping.

5.2 High-Throughput DNA Sequencing

While we can compare the physical and genetic map to differentiate and classify organisms, it is highly desirable to get the complete DNA sequence of the organism. In earlier

days, sequencing complete genomes was prohibitively expensive and therefore, less expensive techniques were used to narrow down the region of interest to small locus of DNA, that is, statistically associated with the phenotype of interest. Only this small region of interest is used for sequencing to avoid the prohibitive cost of full genome sequencing. However, this approach requires a map of genome that could be referred to or compared with others. Advances in sequencing technology have now made it possible to obtain complete DNA sequence of any organism, if we can get its DNA. Initially, the cost of DNA sequencing per base was prohibitive to carry out full DNA sequencing of eukaryotic organisms including human beings. However, this has changed drastically at present. In this section, we will see as to how the DNA sequence technology has evolved over last three decades.

5.2.1 First generation DNA sequencing technology

Initial or first phase of genome sequencing was an elaborate and a multistep procedure. It involved separation of chromosome on agarose gel using Pulse Field Gel Electrophoresis (PFGE). Separated chromosome was then subjected to restriction digestion and the digested fragment were ligated to high-capacity cloning vectors, such as BAC, YAC, PAC, etc. (Refer Section 2.3, Chapter 2). The DNA present in these clones are often overlapping and the clones from such exercises can be organised in a set of minimum clones that carry overlapping set of DNA from the organism.

DNA segments from these clones are physically mapped and sub-cloned into sequencing plasmid for sequencing the cloned DNA using the first generation DNA sequencing techniques.

The first generation DNA sequencing technique used a method called sequencing by chain termination (refer Section 12.9.1 of Class XI *Biotechnology* textbook). In this method, a ladder of single stranded DNA is created, each terminating at a specific base (identified by a specific fluorescence tagged to the terminating base) location. These fragments are separated based on their size using capillary gel electrophoresis technique (in

which the electrophoresis of the charged molecule, i.e., DNA fragments, is done in a capillary filled with gel). A fluorescence detector is positioned near the bottom end of the capillary to read the fluorescence of the single stranded DNA passing in front of the detector. Output of this technology is a chromatogram of fluorescence, where each peak represents a base position and colour of the peak indicates which DNA base is present there. Each capillary in a single run, sequences about 800-1000 base–long DNA. A single DNA sequencer can have as many as 96 capillaries (with 96 different sequencing reactions loaded) operating in parallel in one machine. This type of setup can provide up to 96000 bases per run, per machine.

One of the drawbacks of this technology is that it is time consuming and labour intensive which leads to a prohibitively high cost of genome sequencing. To overcome this challenge, the next generation of DNA sequencing technology was developed.

5.2.2 Next (second) generation DNA sequencing technology

Next generation DNA sequencing technology uses a massively parallel (millions of parallel sequencing reactions in one machine) DNA sequencing approach. This technology does not require time consuming cloning and subcloning steps and therefore, reduces cost required for the genome sequencing project. Compared to the first generation sequencing methods, next generation methods provide advantages, such as simple workflow high data accuracy (due to high depth of coverage) and a broad range of application.

Although there are many approaches to this strategy, we will look at working of one of the most popular Next generation sequencing technologies platform, i.e., **Illumina Sequencing Technology** (Fig. 5.5). It uses a flow cell (glass slide) where DNA fragments are amplified through a special method called **Bridge PCR Amplification**. A flow cell is a solid support, where single stranded small oligonucleotides (8 –10 bases) are attached. Initially, DNA is fragmented into small pieces (~1– 2 kb) and small oligonucleotide adaptors are attached to these DNA fragments. These adaptors are

complementary to the oligonucleotides on the flow cell and hence, help in the attachment of DNA fragments to flow cell. These adaptors are used as primers and new DNA strands are synthesised. During this process, fragments

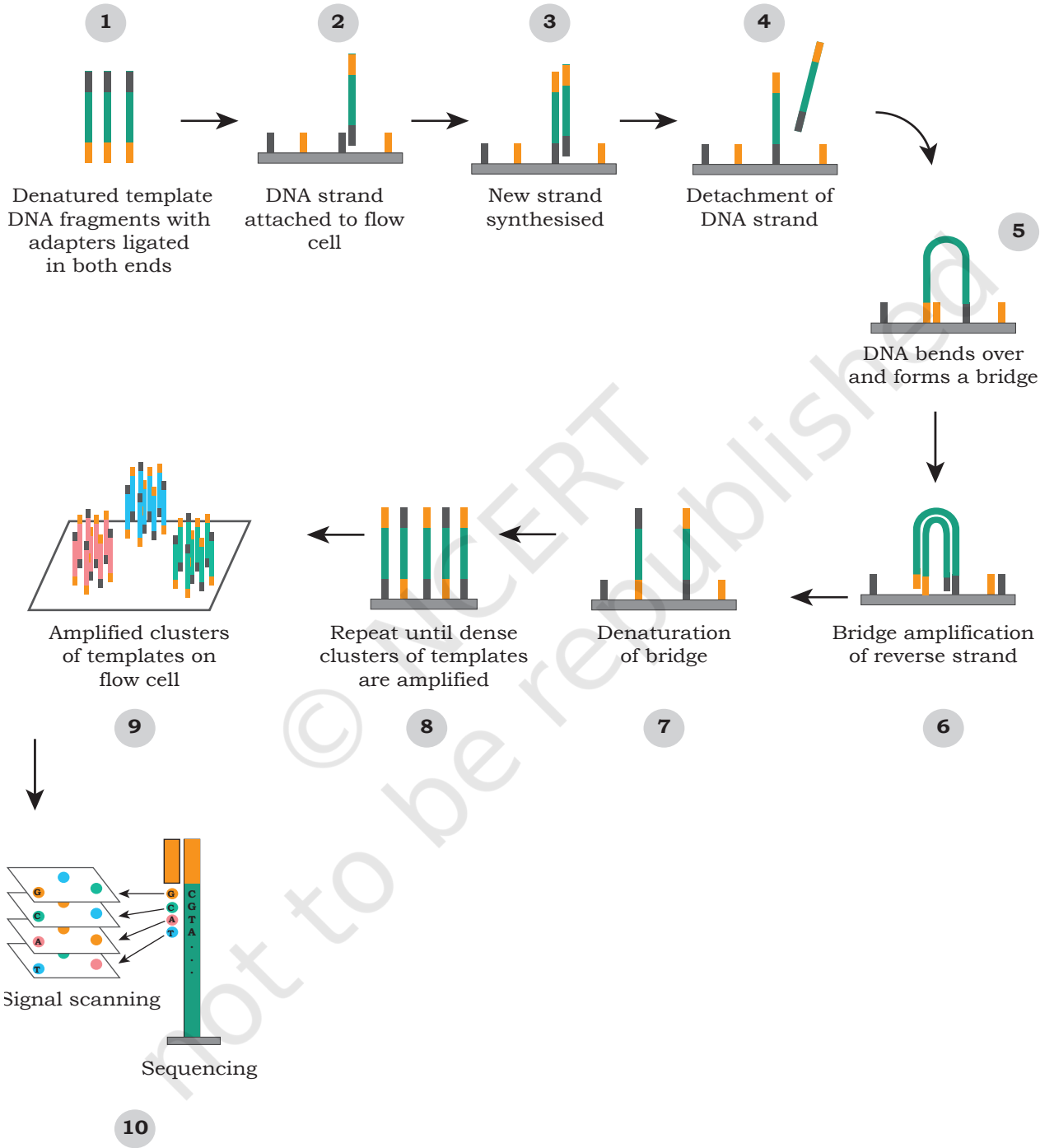


Fig. 5.5: A popular Next generation sequencing technology

make a bridge like structure because of complementarity of adaptors with the oligonucleotides attached to the flow cell. Therefore, it is called **bridge amplification**. It results in a cluster of similar fragments at the same location. After the PCR amplification, sequencing primer is used, which binds to one of the adaptors and each fragment is sequenced at the same time. For sequencing, fluorescently tagged dNTPs are used for the synthesis of complementary strand and each of the bases, i.e., dATPs, dTTPs, dGTPs or dCTPs, is attached with different fluorescent tags. Only one base is added at one time and incorporated bases are read based upon the type of its fluorescence. This is followed by the addition of next base and fluorescence is imaged. In this way, millions of fragments are sequenced parallelly at the same time.

The sequencing reaction is polymerase based and it is carried out in the flow cell by passing labelled base (these bases have unique fluorescence tag). These fluorescent bases would get incorporated depending on the DNA template initially attached to the flow cells. The fluorescence of the newly incorporated base reveal the sequence of the DNA fragment attached on the flow cells (Fig. 5.5). After recording the image of the flow cell, the fluorescent tag of the incorporated base is removed and then next base is passed through the flow cell (Fig. 5.5). The whole sequencing is recorded as an array of fluorescent images, where the DNA sequencing is recorded as fluorescence changes at a given coordinate or spot. Such an array of images reveals the sequence of millions of such spots in parallel.

Although this technology is capable of sequencing millions of DNA in parallel, it generates a very short (75 to 300 base long) length of sequences compared to the first generation technology. Also, additional sequencing strategies are available to overcome some of the limitations posed by short sequencing output.

5.2.3 Some recent advances in DNA sequencing technology

One of the recent advances in DNA sequencing technology is the use of nanopores for DNA sequencing. This is also

considered a third generation sequencing technology. Here, sequencing is carried out by measuring the pattern change in electric current across a nanopore when a specific base in single stranded DNA passes through a nanopore (Fig. 5.6). The current implementation of this technology makes use of two proteins—a DNA helicase and a porins like molecule that is assembled to form nanopore on a synthetic membrane. The DNA helicase captures and unwinds a double stranded DNA and pushes one of its strand through the nanopore. An ionic current is maintained across the nanopore, and it is monitored continuously. When the single stranded DNA bases pass through the pore, the porins like molecule interacts differentially with DNA bases and it disrupts the ionic current. The disruption caused by each base is

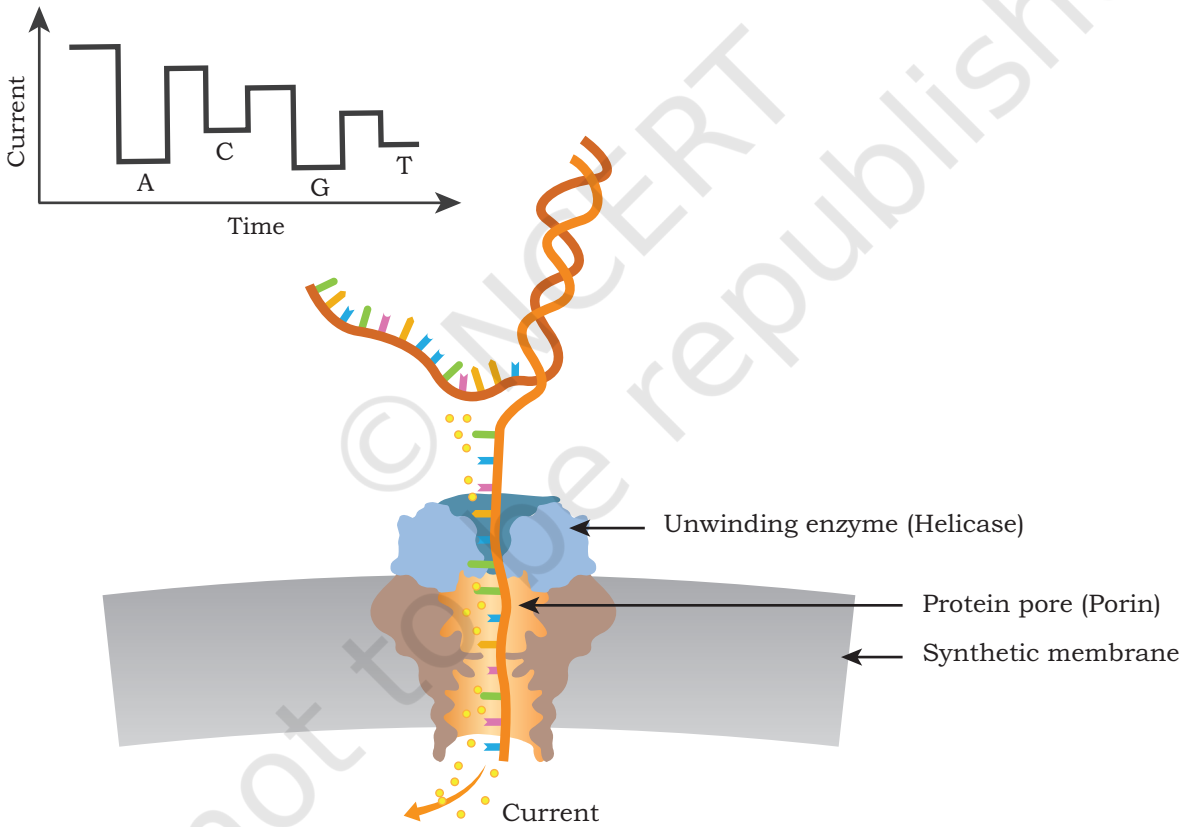


Fig. 5.6: Nanopore based sequencing technology. Yellow dots are ions flowing through a pore creating current across nanopore formed by protein pore. Passage of each base through the nanopore results in specific changes in the ionic current. These changes in the ionic current are measured and the corresponding bases are identified. The sequence read here is ACGT. The unwinding enzyme efficiently captures the DNA, docks on the protein pore and drives one strand of DNA through the nanopore.

very specific and by measuring these signatures of electric current changes, corresponding bases can be identified. The nanopore based sequencing technology offers a rapid and simple sample processing and has the potential to display sequencing results in real-time, making it highly useful for genotyping even in the field setup. Another major advantage of the nanopore sequencing is the production of very long sequencing reads (as long as 1 Mb or even more) at a relatively low cost.

5.3 Other Genome-related Technologies

Genome sequencing comes in multiple flavours or variations. This includes whole genome sequencing (WGS), targeted sequencing, metagenomics etc., as described in the following sections.

5.3.1 Whole Genome Sequencing (WGS)

WGS is a comprehensive method of determining the DNA sequence of the entire genome of an organism. Using WGS, the first genome of *Haemophilus influenzae* was sequenced. The availability of the whole genome sequences is helpful in understanding the genetic regulation of inheritance, identification of the genetic disorders that are inherited and so on. Whole genome sequencing of human genome started in 1990 and complete sequence was published in 2003. With advances in sequencing technologies (as discussed in the previous section), now it is possible to sequence the entire genome in a few days. Whole genome sequence of a large number of microbes, animals and plants has already been obtained. Availability of the WGS can help to create personalised plans to treat human diseases or reading the variations in the cancer cells can help the physician to select the best chemotherapy.

When a whole genome sequencing uses a reference genome sequence (already sequenced and phylogenetically related genome sequence) to assemble its individual sequencing reads into genome sequence it is called reference based genome sequencing. When no such reference is used, it is called *de novo* genome sequencing.

5.3.2 Targeted sequencing

Targeted sequencing project is done to know as to whether targeted regions of a genome have variation or not. Such projects make use of already sequenced genome as reference genome sequence in order to assemble the newly sequenced genome. Targeted sequencing focuses on the sequencing and analysis of a set of selected genes or genomic regions of the genome. It can be used to compare the smaller set of the genomic regions among different genomes to identify variations like mutations, insertions or deletions, etc. For this purpose, target genomic regions is selected, isolated and then sequenced. Usually, PCR is used for this purpose.

Clinical exome sequencing is also very similar to targeted sequencing. Clinical exome sequencing is done for genes that are known to be disease associated (as reported and curated from databases, like OMIM, HGMD, etc.). Genetic diagnostics tests based on clinical exome sequencing are cost effective than whole genome sequencing.

With the development of high-throughput DNA sequencing, DNA sequencing technology itself has been adapted to do much more than just reveal the genome sequence of an organism. One can use this technology to study the biology of an organism at genome level. Some of the advance applications of sequencing technologies are ChipSeq, RNASeq, etc.

ChipSeq sequencing projects are used to map genome-wide DNA binding site of a transcription regulator. Since transcription regulators play an important role in controlling the gene expression of one or more genes, ChipSeq projects are used to know ginome-wide DNA binding sites (as well as target genes) of a transcription regulator.

RNASeq projects are carried out to study the global gene expression profile (transcriptome) of an organism, a tissue or a sample. Total RNA is extracted from the target sample and cDNA is synthesised, which is sequenced using next generation sequencing technologies.

The sequencing read thus generated mapped to various genes on the genome and count of such reads mapping to those genes are in proportion to their expression reads.

5.3.3 Metagenomics: Sequencing of DNA or cDNA present in a microbial community

Metagenomics projects are used to study the genomics of community of organisms. Here, we do not separate the organisms or culture them individually. Metagenomics, in fact, is the study of the total genetic material directly obtained from the microorganisms living in a particular environment, like microbes living in the animal gut or in any particular ecological environment. It has several applications in medical microbiology, agriculture as well as environmental microbiology. In particular, it helps to study the diversity of microbial communities and how the diversity of these microbes changes with changes in the environment. Metagenomics may also help in identifying novel genes or enzymes with the significant industrial applications. For example, the study of microbial communities living in extreme environments, like high temperature sulphur springs can help to identify the novel enzymes that are resistant and functional at such high temperatures.

Thus, metagenomics has applications in understanding the genomes of gut microbes, throat microbes, microbes present on a toilet seat, and many more. It provides an insight about the genome of diverse forms of microbes and their correlations. For example, study of genomes of diverse forms of viruses may provide an understanding about virus-host interaction, epidemiology and also evolution of viruses. Presence of multiple genomes in metagenomics sample creates special challenges in data analysis, hence they require a different set of computing algorithms.

5.4 Genome Engineering

This technology is used to modify the genome. It is often carried out to introduce or remove one or more genes. The goal of this engineering is to introduce a new functionality or to modify (or remove) the existing functionality of a biological organism. In this section, we would get a glimpse of the approaches that had been used to engineer a genome. One of the approaches uses transposon or jumping genes to inactivate or delete a gene(s) in a genome. Another approach is to edit the genome without having any additional unwarranted changes in the genome.

5.4.1 Knock-out and knock-in of a gene by transposon insertion

Transposons are DNA sequences also known as 'Jumping Genes' that move from one location to another on the genome found in almost all organisms (both prokaryotes and eukaryotes). DNA transposon generally moves by a 'cut and paste' mechanism in which the transposon is excised from one location and integrated elsewhere in the genome. Many a times, such movement of DNA segment results in the insertional inactivation of a gene located at the site of insertion. This behaviour of transposon is exploited to *knock-out* an existing gene. In such a strategy, the transposon is engineered, so that it recognises a specific sequence of a target gene, where it gets inserted. Once the transposon is inserted at the target site, the coding frame of target gene is disrupted and therefore, it can no longer produce the original transcript required to code the gene product (Fig. 5.7).

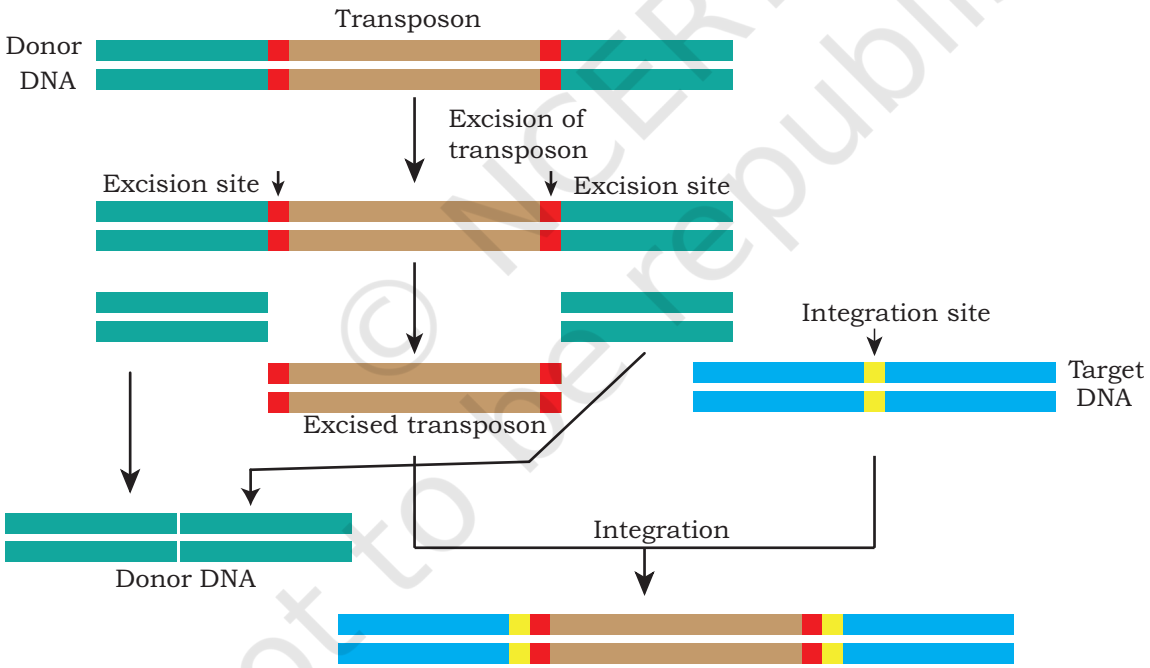


Fig. 5.7: Genome engineering through transposition

In certain cases, when a transposon gets inserted at a particular site, it alters the genetic locus of interest, resulting in the addition of DNA sequence, that was not present on the said genetic locus. This property of

transposon is used to *knock-in* of a functional gene. This *knock-in* and *knock-out* technology helps in the generation of animal/plant models to understand the molecular basis and development of new drugs for the disease.

5.4.2 Genome editing using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas9)

Genome editing is a set of techniques or technologies that can be used to change the DNA of an organism by adding, removing or altering the specific DNA sequence of the genome. Like restriction enzymes, this technique is a biotechnological adaptation or repurposing of a naturally occurring genome editing system present in bacteria. In its natural context, this system is used to memorise the DNA signature of past viral attacks so that any subsequent viral infection with matching DNA signature present on the viral DNA can be countered or destroyed effectively.

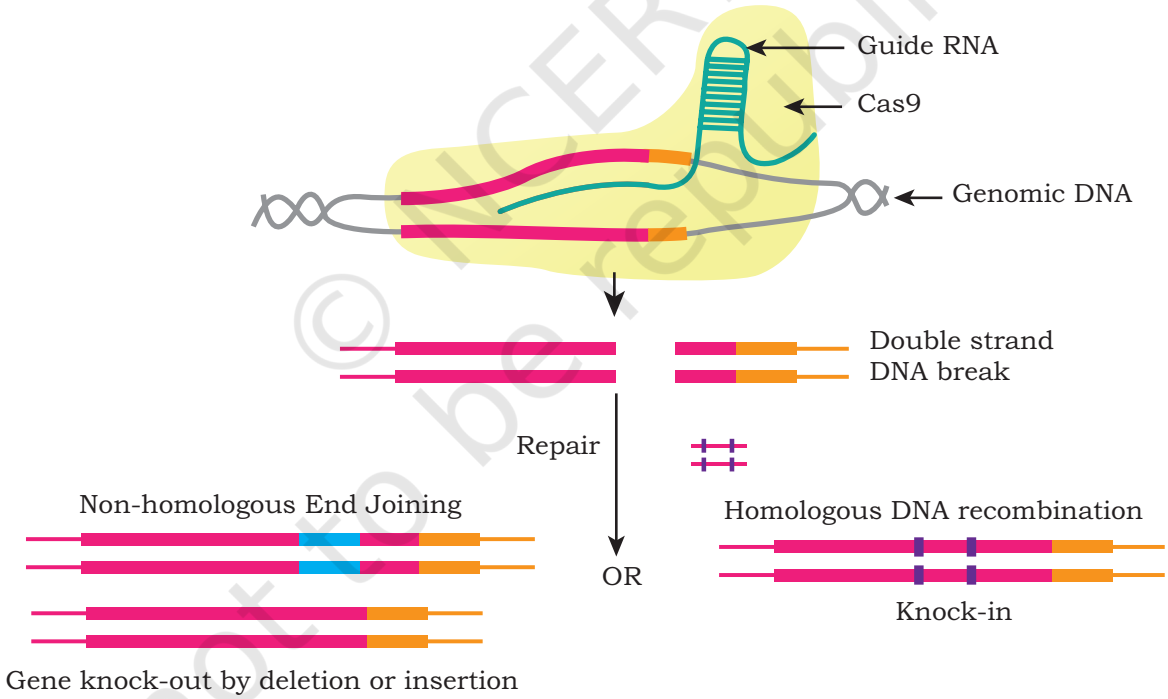


Fig. 5.8: Gene editing using CRISPR-Cas9 technology. Guide RNA position the specific region of genomic DNA with Cas9 protein complex for inducing double strand break. After double strand break, gene knock-out can be created using deletion or insertion of DNA sequence. For gene editing, a desired mutant copy (for correction/replacement around the break site) can be introduced to this region to undergo homologous recombination in the presence of desired mutant template DNA, thereby introducing these mutations in genomic DNA at a specific place.

In this approach, a guide RNA is designed and produced in the cell along with Cas9 endonuclease. The guide RNA are required for positioning of Cas9 endonuclease at a location, where DNA are required to be edited or engineered. Part of the guide RNA is complementary to the DNA sequence, where the sequence needs to be edited. The other part of guide RNA attracts and binds to Cas9 endonuclease. Once this whole complex (the guide RNA bound to target DNA and Cas9) is assembled, a double strand break is introduced in the DNA by the endonuclease (Fig. 5.8). The broken DNA undergoes a template based homologous DNA repair where a modified or edited DNA template is provided that has the desired changes (edited sequence). As a consequence of such double strand break and template guided DNA repair, the repaired genomic DNA now possess an edited DNA sequence.

Box 1 Nobel Prize in Chemistry 2020 for discovering the CRISPR-Cas9 genetic scissors

Emmanuelle Charpentier

Jennifer Doudna

Emmanuelle Charpentier, a researcher at the Max Planck Unit for the Science of Pathogens, in Berlin, Germany; and Jennifer A. Doudna, a biochemist at the University of California, Berkeley were awarded the Nobel Prize in chemistry 2020 for discovering the CRISPR-Cas9 genetic scissors, a gene-editing method.

The concept of CRISPR-Cas9 was modified from naturally occurring ancient bacterial immune systems. Bacteria use CRISPR-Cas systems to recognise and cut the DNA of invading viruses into inactive segments called CRISPR arrays. This effectively disables the virus and contributes to immune 'memory' of the bacteria. While studying *Streptococcus pyogenes* in 2011, Dr. Charpentier discovered a new molecule in the CRISPR-Cas system, called tracrRNA, and started a collaboration with Dr. Doudna to reprogram the CRISPR system. Together, they discovered that CRISPR could be directed to cut DNA at the target sequences using Cas9 protein. This allowed the genetic material to be subsequently added, deleted or changed as desired, using the cell's own DNA repair machinery. Researchers can use this technique to modify the DNA of living organism with extremely high precision. This specialised gene editing tool allows very small and precise changes to be made to the genetic code, it can halt a disease that causes premature ageing in a mouse model and double the lifespan of the treated animals.

5.5 Structural, Functional and Comparative Genomics

Next generation sequencing projects generate large volume of data that require extensive computing for data analysis. Computational genomics is a field that uses high performance computing clusters and workstations to analyse genomics data including the data generated by the sequencing technology. Computational genomics helps in understanding the functions of the genome based on computational and statistical analysis to decipher biology from genome sequences and related data based on DNA and RNA sequences, as well as other experimental data. With the increasing number of genome sequence of different organisms, one major step is the identification of common genes through homology search and gene annotation (assigning functional value). Secondly, sequence comparisons across multiple sequences can be carried out by advanced computational genomics tools, such as BLAST, ClustalW, Phylip to understand the evolutionary relationship. Rapid progress in the field has led to advancements in structural genomics, which deals with the three dimensional information of the proteins encoded by the genome.

Advancements in the area of powerful computer graphics processing units (GPU) and graphic cards have been utilised [General purpose (GP-GPU) and Computer Unified Device Architecture (CUDA) programming] for scientific computing including computational genomics to bring high performance computing for accelerated data analysis.

Genomics work is capital intensive. Since genomics has become a major tool to study biology and has a profound effect on biotechnology, the Department of Biotechnology (under Ministry of Science and Technology, Government of India) has taken an initiative to establish three National Genomics Core facilities in India. These facilities would help Indian scientists and entrepreneurs to execute their genomic projects. These facilities are located within two national laboratories (Centre for DNA Fingerprinting and Diagnostics, Hyderabad and National Institute

of Biomedical Genomics, Kalyani) and one university (University of Allahabad, Prayagraj).

5.5.1 Structural genomics

Structural genomics means two different things to two different sets of people. For those who are interested in protein structure, it refers to 3D structure of every protein encoded by a particular genome. For those who are interested in chromosome organisation, it refers to physical nature of the whole genome.

Earlier, the focus of structural genomics (as defined by structural genomics consortium) was to search for new protein folds or structure in newly sequenced genomic DNA. Through this approach, scientists address the need for solving more protein structures, especially of proteins with sequence that are of unknown fold types.

An expanded view of structural genomics includes the study of structural organisation of DNA region in chromosome and nucleosome status of the genome. This includes the study of large structural changes in genome organisation of related species. An interesting example is to see as to how genes that are common between mice and human are redistributed across different chromosomes of two species.

5.5.2 Functional genomics

Functional genomics aims to understand as to how the physiological function information encoded in genome is executed. A set of genomics based tools and techniques, such as RNAseq, ChipSeq, Metagenomics (of microbial community), etc., are used to understand the physiological and pathological function associated with the state of a cell.

5.5.3 Comparative genomics

Comparative genomics deals with the comparison of genes and genomes of multiple species or within multiple individuals of the same species. It can help in genome annotation of the newly sequenced genome based on its comparison with related well-known genome. It can also help identify the set of common genes that form core

genome and other genes that are unique to a species. The latter may be responsible for unique behaviour or function of a particular species. Comparative genomics may also identify whether a functional molecule is present or absent in a genome. It may also be used as a starting point for genome based taxonomy and phylogenetic lineage study.

5.6 Protein Engineering

In this unit, we would learn about another advanced usage of rDNA technology that can be used to create and produce engineered proteins having additional or extended property as compared to the original protein.

The engineered proteins may have greater stability when exposed to elevated temperature, change in pH, presence of salts or organic solvent. Proteins can also be engineered to develop and produce novel reagents for research, diagnostics and therapeutics.

5.6.1 Applications of protein engineering

To engineer a protein, the critical amino acids responsible for the desired trait is identified. Its codon sequences on the gene are modified by creating point mutations so that it can be mutated or replaced by another amino acid that modulate the desired activity. Let us discuss some examples of engineered proteins.

1) Recombinant protein to improve cleansing action of detergent: Subtilisin is a proteolytic enzyme that is added to detergents to hydrolyse the protein component of dirt. This improves the cleansing action of the detergent. The catalytic triad of subtilisin is Ser 221, His 64 and Asp 32. However, in the presence of bleach, Met 222 is oxidised, this leads to reduced action of subtilisin. If the codon for Met 222 is replaced by codon for Ala by point mutation by site directed mutagenesis (a process

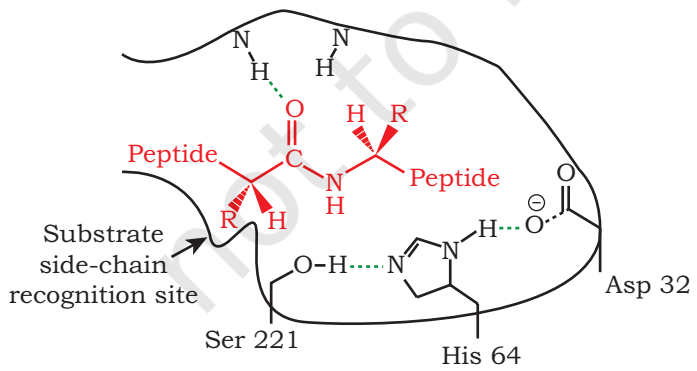


Fig 5.9 : Catalytic Triad in Subtilisin

by which point mutation can be introduced at a specific site), the engineered protein becomes stable even in the presence of bleach (Fig. 5.9).

2) Recombinant protein for protein purification: A peptide with six consecutive His amino acids called 6-His-Tag, can bind to metal ions, like Nickel, Zinc and Cobalt with great affinity at mildly alkaline pH. This property of 6-His-tag is exploited for the purification of recombinant protein. DNA encoding for 6-His-tag is fused with the gene sequence of recombinant protein of interest. During protein purification by affinity chromatography, the 6-His-tagged recombinant protein will be immobilised by the presence of metal ions in the agarose gel. All other proteins will be removed by washing the gel. 6-His-tagged recombinant protein can then be eluted by using an elution buffer (Fig. 5.10).

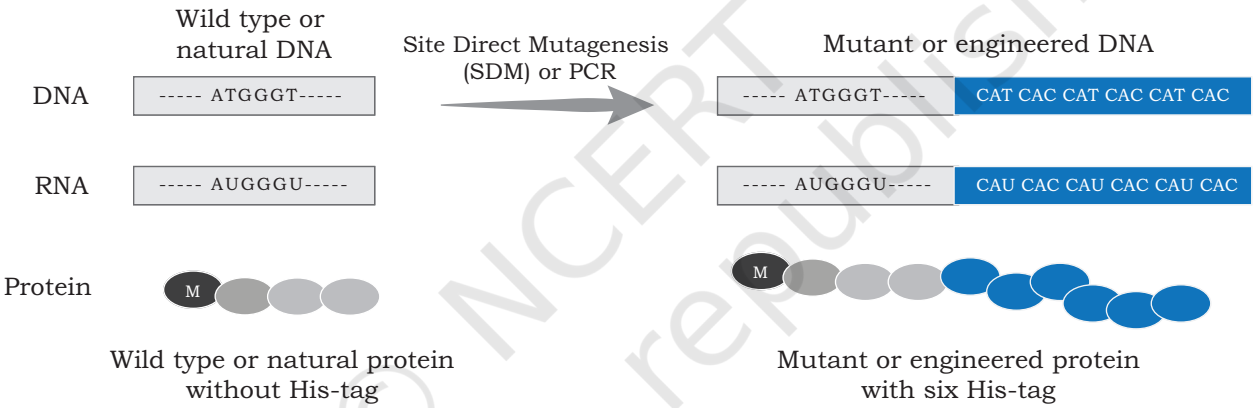


Fig. 5.10: Protein engineering for protein purification. The engineered protein with 6-His-tag are easily and efficiently purified on Nickel ion columns

3) Recombinant protein for tracking the location of the protein in a cell: The gene encoding for green fluorescent protein (GFP) obtained from jelly fish, *Aequorea victoria*, is fused with the DNA sequence of interest. This chimeric DNA now encodes for an engineered protein that appears as a green fluorescent molecule when exposed to UV light, thus revealing its location inside the cell (Fig. 5.11).

4) Recombinant protein as immunotoxin: Recombinant immunotoxins are rationally engineered protein agents (often anti-cancer agents) that combine the selectivity of antibody with cell-killing potency of toxin.

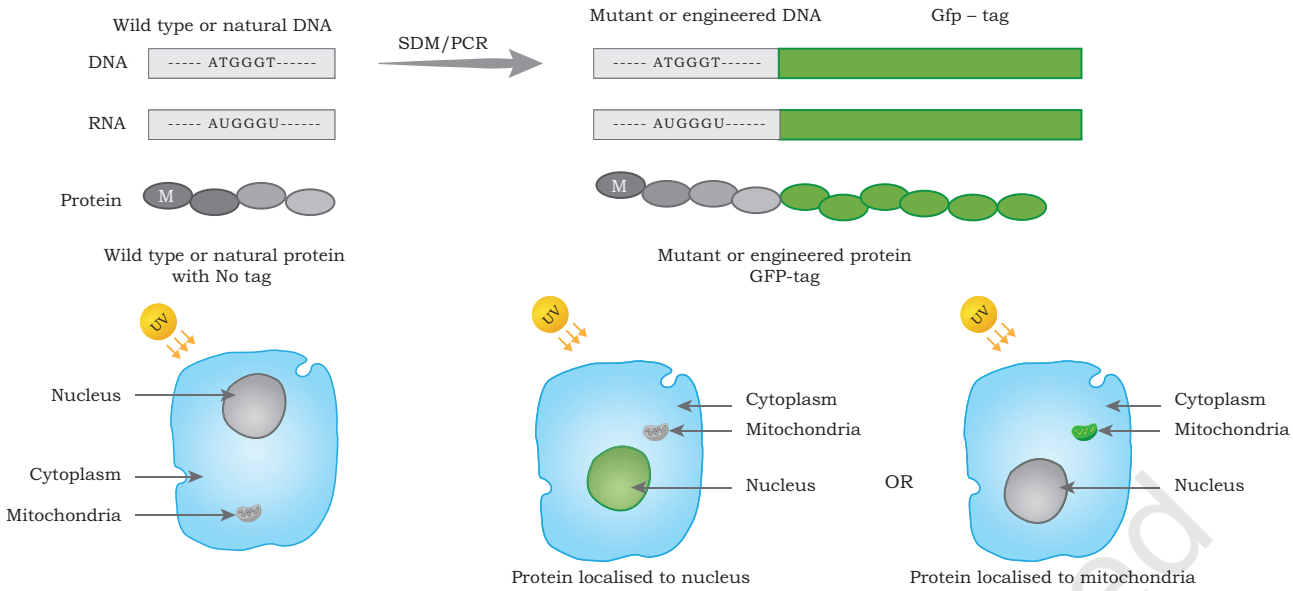


Fig. 5.11: Protein engineering for protein localisation. The engineered protein with Green Fluorescent protein (GFP) tags are easily visualised and localised in the specific regions of cell using epifluorescence or confocal microscopy. The nucleus localised GFP tagged protein appears green in the presence of UV light.

Box 2 Recombinant Immunotoxin

Recombinant immunotoxins are engineered proteins in which the selectivity of antibody is combined with the cell killing potency of toxins. Antibodies are in fact multi-chain proteins having two heavy and two light chains. The function of each fragment of antibody is well defined i.e., the antigen binding activity (present in Fab fragment) and the effector function (in the Fc fragment). The Fab fragment is further divided into the Fv fragment, the smallest fragment which is responsible for antigen binding *via* contact with both the heavy and light chains. For the production of recombinant immunotoxins, recombinant single-chain antibodies, also known as single-chain variable fragments (scFv) are produced through rDNA technology. These antibodies consist of the variable regions of the heavy (VH) and light (VL) chains of immunoglobulins, linked by a short flexible peptide linker. Such scFv can be conveniently produced in bacteria as it can be encoded by a single gene with genetic manipulation and is expressed as a single-function polypeptide chain. During production of recombinant immunotoxins, scFv that specifically binds to the target antigen on the surface of cancer cells are designed. Then toxin fragment that can effectively kill the target cells are selected (such as *Pseudomonas* exotoxin A (PE), *Diphtheria* toxin (DT), or ricin). The designed scFv with the toxin is finally fused with a linker, which allows for proper folding and activity of both the components (Fig. 5.12). This engineered protein uses the antibody part for selectivity targeting the cancer cells displaying their antigens and gets internalized. The toxin part gets released from the endosome and effectively kills the cells.

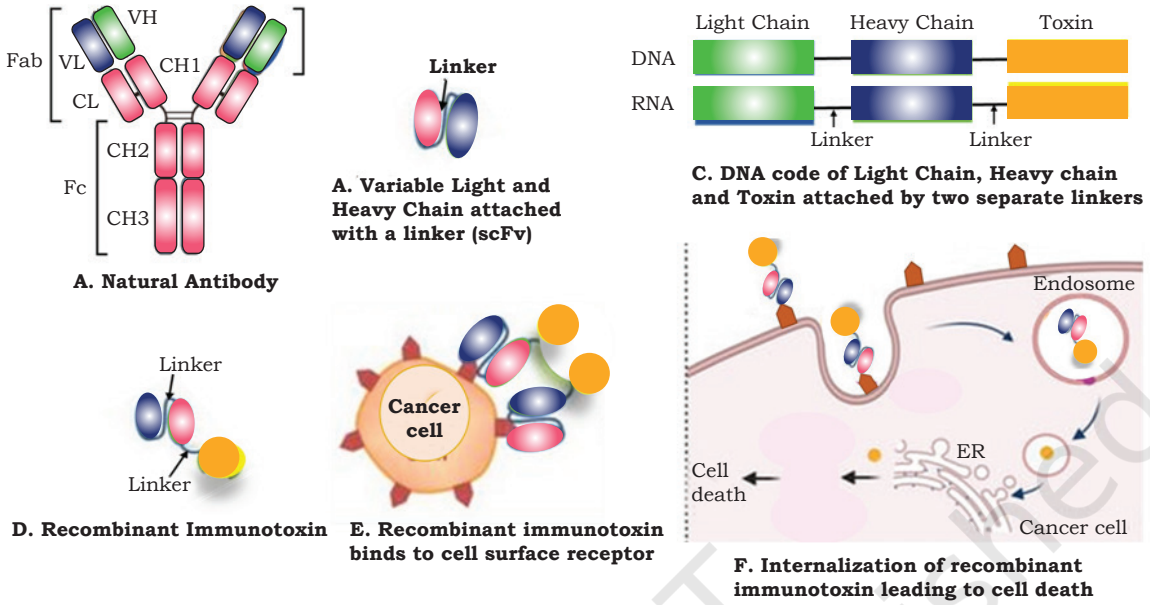
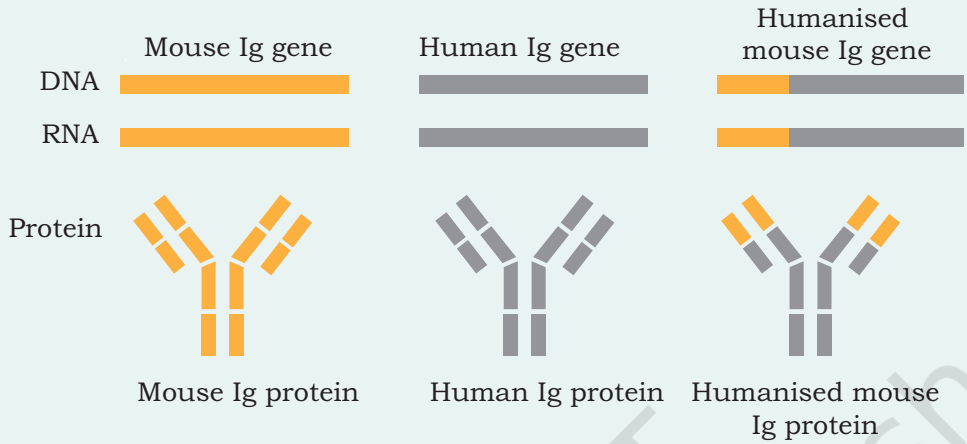


Fig. 5.12: Production of recombinant immunotoxin (A) Natural Antibody (B) Single chain variable fragment joined with a linker (C) Variable part of light chain coding DNA (blue) is fused with the equivalent part of heavy chain coding DNA (green) and toxin coding DNA (yellow) through a flexible linker DNA code (black) giving rise to an engineered single chain recombinant immunotoxin; [D] Recombinant immunotoxin; [E] The antigens expressed on the cancer cell are the antigen binding sites on which the recombinant immunotoxin binds. [F] After binding it gets internalized by endocytosis and the toxin is released in the cytosol from the endosome that kills the cancer cells.

Box 3 Humanised monoclonal antibody

Monoclonal antibodies are very useful reagents that bind their targets in a well-defined way. These antibodies are easily produced using Mouse hybridoma technology (refer Section 4.5, Chapter 4), a biotechnological tool that exploits normal mouse immune response to develop a specific antibody producing immortalised mouse plasma cells by fusing it to mouse myeloma cells. Such immortalised hybrid cells become cell factories for producing specific antibodies. While these mouse monoclonal antibodies are useful as reagents but the same cannot be used as immune-therapeutics in humans. This restriction is due to the ability of human immune system to recognise the mouse antibody as a foreign antigen leading to anti-mouse monoclonal antibody immune response. Raising monoclonal antibodies is not practical in humans. As a result, a biotechnological strategy of protein engineering is applied to humanise the mouse monoclonal antibody. In this strategy, the part of mouse antibody involved in antigen binding is identified and transferred to a human antibody,

which again is done at the DNA level. The DNA coding sequence that codes part of mouse antibody involved in binding the antigen is transferred to the equivalent position in a human antibody coding genes using recombinant DNA techniques, thereby producing a humanised version of mouse monoclonal antibody (Figure). The humanised antibody is often considered self and therefore, has good immune acceptance in human beings when administered for therapeutic purposes.



Humanised mouse Ig using protein engineering

SUMMARY

- The genome of an organism is the complete DNA content, which includes DNA present in the nucleoid in prokaryotes and the DNA present in the chromosomes of the nucleus, mitochondria and plastids in eukaryotes.
- There are two different approaches of genome mapping, physical mapping and genetic mapping.
- A genetic map estimates genetic distances between genetic loci, which are responsible for a set of well-known phenotypes. Genetic distances are measured in centimorgan or map unit (a unit to measure genetic linkage of two phenotypes).
- Physical mapping is based on map features, such as restriction enzyme sites (restriction mapping) and sequence tagged sites (STS).
- In restriction mapping, restriction enzymes cut DNA at a specific base sequence, resulting in fragments of DNA that can be visualised by agarose gel electrophoresis in which DNA fragments are separated based on their sizes.
- STS are unique DNA sequences (200–500–bp) with well–known location, these serve as a useful landmark in the creation of a physical map of a genome. STS can be demonstrated by PCR.

- First generation DNA sequencing technology was a multistep procedure involving separation of chromosomes, restriction digestion and ligation to high capacity cloning vectors.
- First generation DNA sequencing technique used a method of sequencing by chain termination, where a ladder of single stranded DNA with specific fluorescence tagged terminating bases are separated and detected by a fluorescence detector. By this method, base position and type of DNA bases are identified.
- Next generation DNA sequencing technology depends on advance computing algorithms to take millions of short sequence outputs, assembling in large contigs, representing DNA sequences of large genomes.
- Next generation sequencing includes techniques, like whole genome sequencing (WGS), Targeted resequencing, clinical exome sequencing, etc., and Chipseq, RNAseq are some of the advance applications of sequencing technologies.
- Nanopore sequencing technology uses two proteins: DNA helicase and porins like molecules to bind, unwind double stranded DNA and push single stranded DNA through porins like molecules to identify base pattern. This technique is simple, rapid, cost efficient, and displays results in real-time, and useful for genotyping and high mobility testing.
- Metagenomics involves sequencing of DNA or cDNA present in a microbial community.
- Computational genomics involves usage of high performance computing clusters and workstations to analyse genomics data.
- Genome engineering is a technology to modify a genome (such as inactivate, delete, integrate, transduce and edit the genome).
- Transposons are DNA elements with the ability to move from one position of DNA to another, and can be exploited to engineer a knockout of the existing genes.
- Genome editing using CRISPR-Cas9 (use guide RNA along with Cas9 endonuclease) involves double strand break, homologous DNA repair to generate the edited DNA sequence.
- Structural genomics includes the study of structural organisation of DNA region in chromosome and nucleosome status of genome.
- Comparative genomics identify a set of common genes that form core genome and other genes that are unique to a species. So, it is a starting point for genome based taxonomy and phylogenetic lineage study.
- Functional genomics aims to study the physiological and pathological function associated with the state of a cell.

- Protein engineering is applied to develop novel reagents, diagnostics and therapeutics. It is used to develop recombinant protein with 6 histidine-tag (6-His-tag) for the ease of purification and to develop fluorescent proteins to track the cellular localisation of proteins.
- Protein engineering technology is also involved in the production of humanised monoclonal antibody, single chain antibody and a recombinant immunotoxin which is an engineered antibody that delivers toxin to specific cellular targets.

EXERCISES

1. What is a genome? How is the genome of prokaryotes different from eukaryotes?
2. What are the types of genome mapping? Explain each with a comparative approach.
3. Suppose a DNA is extracted, purified and digested with enzyme BamH1. What type of mapping can be achieved? Mention the application of this type of mapping.
4. What is STS and its role in genome mapping?
5. Give a brief insight about the development of DNA sequencing technology and genomic workflow.
6. Discuss how next generation DNA sequencing technology has overcome the drawbacks of the first-generation DNA sequencing technology. Elaborate the methodology.
7. What is a unit of physical mapping? Discuss in detail.
8. Discuss the methodology and applications of third generation sequencing technology.
9. What are the different variations of Next Generation Sequencing? Explain in brief.
10. What are the applications of advanced sequencing technologies? Elaborate.
11. Discuss the applications of metagenomics.
12. What are the goals of genome engineering? Explain each in detail.
13. One of the applications of genome engineering is to achieve high level recombinant protein production. How can we achieve this? Explain in detail.

14. What is a genome editing? Why is there a need to edit a genome? Explain the methodology of editing using CRISPR-Cas9.
15. What are the structural, functional and comparative genomics?
16. What are the applications of protein engineering? Discuss in brief.
17. If you are given a recombinant protein with 6-His-tags, how are you going to use it? Explain the methodology and application in detail.
18. How do you apply protein engineering to track cellular localisation of proteins?
19. How to generate a recombinant immunotoxin? Why do you think there is a need for generation of an immunotoxin? Mention its mechanism of action.
20. **Assertion:** RFLP is variation in length of DNA fragments when cleaved by same restriction endonuclease.

    Reason: Genome of every individual has different position of restriction sites.

    (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.

    (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.

    (c) Assertion is true but reason is false.

    (d) Both assertion and reason are false.
21. **Assertion:** Recombinant immunotoxins are rationally engineered protein agents.

    Reason: It is prepared by fusing DNA-coding region of antibody with the DNA code of toxin.

    (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.

    (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.

    (c) Assertion is true but reason is false.

    (d) Both assertion and reason are false.

UNIT III

Microbial, Plant, Animal Cell, Organ Cultures and Bioprocessing

Gottlieb Haberlandt
(28 Nov 1854 – 30 Jan 1945)

Gottlieb Haberlandt was an Austrian Botanist. He was the son of European 'soybean' pioneer Professor Friedrich J. Haberlandt. Haberlandt first pointed out the possibilities of the culture of isolated tissues and plant tissue culture. He suggested the potentialities of individual cells via tissue culture and also suggested that the reciprocal influences of tissues on one another could be determined by this method. Haberlandt's original assertion methods for tissue and cell culture have been realised, leading to significant discoveries in Biology and Medicine. His original idea presented in 1902 was called totipotentiality: 'Theoretically all plant cells are able to give rise to a complete plant.' The term Kranz (German for wreath) anatomy was given by Gottlieb Haberlandt in 1904 to describe the specialised leaf anatomy found in more efficient C4 photosynthesis in land plants.

Chapter 6 Microbial Culture

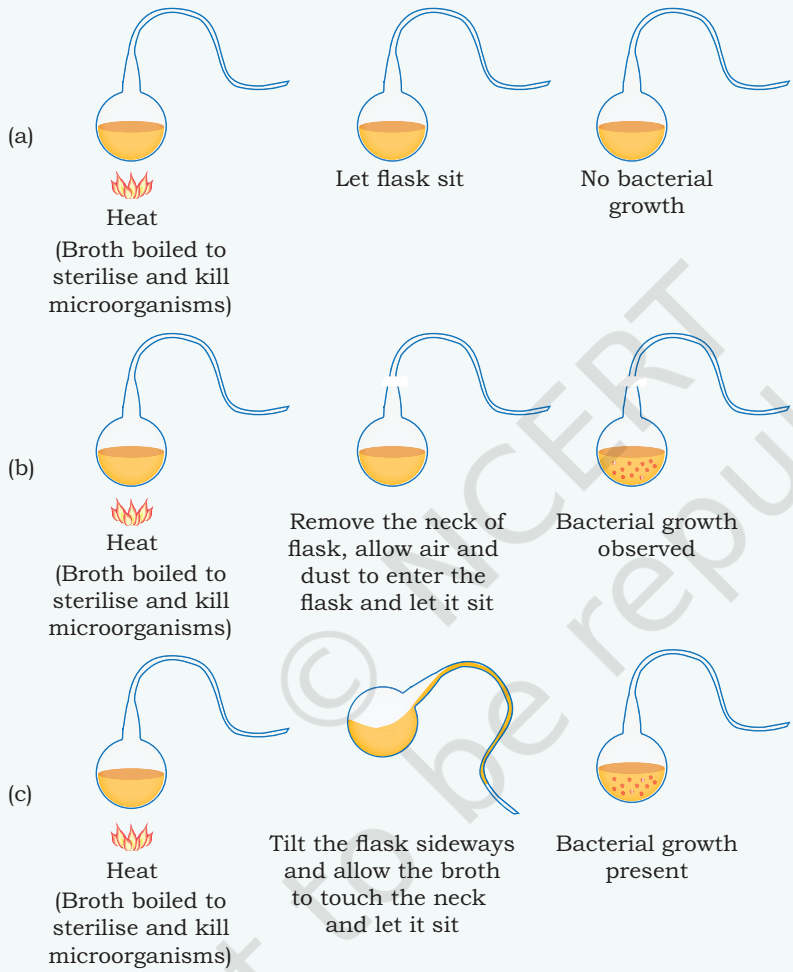
The world of microorganisms has unusual diversity pertaining to their structure, function, habitat and applications. They are ubiquitous in nature, i.e., they are present everywhere. Microbiology is the study or science (logos) of the small (micro) organisms (bios). Microbiology has gained a dominant position in fundamental research, agriculture, pharmaceutical industry, medicine, environmental science, food technology and genetic engineering, etc. In this chapter, you will learn about the nutrition of microorganisms, culture media, sterilisation techniques, and growth curve.

6.1 Historical Perspective

Discovery of microscope during the mid 1600s laid the foundation of microbiology. In the 1670s to 1680s, a Dutch merchant, Antonie van Leeuwenhoek developed the microscope and observed the microscopic organisms and coined the term 'animalcules'. After the era of Leeuwenhoek, the developments in microbiology were slow, due to rare availability of microscopes and lack of interest towards microorganisms. Scientists at that time

believed that the microorganisms originate from lifeless matter. But, on the contarary, Lazzaro Spallanzani observed that there were no microscopic forms of life in boiled broth. In the middle and late 1800s, Louis Pasteur had performed experiments to prove the importance of microorganisms in everyday life and encouraged scientists to think about the role of bacteria in human illness.

Box 1 Pasteur's Experiment

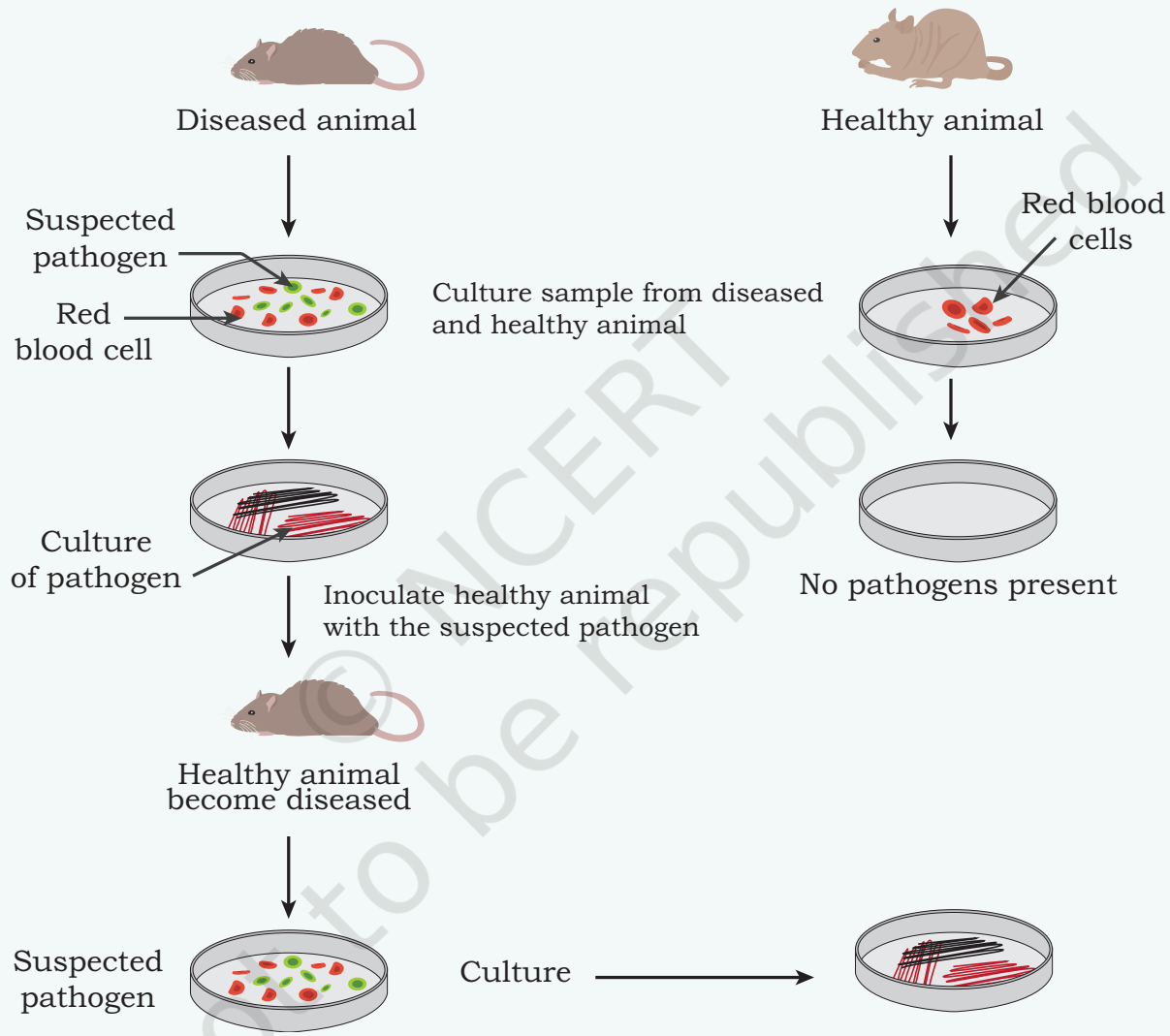


Louis Pasteur's experiment that illustrates the idea of germ theory of disease. (The unique design of neck allowed air to enter the flask but prevented the entry of fungal spores and bacteria)

Pasteur exposed the boiled broths to air in a filter containing flasks to prevent all the particles from penetrating to the growth medium. He also put broth in the flasks with a long twisted 'S' shaped neck that would not allow dust particles to pass. He observed that nothing grows in the broth [Fig. (a)]. He then broke the long neck of the flask and bacterial growth was observed [Fig. (b)]. He also tilted the flask sideways and allowed the broth to touch the neck, thereby exposing the broth to the outside environment. After letting it sit, he found bacterial growth [Fig. (c)]. These [Fig. (b) and (c)] prove that the living organisms grow in such broths due to the agents coming from outside, rather than spontaneously generated within the broth. Thus, his pioneer work further proved that the microorganisms were present in the air, and could cause diseases. On the basis of on his experiments, Pasteur disproved the theory of spontaneous generation and postulated the germ theory of disease, which states that 'the microorganisms were the cause for infectious diseases.

The German scientist, Robert Koch further proved the germ theory by injecting pure cultures of *Bacilli* into mice and showed that the *Bacilli* caused anthrax. Accordingly, Koch postulated (Fig. 6.1) and provided a set of principles that implicated the microorganisms to be the causative agents for diseases.

Box 2 Koch's Experiment



Pictorial representation of Koch's experiments

In the course of development of microbiology, based on the suggestions made by Pasteur and Koch, the period of late 1800s and the early decade of the 1900s is referred to as the Golden Age of Microbiology. During this period, various causative agents responsible for microbial diseases were discovered.

In the 19th century, the bacteriologists have attempted the use of food or material to grow the microbes. In 1860, Louis Pasteur used a media containing yeast, ash, and ammonium salts. This medium contained the basic nutrients for microbial growth, such as carbon source (e.g., sugar), nitrogen (e.g., ammonium salts), and vitamins (e.g. ash). In order to develop more versatile basal medium, Ferdinand Cohn further refined the Pasteur's medium by varying the types of sugars. Robert Koch observed that broths based on fresh beef serum or meat extracts could be used for optimal growth.

The use of solid media instead of liquid medium also proved an important milestone in the development of bacteriology. In 1881, Robert Koch had recognised the difficulties of using broth media for the isolation of pure cultures and considered the use of solid media alternatives. During the isolation of *Bacillus anthracis*, Koch evaluated media with coagulated egg albumin, starch paste and an aseptically cut slice of a potato, but it proved to be relatively poor at recovering the pathogenic bacteria. After that, Koch further developed medium with meat extract and gelatin (derived from the collagen of skin and bones). During the course of development, finally agar replaced gelatin, as agar provides the requisite solid support for microbiological media, but it does not provide the nutrients necessary for the bacteria to grow. On the basis of the observation of the growth of bacteria on solid media, Koch coined the term 'colony' to describe the pure and discrete growth.

Box 3 Petri dish

Julius Richard Petri in 1887 improved microbial culturing conditions by using a shallow, circular glass dish with a losse fitting cover termed Petri dish instead of the flat glass plates.

Most of the media used during the 19th century was non-selective and was designed to grow a range of bacteria. In the beginning of the 20th century, further advances with in the culture media occurred which led to isolation of pure cuiture on media called selective media, such media contained compounds which felicitate the growth of only a specific type of micro organisms.

By the 1930s, the researchers started to consider the importance of growth factors upon bacterial nutrition. In the 1940s, with the invention of electron microscope, the study and culture methods for viruses were introduced, and the knowledge of viruses developed rapidly. By 1950s, the mechanisms of coenzymes in bacterial growth was found to be useful. Another important development was the use of antibiotics in media and by 1960s, antibiotics were used in culture media as selective agents.

After World War II, further advancement of microbiology led to the discovery of antibiotics as medicine for various diseases, such as pneumonia, tuberculosis, meningitis, syphilis, etc. During the period of 1950s and 1960s, many vaccines against viral diseases, such as polio, measles, mumps, and rubella were developed.

Modern microbiology has application in various fields, such as development of pharmaceutical products, food and dairy products, the control of disease-causing microorganisms, and the industrial exploitation of microorganisms. Thus, microorganisms were used to produce vitamins, amino acids, enzymes, growth supplements as well as various foods, such as fermented dairy products (sour cream and buttermilk), pickles, breads and alcoholic beverages.

In biotechnology, applied microbiology has a dominant role as the microorganisms are used as living factories to produce pharmaceuticals, including human hormone insulin, antiviral protein interferon, numerous blood-clotting factors and clot-dissolving enzymes, including a number of vaccines. For rDNA technology, genetically engineered microorganisms are used as host vectors in order to develop genetically modified organisms (GMOs) with improved traits.

Nutritional categories of microbes

Microorganisms can be categorised based on the means of obtaining their carbon and energy. Most microorganisms obtain their carbon in the form of organic molecules, derived directly or indirectly from other organisms, and are described as heterotrophs that include all the fungi and protozoans, as well as most types of bacteria. Microorganisms that obtain their carbon from carbon dioxide are called autotrophs that include a significant number of bacteria and about all of the algae.

Autotrophs may further be of two categories. **Chemoautotrophs** obtain their energy as well as their carbon from inorganic sources (by the oxidation of inorganic molecules, such as sulphur or nitrite), while **photoautotrophs** have photosynthetic pigments and use them to convert light energy into chemical energy.

Majority of the heterotrophs obtain energy by chemical oxidation of organic nutrient molecules, and thus are called **chemoheterotrophs**. Although, some heterotrophs (such as the green and purple non-sulphur bacteria) obtain light energy from photosynthesis, they get carbon from organic nutrients, hence known as **photoheterotrophs**. A **lithotroph** is an organism that uses inorganic molecules, such as H_2O, H_2S or ammonia as a source of electrons, while an **organotroph** uses organic molecules for the same purpose.

6.2 Nutritional Requirements and Culture Media

Microorganisms require nutrients that are essential for energy production as well as their growth and multiplication.Carbon, oxygen, hydrogen, nitrogen, sulfur, phosphorus, potassium, calcium, magnesium and iron are called macronutrients and they are required in relatively large amounts. Among them, C, O, H, N, S and P are the components of carbohydrates, lipids, proteins and nucleic acids, and K, Ca, Mg, Fe play a variety of roles being involved as cofactor of various enzymes. Besides macronutrients, some micronutrients or trace elements, such as Mn, Zn,

Co, Mo, Ni, and Cu are also required for microbial growth in a small amounts. These micronutrients are ubiquitous in nature and already exist with various regular media components, so they are not usually supplied separately. The essential nutrients have to be supplied in the form of nutrient media. The major components required for the medium are as follows:

(a) Carbon source: Carbon is required for the skeleton or backbone of all organic molecules, and the carbon sources usually also provide oxygen and hydrogen atoms. In the microbial culture media, variety of a carbon sources are used. For example, glucose, lactose, sucrose, starch, glycogen, cellulose, a mixture of various carbohydrates, such as cereal grain powders and cane molasses etc.

(b) Nitrogen, phosphorous and sulphur sources: Nitrogen source is required for the biosynthesis of amino acids, nucleic acids and enzymes etc. The ammonium salts, urea, animal tissue extracts, amino acid mixtures and plant-tissue extracts are the main nitrogen sources that are used in culture media. Phosphorus is present in nucleic acids, phospholipids, nucleotides, several cofactors, and other cellular components. Almost all the microorganisms use inorganic phosphate as their phosphorus source. Sulfur is required for biosynthesis of various amino acids, such as cysteine and methionine, some carbohydrates, etc. Most of the microorganisms use sulfate as a source of sulfur.

(c) Growth factors: Growth factors are organic compounds (e.g., certain amino acids, purines pyrimidines and vitamins) that cannot be synthesised in the cells and are required for the growth and multiplication of cells.

(d) Anti-foams: Anti-foams are not the nutritional component. However, the media having nutritional components including starch, protein, and other organic compounds as well as products formed during cell growth may have excessive foaming due to the agitation of culture media. To prevent this foam formation, some anti-foaming agents including olive oil, sunflower oil and silicones are included in the media.

(e) Water: Water is an essential component/base of any culture media (liquid or solid). In solid culture media, the quantity of water used is comparatively less than the liquid media.

A culture media has all the nutrients, growth factor, energy source, etc., to support the growth of microorganisms.

Though all the microorganisms need sources of energy, carbon, nitrogen, phosphorus, sulfur and various minerals, the specific composition of a suitable medium will depend on the particular species, which is to be grown in the laboratory. Thus, for selecting an appropriate culture medium for the microorganism, prior knowledge of the habitat of the microorganism is useful, because its nutrient requirements are based on its natural surroundings.

Types of media

(A) On the basis of chemical composition, the culture media are classified into two types:

(i) Synthetic or chemically defined media

Media in which all chemical components are known is called defined media or synthetic media. Some microorganisms, specifically photolithotrophic autotrophs (e.g., cyanobacteria) can be cultured on a media containing CO_2 as a carbon source, nitrate or ammonia as a nitrogen source, sulfate, phosphate, and a number of minerals. A variety of heterotrophs may be cultured in a media with glucose as a carbon source and an ammonium salt as a nitrogen source. Defined media are widely used in research and prepared by mixing various components according to the requirements for experimental microorganism. M9 media is widely used for the cultivation and maintenance of *Escherichia coli* (*E. coli*) cultures, and BG11 media is used for the cultivation and maintenance of blue green algae (cyanobacteria).

(ii) Complex media

Media that contain some constituents with unknown chemical composition are called complex media. These

types of media are useful, because even a single complex medium may have almost all the constituents to meet the nutritional requirements of a number of different microorganisms. The undefined components in the complex media are peptones, beef extract and yeast extract etc. Peptones are protein hydrolysates prepared by the partial proteolysis of meat, casein, gelatin, and other protein sources and serve as a source of carbon, energy and nitrogen. The beef extract and yeast extract are aqueous extracts of beef and brewer's yeast, respectively. The beef extracts have amino acids, peptides, nucleotides, organic acids, vitamins, and minerals, whereas yeast extract is a rich source of vitamin, nitrogen as well as carbon compounds. Examples are nutrient broth, tryptic soy broth, and MacConkey agar. Lysogeny broth (Luria-Bertini) is a commonly used medium for culturing bacteria, such as *E. coli*. The LB medium, first developed by Giuseppe Bertani (1951) consists of 10% tryptone/peptone, 5% yeast extract, and 10% sodium chloride. Potato dextrose agar (PDA) is used for the identification, cultivation and enumeration of various fungi including yeast and moulds.

(B) On the basis of consistency, the culture media are of three types

(i) Liquid media or broth

In liquid media, agar is not added. After inoculation and later incubation, the growth of cells becomes visible in the broth.

(ii) Solid media

For the surface cultivation of microorganisms, solid culture media is required. For preparation of solid media, the liquid media is solidified with the addition of 1.0–2.0% agar. Agar is a sulphated polymer composed of D-galactose, 3, 6-anhydro-L-galactose, and D-glucuronic acid, generally extracted from red algae. Solid media are used for making agar slants, slopes and agar stab. Agar is preferred as a solidifying agent as most of the microorganisms cannot degrade it. Although, other solidifying agents, such as silica gel can also be used.

(iii) Semi-solid agar media

The semi-solid media are prepared by adding 0.5% of agar in the media. This type of media may be selective as it promotes the growth of one organism and may retard the growth of the other organism.

(C) On the basis of their application(s) and functions, the culture media are classified into the following types

(i) Selective media

The selective media favours the growth of a particular type of microorganism. They provide nutrients that increase the growth of a particular type of microorganism and suppresses all other microorganisms that are present in the culture. This medium is useful for isolating a specific microorganism from a mixed population. Thus, for the isolation of cellulose-digesting bacteria, a medium containing only cellulose as a carbon and energy source is used. Endo agar, eosin methylene blue agar, and MacConkey agar media are used for the growth of *E. coli* and similar bacteria. Certain media that contain bile salts or dyes, such as basic fuchsin and crystal violet are used to suppress the growth of Gram-positive bacteria but encourage the growth of Gram-negative bacteria.

(ii) Differential media

Differential media are used to distinguish different groups of microorganisms based on the appearance on the medium as well as their biological characteristics, which permit the identification of microorganisms. For example, if a mixture of bacteria is inoculated on to a blood-containing agar medium (blood agar), some of the bacteria may hemolyze (destroy) the red blood cells, and others do not. Thus, this medium distinguishes between hemolytic and non-hemolytic bacteria. Therefore, blood agar medium is both a differential medium and an enriched one. MacConkey agar is also kept under both differential and selective media. Certain indicators are present in this medium, which may allow such differentiation. For example, the MacConkey's agar containing lactose and a dye (which changes colour when pH drops). The microorganisms that ferment lactose,

produce an acid as the end product, which lowers the pH and produces a red colony. Microorganisms that fail to ferment lactose produce colourless colonies. The dye in MacConkey's agar also inhibits the growth of Gram-positive bacteria. This medium is, therefore, both selective and differential.

(iii) Enrichment media

In enrichment medium, the nutritional environment is adjusted in order to selectively enhance the growth of certain microorganisms within the mixed inoculum. For example, the addition of plant and animal tissue extract into nutrient broth and nutrient agar media provides additional nutrients, which enhance the growth of fastidious heterotrophic bacteria. Blood agar is also an enrichment media.

6.3 Sterilisation Methods

In any microbial study, all media, working surface, glassware and plasticware should be free from contaminants by sterilisation.

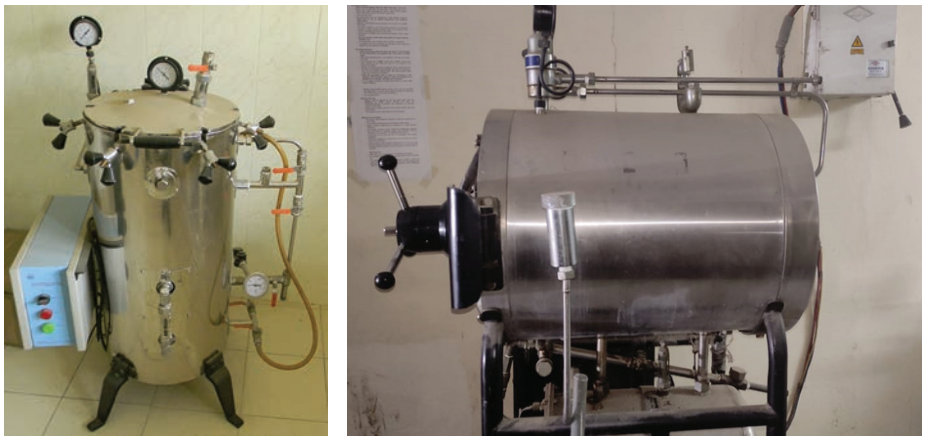
Usually, sterilisation can be achieved by both physical and chemical methods. In physical methods, sterilisation can be done by heat, radiation and filtration, while in chemical methods, sterilisation can be achieved using chemicals.

Sterilisation by Heat

Sterilisation by heat is considered to be the most effective and rapid method for articles that can withstand heat. Heat acts by denaturation, coagulation of proteins, by exerting oxidative effect and interfering with the metabolic reactions that lead to the death of cells. The sterilisation by heat is performed by various methods:

(a) Boiling: Sterilization can be done at 100°C for 30 min in boiling water bath. The syringes, rubber goods and surgical instruments may be sterilised by this method.

(b) Autoclaving: Autoclaving is one of the most common methods for sterilisation in almost all the laboratories. In this method, sterilisation is done by steam at temperature higher than 100°C under pressure. At atmospheric pressure, water boils



(a) (b)

Fig. 6.1: (a) Vertical Autoclave (b) Horizontal Autoclave (Photo courtesy: Dr. Veda P. Pandey)

at 100°C but if the pressure is raised, the boiling temperature also increases. In an autoclave, the water is boiled in a closed air-tight chamber. At a pressure of 15 lb per square inch (psi) inside the autoclave, the temperature is raised to 121°C. In an autoclave, the articles are exposed to the steam released at this temperature and sterilisation is achieved. Autoclaves are of vertical or horizontal design and they may be jacketed and non-jacketed (Fig. 6.1). In the jacketed autoclave, the steam flows surrounding the jacket in which the articles/media are kept and they are not directly exposed to steam, while in non-jacketed autoclave, the articles/media are directly exposed to the steam. The **holding time** in the autoclaving is the time for which the entire load in the autoclave is exposed and actual sterilisation takes place. The holding time depends on the size and types of the load and generally exceeds 30 min.

(c) Pasteurisation: This process is used in food and dairy industry. Actually, pasteurisation is the heating of milk to a temperature for a period of time to kill the pathogenic microorganisms that may be present in milk. It does not fully sterilise the milk, so many living organisms including spores are not destroyed. There are two methods of pasteurisation,

the holder method (low temperature long time, LTLT, heated at 63°C for 30 min) and flash method (high temperature short time, HTST, heated at 72°C for 15 sec) followed by quick cooling. Other pasteurisation methods are also available, such as Ultra-High Temperature (UHT) at 140–150°C for 1–3 seconds.

(d) Heating and flaming: Many objects are sterilised in the absence of water by dry heat sterilisation. The articles, such as inoculation loops, straight wires, tips of forceps and searing spatulas are sterilised by holding them in Bunsen flame till they become red hot. While, some articles, such as mouth of test tubes, flasks, glass slides, etc., are passed through the flame for a few times without allowing it to become red hot. Hot air oven is also used to sterilise glassware.

Sterilisation by Radiation

Two types of radiations, namely, ionizing and non-ionizing radiations are used for sterilisation. As radiation does not generate heat, so sterilisation by radiation is termed as 'cold sterilisation'. Non-ionizing radiations, such as UV rays have the germicidal activity in the wavelength range of 200–280 nm, with 260 nm being the most effective. UV rays induce the formation of thymine—thymine dimers, which ultimately inhibits DNA replication and induces mutation in cells. UV radiation is useful for surface disinfection, i.e., to disinfect culture lab, laminar hood, operation theatres, virus laboratories and etc. The disadvantages of UV rays include their harmful effect on skin and eyes, and reversal action of damage due to DNA repair bacterial enzymes. Ionizing radiations are of two types, particulate (electron beams) and electromagnetic rays (gamma rays). Electron beams are used to sterilise syringes, gloves, dressing packs, foods and pharmaceuticals, etc. Electromagnetic rays have more penetrative power than electron beam, but require longer time of exposure. These radiations damage the nucleic acid of the microorganism. Electromagnetic radiations are used to sterilise disposable Petri dishes, plastic syringes, antibiotics, vitamins, hormones, glassware and fabrics.

Sterilisation by Filtration

Filtration is used to sterilise the heat labile substances in solutions, such as serum, antibiotics, sugars, urea, etc. Filtration does not kill microbes but it separates them out based on the size. Membrane filters with pore sizes between 0.2–0.45 µm are commonly used to remove microbes from solutions. Air can be filtered using **High Efficiency Particulate Air** (HEPA) filters. HEPA filters are 99.97 percent efficient for removing particles >0.3 µm in diameter. They are usually used in biological safety cabinets.

Sterilisation by Chemicals

Sterilisation/disinfection can also be achieved by certain chemicals, such as alcohols, aldehydes, heavy metals, etc. Alcohols sterilise the surface through cell dehydration, membrane disruption and protein coagulation. 70 percent ethyl alcohol is used as antiseptic on skin. Isopropyl alcohol can be used to disinfect surfaces and clinical thermometers. Aldehydes such as 40 percent formaldehyde (formalin) is used for surface disinfection and fumigation of rooms, chambers, operation theatres, biological safety cabinets, etc. Heavy metals such as mercury, silver, arsenic, zinc, and copper are used as germicides. They cause precipitation of proteins. Hydrogen peroxide solution is used for skin disinfection of wounds and ulcers and deodorising wound dressings.

6.4 Pure Culture Techniques

Bacteria may be cultured using either liquid or solid media. Solid media are particularly useful in the isolation of bacteria and are also used for their long-term storage, while liquid (broth) cultures are used for rapid and large-scale production of bacteria.

As you know, the microorganisms in the natural habitat, exist as a part of complex ecosystems comprising a numerous other microorganisms. Obtaining a pure

culture, is important for studying a particular microbe. A culture consisting of entirely one strain of organism is called pure culture.

The standard method of obtaining a pure bacterial culture is the **streak plate method** (Fig. 6.2). An inoculating loop is used to streak out a drop of bacterial suspension or bacterial colony on a solid agar plate in such a way that it becomes progressively more dilute. After proper incubation at an appropriate temperature, due to cell divisions, visible bacterial colonies are developed. Colonies are formed because all the progeny stay in the same place and the movement of daughter cells is not possible on the solid surface. In other words, a clump of cells, deposited together, gives rise to a colony. Near the end of streak, isolated colonies are obtained.

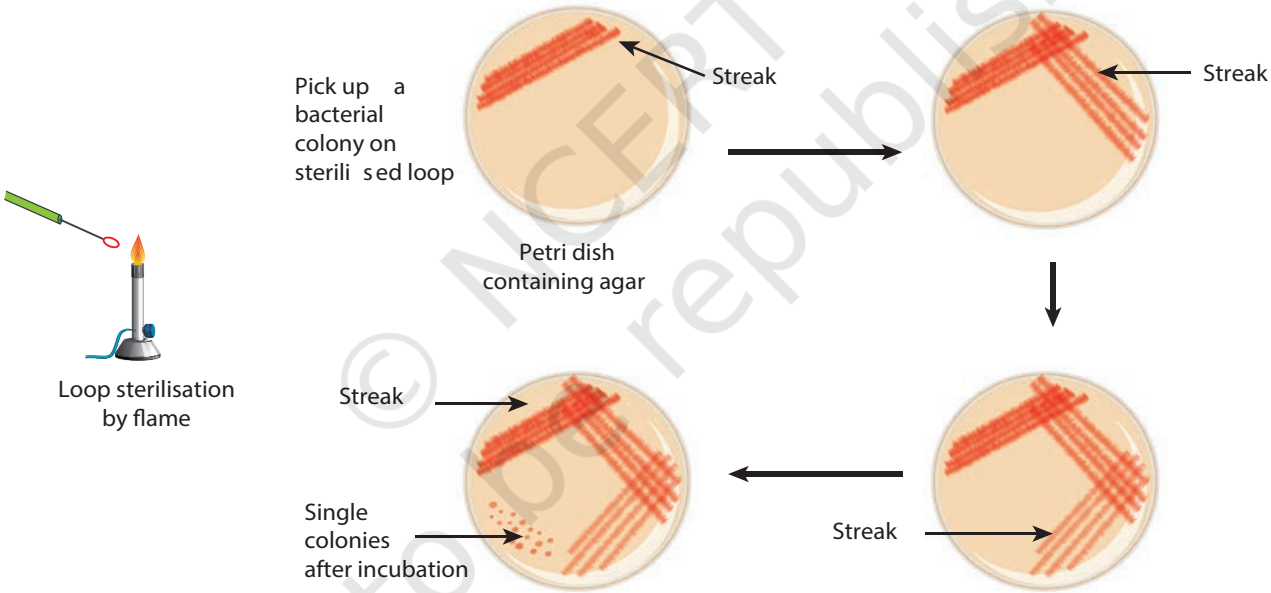


Fig. 6.2: The streak plate method

An alternative method for the isolation of pure cultures is the **pour-plate method** (Fig. 6.3). In this method, the original microbial sample is diluted several times to dilute the population sufficiently in order to obtain separate colonies upon plating. The small volumes (1 mL) of each dilution is then poured on the bottom of a Petri plate/dish. After that, the melted medium with agar is poured on to the Petri plates and mixed gently with a circular motion. After incubation at an appropriate temperature, the isolated cells grow into colonies, which can be used to establish pure cultures.

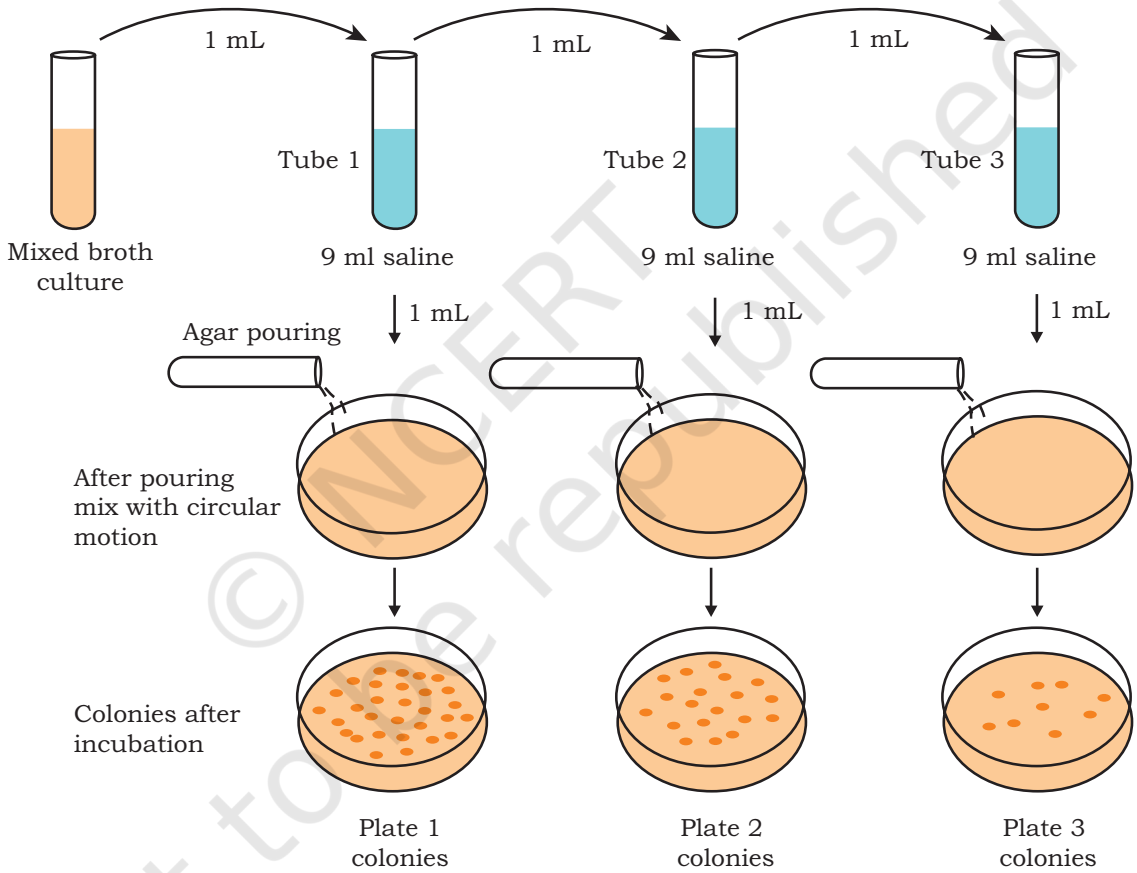


Fig. 6.3: The Pour-Plate method

The **spread-plate technique** is another easy and direct way of achieving a pure culture (Fig. 6.4). In this technique, a small volume of the diluted bacterial mixture is transferred to the centre of a solidified agar plate and spread evenly over the surface with a sterile, L-shaped

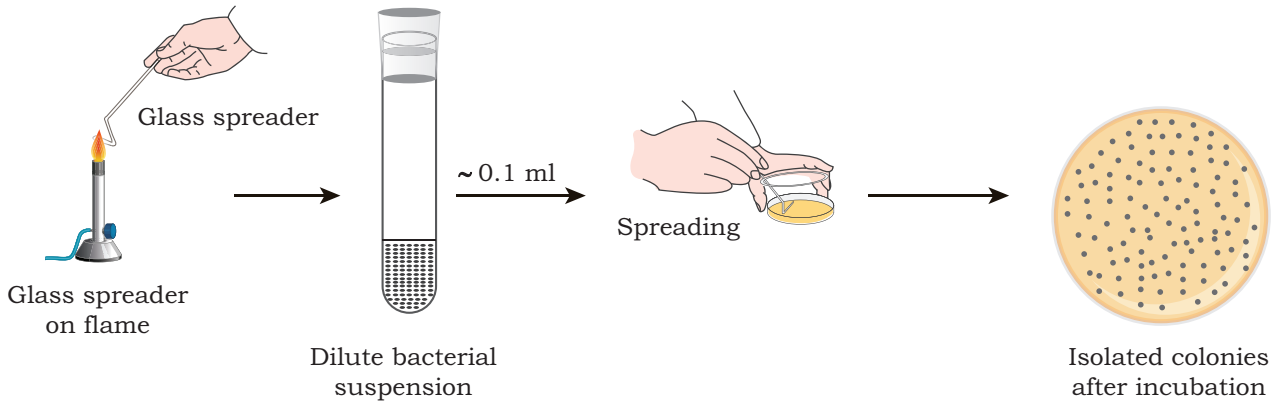


Fig. 6.4: The spread-plate technique

glass spreader. The glass rod is usually sterilised by dipping in alcohol and flamed to burn off the alcohol. After proper incubation at an appropriate temperature, some of the dispersed cells develop into isolated colonies.

6.5 Factors Affecting Microbial Growth

Microbial growth may be affected by a number of factors. The major factors are described below:

Temperature

Microorganisms are able to grow over a wide range of temperatures. The minimum and maximum growth temperatures for a microorganism is typically about 25–45°C. The growth rate increases with temperature until the optimum temperature is achieved, and beyond that the growth rate declines again due to loss of enzymatic activity. The majority of microorganisms achieve optimal growth in the range of about 20–45°C and are called **mesophiles**. Some microorganisms would be capable of growing within a range of about 40–80°C, with an optimum temperature around 50–65°C and are called **thermophiles**. In addition, some are **extreme thermophiles** that can tolerate temperatures above 100°C.

pH

The growth of microorganisms is strongly influenced by the variation in pH of their surroundings. Fungi can tolerate a wide range of pH variation when compared to bacteria. Most of the microorganisms grow best around

neutrality (pH 7). A number of bacteria prefer slightly alkaline conditions, but a few are tolerant to acidic conditions. On the contrary, fungi generally prefer slightly acidic conditions and therefore tend to dominate bacteria when these prevail together. The pH of growth media is adjusted to the desired value depending upon the type of microorganisms by the addition of acid or alkali during its preparation.

Oxygen

Oxygen is present as one of the major constituents (21%) of our atmosphere, and the microorganisms that are dependent on oxygen for survival, are called **aerobes**. Some microbes are also able to survive in the absence of oxygen and called **anaerobes**. During the culture of aerobic organisms, oxygen must be provided. For a shallow layer of medium such as that in a Petri dish, sufficient oxygen is available as dissolved in surface moisture. But in a flask of broth, aerobes will only grow on the surface layers, therefore, additional oxygen is provided by shaking or mechanical stirring to facilitate uniform growth. **Obligate anaerobes** cannot tolerate oxygen and they are cultured in special anaerobic chambers, where oxygen is excluded from all liquid and solid media. The **facultative anaerobes** can survive like aerobes in the presence of oxygen, and are also able to survive in anaerobic condition. **Aerotolerant anaerobes** are the organisms that are generally anaerobic, but they are not inhibited by the atmospheric oxygen as they do not utilise it.

Carbon dioxide

The autotrophic organisms are able to use carbon dioxide as a carbon source and when they are grown in culture, CO_2 is provided as bicarbonate in their growth medium or incubated in a CO_2 enriched atmosphere.

Light

Phototrophic microorganisms require light in order to carry out photosynthesis. In the laboratory, during culturing of microorganism, one should ensure that light of the correct wavelength is used.

6.6 The Growth Curve

When single cell microorganisms are grown in a suitable nutrient media with initial limited amount of nutrients, cells start growing in number and size at their maximum. The growth of cells in an artificial media depends on various factors including nutritional factors, environmental factors, etc. The growth of cells in nutrient media depends on cell concentration and the outcome of this process is cellular reproduction. Unicellular organisms divide by binary fission, replicate their genetic material, then divide into two identical cells, which then divide into four, four into eight and so on, leading to an exponential increase in the number of cells. This growth of the cultured microorganisms can be analysed by plotting the logarithm of the number of viable cells versus the incubation time, which results in a curve with four distinct phases as lag phase, exponential phase, stationary phase and decline phase (Fig. 6.5).

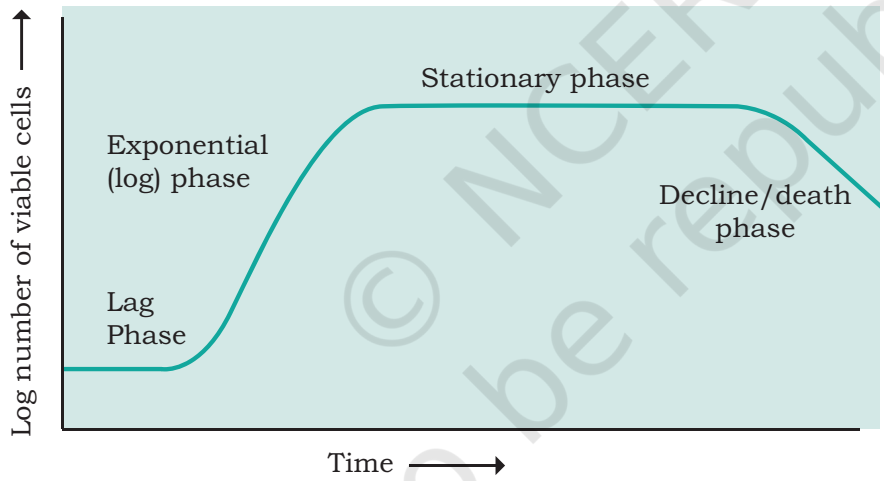


Fig. 6.5: Microbial growth curve

Lag Phase

Initially, when microorganisms are introduced into fresh culture medium, usually increase in cell number is not observed immediately, and therefore, this period is called lag phase. Due to the lack of cell division, during lag phase, there is no net increase in mass, the cell is synthesising new components required for cell division. The duration

of lag phase varies with the condition of the inoculated microorganisms and the nature of the medium. This phase may be long if the inoculum is taken from an old culture or refrigerated culture. On the contrary, when a young or growing microbial culture is inoculated to fresh medium, the lag phase may be short or even absent.

Exponential Phase

During the exponential or log phase, the microorganisms are growing and dividing at the maximal rate. The microorganisms are doubling in number at regular intervals during the exponential phase and the growth rate is constant. In this phase, the population will double in number during a specific time period, which is called the generation time or doubling time. For example, the doubling time for *E. coli* is 20 minutes. During exponential growth, the doubling time can be expressed as:

$$t_d = \frac{0.693}{\mu}$$

μ = Specific growth rate $\left[\mu = 2.303 \frac{(\log X_t - \log X_0)}{t}\right]$

X_t = Cell concentration at time t

X_o = Initial cell concentration

t = Time taken

t_d = Doubling time

During this phase, the population is most uniform in terms of chemical and physiological properties, and therefore, the microorganisms of this phase are usually used for biochemical and physiological studies.

Stationary Phase

After a certain time of log phase, the population growth ceases and the growth curve becomes horizontal. This depends on the overall nutrient availability and other factors, as well as the type of microorganism being cultured. During the stationary phase, the total number of viable microorganisms remains constant due to the development of a balance between cell division and cell death. There are several factors for the microbial populations to enter into stationary phase. One important factor is the nutrient

limitation; if any essential nutrient is severely depleted, then the population growth will be slow.

Death Phase

The unfavourable environmental conditions, such as nutrient deprivation, accumulation of toxic wastes, etc., lead to further decline in the number of viable cells, which is the characteristic of decline/death phase. During this phase, the death of a microbial population is usually

Box 2

Measurement of microbial growth

A microbial cell culture undergoing balanced growth is like a chemical reaction where substrates present in media are converted to cell mass.

The parameter that characterises microbial growth is the doubling time, also called **generation time t_d.** It is time (t) required in minutes/hours for cell mass or cell number to double its original value during balanced growth.

$$t_d = t/n \qquad (n = \text{number of generations})$$

Specific growth rates μ, is an index of growth of microbes in a particular environment. It is defined as the rate of increase of biomass of a cell population per unit of biomass concentration. Value of μ is characteristic for each microbe. It depends upon the temperature, pH, media composition and O_2 dissolved in the media.

As the value of μ increases, the microbial culture having specific growth rate has less doubling time.

On the basis of value of μ and t_d of an organism, the media composition and fermentation batch time is decided to optimise the production in commercial fermenters.

Example

If a culture contains 10^4 cells/mL at t_o and 10^8 cells/ml after 2 h, calculate the specific growth rate and generation time of the culture.

Solution:

$X_0 = 10^4$ cells/mL

$X_t = 10^8$ cells/mL

$t = 2$ h

$$\text{(i) } \mu = 2.303 \frac{(\log 10^8 - \log 10^4)}{2}$$

$$= 2.303 \frac{(8 - 4)}{2}$$

$$= 2.303 \frac{(4)}{2} = 4.606\ hr^{-1}$$

$$\text{(ii) } t_d = \frac{0.693}{\mu} = \frac{0.693}{4.606} = 0.15\ h$$

logarithmic (as it is growth during the exponential phase). The accumulation of metabolic waste products may often become a factor for the decline of population growth. For example, *Streptococci* can produce lactic acid and other organic acids from sugar fermentation, which results in the medium becoming acidic and growth is inhibited.

SUMMARY

- Microbiology is the study about small life forms, i.e. microorganisms.
- The microorganisms require a source of energy, carbon, nitrogen, oxygen, iron, other minerals, micronutrients, and water for growth and multiplication.
- Selection of appropriate culture medium for the microorganisms depends on the prior knowledge of the habitat of the microorganisms.
- Culture media can be categorised based on the chemical composition. Media are classified into two types: synthetic and complex media. While on the basis of consistency, the culture media may be solid, liquid, and semi-solid. Whereas, on the basis of their application and functions, the media may be divided into selective, differential, and enrichment media.
- Sterilisation is of prime importance for any microbial study and it is the process, where all the living microorganisms, including bacterial spores are killed or removed.
- Sterilisation can be achieved by physical (heat, radiation and filtration) and chemical methods.
- The microbial growth may be affected by a number of physical factors, such as temperature, pH, oxygen, etc.
- The growth of the cultured microorganisms can be analysed by plotting the logarithm of the number of viable cells versus the incubation time, which results in a curve with four distinct phases, namely lag, exponential, stationary, and death phase.

EXERCISES

1. Describe the nutritional requirements of microorganisms.
2. What is culture media? Classify the culture media.
3. Write an account of the growth curve analysis of a microorganism.
4. Discuss any two methods to isolate a pure culture.
5. Define sterilisation, disinfection, and sanitisation.

6. Give a detailed account on the various methods of sterilisation.
7. Bacterial strains that do not require any organic supplement are called:
   (a) Auxotroph
   (b) Prototroph
   (c) Heterotroph
   (d) Chemotroph
8. Who was the first to develop the process of colony purification on solid media?
   (a) Louis Pasteur
   (b) Robert Koch
   (c) Fannie Hesse
   (d) Richard Petri
9. HTST and UHT methods belong to:
   (a) Pasteurisation
   (b) Isolation of pure culture
   (c) Staining of bacteria
   (d) Culture of bacteria
10. Spontaneous generation was suggested by:
   (a) Francesco Redi
   (b) Lazzaro Spallanzani
   (c) Robert Koch
   (d) Louis Pasteur
11. Germ theory of disease was suggested by:
   (a) Francesco Redi
   (b) Lazzaro Spallanzani
   (c) Robert Koch
   (d) Louis Pasteur
12. Calculate the specific growth rate and generation time of a bacterial population in which the number of bacteria increases from 10^4 cells/mL to 10^7 cells/mL, during 4 hours of exponentail growth.

   (Ans: $\mu = 1.72\ hrs^{-1}$, $t_d = 0.4$ h or 24 min)
13. **Assertion:** Alcohol production by a batch culture of *Saccharomyces* starts declining steadily even though conditions like temperature are optimum.

   Reason: Alcohol concentration of around 13% is toxic for yeast cells.
   (a) Both assertion and reason are true and the reason is correct explanation of the assertion.
   (b) Both assertion and reason are true but reason is not the correct explanation of the assertion.

(c) Assertion is true but reason is false.

(d) Both assertion and reason are false.

14. **Assertion:** A selection medium containing Ampicillin is sterilised by autoclaving. Both amp^S and amp^R microbes show growth on the medium.

Reason: To inhibit the growth of amp^S microbe ampicillin should not have been autoclaved but sterilised by using micro-filters before adding to the medium.

(a) Both assertion and reason are true and reason is correct explanation of the assertion.

(b) Both assertion and reason are true but reason is not the correct explanation of the assertion.

(c) Assertion is true but reason is false.

(d) Both assertion and reason are false.

15. **Assertion:** Microorganisms are able to grow and multiply over a wide range of temperatures.

Reason: Extreme thermophiles can tolerate temperatures of above 100°C.

(a) Both assertion and reason are true and reason is the correct explanation of the assertion.

(b) Both assertion and reason are true but reason is not the correct explanation of the assertion.

(c) Assertion is true but reason is false.

(d) Both assertion and reason are false.

Chapter 7 Plant Tissue Culture

Plant tissue culture (PTC) refers to the cultivation of undifferentiated plant cells, tissues or organs on synthetic media under aseptic environment and suitable controlled physical conditions. It is an important tool for both basic research as well as commercial applications. Plant tissue culture is based upon the unique characteristic of a plant cell, i.e., totipotency. Totipotency is the ability of a vegetative cell to divide and differentiate into any type of specialised cell or to regenerate into a whole plant.

7.1 Historical Perspective

In 19th century, German scientists Theodor Schwann and Matthias Schleiden, drew attention to the fact that a cell is the basic unit of life and has the capacity to divide and grow. During 1890's, **Gottlieb Haberlandt** (German Botanist), pioneered the field of PTC with his idea to achieve continuous cell divisions in plant cells on nutrient media. He attempted to culture fully differentiated plant cells on simple nutrient media. He laid down several principles of plant tissue culture in 1902, like plant cells are capable of resuming uninterrupted growth, or it is possible to

regenerate embryos from vegetative cells. Later, all of the predictions made by him were found to be true as they were confirmed experimentally by other researchers. This is the reason he is regarded as the '**Father of Plant Tissue Culture**'.

During 1902–1930s, several attempts were made to culture the isolated plant tissues, like root or shoot tips, and continuously growing plant cell cultures were established. The discovery that vitamins and natural auxins are necessary for the growth of plant tissues on synthetic media gave a significant boost to PTC. During 1940s –70s, extensive studies were performed to improve the existing techniques for developing new methods to optimise the nutrient media components for culturing plant tissues. Coconut water stimulated the development of young embryos and was used with nutrient media for *in vitro* cultures. Later, other natural supplements, like corn milk, orange juice, etc., were used for developing callus culture of several species, like woody plants and herbaceous dicot plants. During 1950s, adenine, kinetin and high levels of phosphate were used in nutrient media that resulted in the successful initiation of cultures from non-meristematic tissues and production of shoots or roots. It was also established during this time that the morphogenic fate of cultured cells was directly influenced by the exogenous balance of auxins and kinetin. It was suggested that a relatively high level of auxin to kinetin causes rooting, while the reverse led to shooting and intermediate levels were responsible for the proliferation of callus. This led to the regeneration of new plants through tissue culture using various plant tissues or organs as starting material in several commercial applications. An artificial culture medium developed by Murashige and Skoog (MS) was successful for culturing plants and resulted in the initiation of tissue culture from a large number of plant species. MS media composition is the most widely used nutrient media in plant tissue cultures.

Box 1

Major landmark discoveries in plant tissue culture and its applications

1902	Gottlieb Haberlandt proposed that plant cells can be cultured on artificial media and developed the concept of *in vitro* cell culture.
1904	Hanning initiated work on excised embryo culture and later cultured embryos from several cruciferous species.
1922	Kotte and Robbins suggested root and stem tips as possible explants to initiate *in vitro* tissue culture.
1926	Went discovered the first plant growth hormone, i.e., Indole Acetic Acid (IAA).
1934	The role of vitamin B as growth supplement in plant tissue culture was reported by White. He could successfully establish continuous growing cultures from tomato root tips.
1937	White formulated the first synthetic plant tissue culture medium (WM).
1941	Johannes Van Overbeek introduced coconut water as a media component and demonstrated its beneficial effects on *in vitro* tissue culture.
1946	Ball raised whole plants from shoot tips of *Lupinus*.
1954	Muir successfully induced cell division in mechanically isolated single cells.
1955	Skoog and Miller reported the discovery of Kinetin, which is a type of cytokinin and promotes cell division.
1957	Skoog and Miller described chemical control hypothesis of root and shoot differentiation by manipulating the ratio of concentrations of auxin and kinetin.
1962	Murashige and Skoog formulated MS medium with higher salt concentrations.
1964	Guha and Maheshwari produced the first androgenic haploid *Datura* plant by anther culture.
1971	Protoplasts were subcultured *in vitro*, and plants were regenerated from their culture.
1972	Protoplast from two different species of *Nicotiana* were isolated, fused together and somatic hybrids were generated successfully.
1976	Gynogenic haploid plants were successfully cultured from unfertilised ovaries of barley by San Noeum.
1978	Melchers and colleagues produced 'Pomato', which was a hybrid of potato and tomato, and was produced through somatic hybridisation.

1981	The term 'Somaclonal Variations' was introduced by Larkin and Scowcroft, for the genetic variations introduced during the plant tissue culture.
	Horsh and colleagues produced transgenic tobacco plants. Leaf discs were used as explants and were cultured with *Agrobacterium tumefaciens*.
	Klien and colleagues developed high-velocity microprojectile-based DNA delivery 'biolistic gene transfer' method for plant transformation.
1987	Y. Fujita and Mamoru Tabata established *Lithospermum erythrorhizon* cell cultures for the shikonin production and commercialised it.
	Transgenic Bt-cotton was produced by Monsanto and in 2000, it was approved by the Government of India for commercial production in India.
1993	Kranz and Lorz produced fertile maize plants through *in vitro* fertilisation.
	Plant tissue culture free plant transformation method '*Arabidopsis* Floral-dip' was developed.
	Transgenic rice engineered for the production of provitamin A (beta-carotene) in rice endosperm was developed and is called 'Golden Rice'.
	The first plant produced enzyme was approved for human use. It is used to treat a rare lysosomal storage disease called Gaucher's Disease.
	Somatic embryogenesis was introduced in plant transformation through the embryonic genes.

7.2 Plant Cell and Tissue Culture Techniques

Virtually any part of the plant like leaf, apical meristem, embryo, cotyledon, hypocotyl, etc., can be used as a starting material called explant. These explants are transferred on to the nutrient media and whole plants can be regenerated through *in vitro* culture. It has been observed in various research experiments that different plant organs of different plant species respond in different ways as per their nutritional requirement and physical conditions under *in vitro* culture conditions. However, the response of different plant organs varies for *in vitro* culture. For example, immature embryos are more responsive than apical meristem, which are generally more responsive than leaf explants on a particular tissue culture media and culture conditions.

Plant regeneration in cultures can mostly be achieved by two morphogenetic pathways—organogenesis and somatic embryogenesis. Inducing the formation of various vegetative organs from cells or tissues in plant tissue culture is called **organogenesis**. First, the specialised cells of explants start dividing under certain specific conditions and form a mass of undifferentiated cells. This process is called **dedifferentiation**. This is followed by the formation of organ primordia, like shoot or root, and is called **redifferentiation**. Relative concentration of growth hormones (especially auxins and cytokinins) play an important role in organogenesis.

The process of formation of an embryo from somatic cells is called **somatic embryogenesis**. Resulting embryos are called somatic embryos. Somatic embryogenesis follows embryogenic pathways of zygotic embryogenesis. Somatic embryos are very similar to zygotic embryos, except in their place of origin and being larger in size.

Following is the step-by-step procedure that explains the *in vitro* plant tissue cultures using tomato cotyledons as explants (Fig. 7.1).

Some of the basic requirements for plant tissue culture are:

(i) Washing area—the place to wash glassware, plasticware and other labware used in PTC. Washed labware need to be stored at a clean and dry place.

(ii) Various media components for the preparation of Nutrient Media.

(iii) Facility to sterilise nutrient media and store media at low temperatures.

(iv) Facilities for maintenance of cultured tissues in aseptic conditions under controlled environment, i.e., light, temperature and humidity.

Steps of plant tissue culture

(i) Selection of a suitable nutrient media and its sterilisation by autoclaving or passing through micropore filters to avoid microbial contamination.

(ii) Selection of a starting desired material for tissue culture, i.e., explants. Any plant tissue , organ or part, which is used in plant tissue culture to regenerate mass of dedifferentiated cells, tissues, organ or whole plant, is called explant. Most

commonly used explants are root or shoot apical meristems, leaves, cotyledons, hypocotyls and immature embryos. Fig. 7.1 (a) shows the aseptically grown tomato seedlings, which can be used for preparing explants.

(iii) Surface sterilisation of the explant is done using suitable disinfectants followed by washing with sterile distilled water. Sodium hypochlorite is the most commonly used disinfectant for sterilisation of explants. Explants harvested from seedlings grown in sterile conditions [Fig. 7.1 (a)] need not to be sterilised again.

(iv) Inoculation of explants takes place onto the nutrient media (Fig. 7.1 (b)).

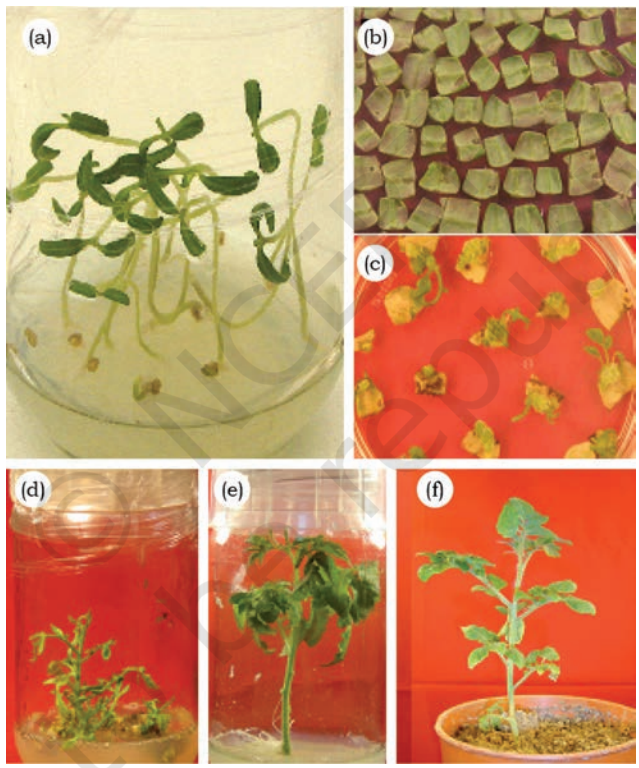


Fig. 7.1: Various stages of regeneration of tomato tissue culture plant: (Photo Courtesy: Dr. Manoj Sharma)

(a) Germination of seeds on the culture medium in a culture bottle.
(b) Explant preparation from germinated seedlings.
(c) Regenerating callus (calli with shoot buds) from inoculated explants.
(d) Regenerating shoots ready to be transferred to rooting media.
(e) Rooting in the inoculated Shoots.
(f) A plantlet transferred to the soil in pot.

(v) **Growing the cultures** in the plant tissue culture room under suitable physical conditions like light, temperature and humidity [Fig. 7.1 (c)], small calli are regenerated on cotyledon explant on suitable tissue culture media.

(vi) **Transfer of growing cultures** on to suitable media to regenerate shoots, and their elongation.[Fig. 7.1 (d)],

(vii) **Excision of regenerated shoots** and transfer onto the rooting medium [Fig. 7.1 (e)].

(viii) **Transfer of plantlets to sterilised soil in pots** for hardening in green house or growth room [Fig. 7.1 (f)] followed by their transfer to the field conditions.

7.3 Nutrient Media

A variety of nutrients and suitable environmental conditions are required for optimal growth and development of an explant. Depending upon the type of plant species, like monocot or dicot; domesticated or wild, etc., composition of the culture media varies. Even different tissues from the same plant may have different nutritional requirements for optimal growth. Therefore, success of *in vitro* culture of plants mainly depends upon the selection of the right composition of culture medium.

Culture media used for *in vitro* plant cultures broadly contain the following components:

1. Inorganic components
2. Organic supplements
3. Carbon source
4. Plant growth hormones
5. Gelling agents
6. Antibiotics

Many **inorganic components** are required in large amounts (milli molar concentrations) and are categorised as **macronutrients**. These include carbon (C), calcium (Ca), hydrogen (H), potassium (K), magnesium (Mg), nitrogen (N), oxygen (O), phosphorus (P) and sulphur (S). Several other essential inorganic components are required in small amounts (micro molar concentrations) and are categorised as **micronutrients**. These include boron (B), cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo)

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and zinc (Zn). Amino acids and vitamins are common **organic supplements** that are used in culture media.

Amino acids serve as nitrogen source and commonly used amino acids are arginine, asparagine, glycine or proline. **Vitamins** added in the culture medium are thiamine (Vitamin B1), nicotinic acid (Vitamin B3) and pyridoxin. For *in vitro* cultures, sucrose is considered as the best carbon source and is used at concentration of about 2–5 percent. Other sources for carbon are glucose, fructose and mannose.

Plant growth hormones play a vital role in plant's growth and development, and are critical components of media. These are required in minute quantities in the nutrient media. There are five main categories of growth hormones i.e., Auxin, Cytokinin, Gibberellin, Abscisic acid and Ethylene. However, auxins and cytokinin are most commonly used in plant tissue culture. Ratio of their concentrations determines the type of organs produced from the cultured cell or tissues. For example, higher concentration of cytokinin results in shoot regeneration in general. Further, different plant tissues need different amount of hormones for their growth and therefore, depending upon the type of explant, their concentration may vary in the nutrient media. **Gelling agent** is required for solid culture media. Agar is the most commonly used gelling agent and is ideal for routine applications.

Further, pH of the nutrient media is usually adjusted to about 5.8 to 6.0. Increase in the pH increases the hardness of the medium and decrease in pH leads to poor solidification of the media. pH of the nutrient media also affects the uptake of the nutrients by plant cells and solubility of the media salts. Antibiotics can be used to suppress the bacteria and antifungal agents for mould infections in cultures. The recipe of plant tissue culture media is usually directed by plant species, however (MS) / media is the most commonly used media composition in plant tissue culture.

7.4 Culture Types

Plant Tissue Culture may be categorised as organ culture, callus culture, cell suspension culture, protoplast

Box 2

Composition of commonly used Plant tissue culture media (White's and MS Media)

Components	White's	Murashige and Skoog (MS)
	Amount (mg l^{-1})	
Macronutrients		
$MgSO_4 \cdot 7H_2O$	750	370
KH_2PO_4	-	170
$NaH_2PO_4 \cdot H_2O$	19	-
KNO_3	80	1900
NH_4NO_3	-	1650
$CaCl_2 \cdot 2H_2O$	-	440
Micronutrients		
H_3BO_3	1.5	6.2
$MnSO_4 \cdot 4H_2O$	5	22.3
$ZnSO_4 \cdot 7H_2O$	3	8.6
$Na_2MoO_4 \cdot 2H_2O$	-	0.025
$CuSO_4 \cdot 5H_2O$	0.01	0.025
$CoCl_2 \cdot 6H_2O$	-	0.025
Kl	0.75	0.83
$FeSO_4 \cdot 7H_2O$	-	27.8
$Na_2EDTA \cdot 2H_2O$	-	37.3
Sucrose	20	30
Organic supplements		
Vitamins		
Thiamine HCl	0.01	0.5
Pyridoxine (HCl)	0.01	0.5
Nicotinic acid	0.05	0.5
Others		
Myoinositol	-	100
Glycine	3	2

culture, etc. Cultivation of plant organs like roots, anther, ovary, embryo, endosperm, seeds, etc., under laboratory conditions is called **organ culture**. Depending upon the type of organ, the culture is called root culture, anther culture, embryo culture and so on. Various plant parts may induce dedifferentiation and form unorganised mass of cells called callus. This culture is called **callus culture** and is commonly used for plant regeneration and genetic transformation studies.

Single cells can be isolated either from callus or other plant parts by mechanical or enzymatic methods. These single cells can be cultured in the liquid medium and therefore referred to as **cell suspension** culture. Single cells from the cell suspension culture can be used for genetic transformation studies, production of secondary metabolites or induction of somatic embryos and plants.

7.5 Applications of Plant Cell and Tissue Culture

Plant cell and tissue culture are routinely used for several applications in plant science. Fig. 7.2 lists some of the major commercial applications of plant tissue culture.

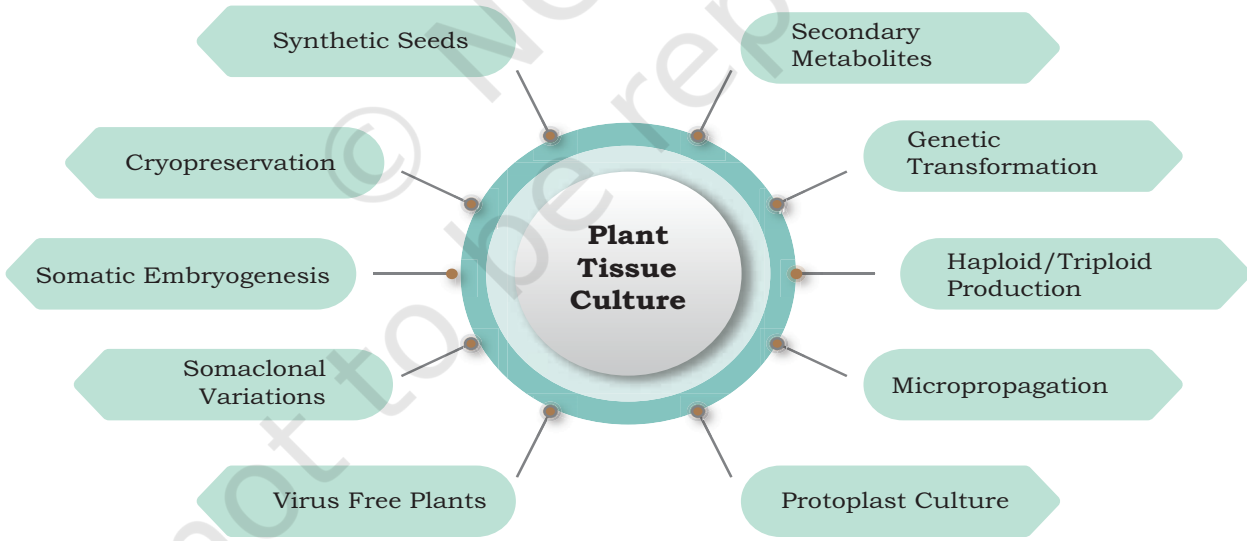


Fig. 7.2: Various applications of plant tissue culture

Micropropagation

It is a tissue culture technique that is used to multiply plants without sexual reproduction or seed formation. Each of the new plant is genetically identical to its parent and can be called clone. Traditionally, it is done by using cuttings, budding, grafting, corms, tubers or other vegetative propagules. However, these traditional procedures are laborious, dependent on environmental conditions and the success rate is also low. Micropropagation can be used to address the above mentioned problems. It results in rapid multiplication of plants within a short period of time in a small space. Since it is performed under controlled environmental conditions, micropropagation is not season dependent. This method is useful for the multiplication of non-fertile plants, rare plants, endangered plants or other plants for which the character of choice cannot be maintained by sexual reproduction (elite plants). Micropropagation has been employed successfully in agriculture, horticulture and forestry like potato, banana, carnation, chrysanthemum, etc. Banana tissue culture technology is very popular for the supply of disease-free quality planting materials and has been proven to be profitable to the farmers. Banana is one of the major tissue culture-raised crops in India. More than 400 million plantlets are regenerated annually through tissue culture.

Artificial (synthetic) seed production

Artificial seeds are also called synthetic seeds or somatic seeds. Using nutrient media with suitable hormones, somatic embryos are produced on the callus tissue. They are produced by artificial encapsulation of the somatic embryos with a protective coating of suitable matrix, like calcium alginate. In addition, nutritional components and growth regulators are also added, which provide nutrition for the embryos and mimic the structure of the conventional seed (Fig. 7.3). Suitable explants are selected for establishing callus culture.

Artificial seeds can be stored for a long time in suitable conditions and can be utilised for the rapid and mass propagation of elite plants species, as well as hybrid varieties. Artificial seed production is also helpful for asexually propagating plants. For example, artificial seed production has been successfully used in carrot, grapes, sandalwood, etc.

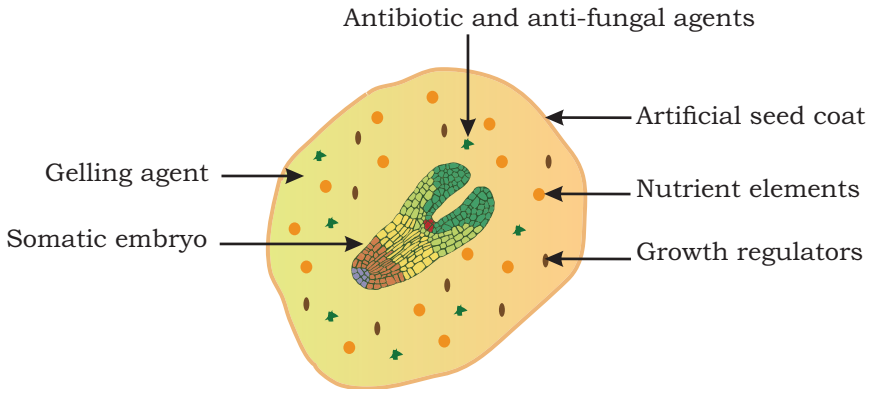


Fig. 7.3: Schematic representation of an artificial seed

Haploids or triploid productions

Unlike diploid plants, which have two sets of chromosomes (2n), haploid plants have only one set of chromosomes, like the number of chromosomes in the gametes of a diploid plant. Haploids are used to produce genetically homozygous diploid plants by doubling the chromosome number through the colchicine application. These haploid plants, after doubling their chromosome number, are called double haploids. This enables the recessive traits to express too. These homozygous diploid plants are frequently used in cross breeding. They can be produced through anther culture, pollen culture or ovary culture. Many varieties of broccoli, brassica, sorghum, rice and tobacco are a few examples.

Somatic hybrids

Sexual reproduction involves the fusion of haploid gametes to produce zygote, which develops into a new individual. However, it is limited to similar individuals as inter-specific or inter-generic crosses do not occur because of species barrier. Therefore, good characters from one species cannot be transferred to another species through sexual reproduction. Somatic hybridization provides an excellent alternative for obtaining hybrids from distantly related species.

Essentially, the plant cell wall is digested with enzymes like cellulases and pectinases, and protoplasts are isolated. When brought in close contact, these protoplasts tend to fuse with each other with help of poly ethylene glycol (PEG)

irrespective of the source of protoplasts. This method of fusion of protoplasts from somatic cells is called **somatic hybridisation**. Resulting fusion products are regenerated to form hybrid plants and called somatic or parasexual hybrids (Fig. 7.4). Inter-specific somatic hybrids were generated for the first time in 1972 by Carlson and his associates. Since then, several somatic hybrids have been produced in a number of crops like potato, rice and brassica.

Several traits are controlled by genes located in the cytoplasmic organelles, like cytoplasmic male sterility is controlled by mitochondrial genome whereas, chloroplast encodes many key proteins involved in photosynthesis. In sexual reproduction, cytoplasmic genomes are mostly contributed by the female parent. However, protoplast fusion provides an opportunity to combine nuclear genome of one parent with mitochondrial or plastid genome of another parent. This process of combining cytoplasmic genomes of one parent with nuclear genome of the other parent is called **cybridisation** and resulting hybrids are called **Cybrids** (Fig. 7.4).

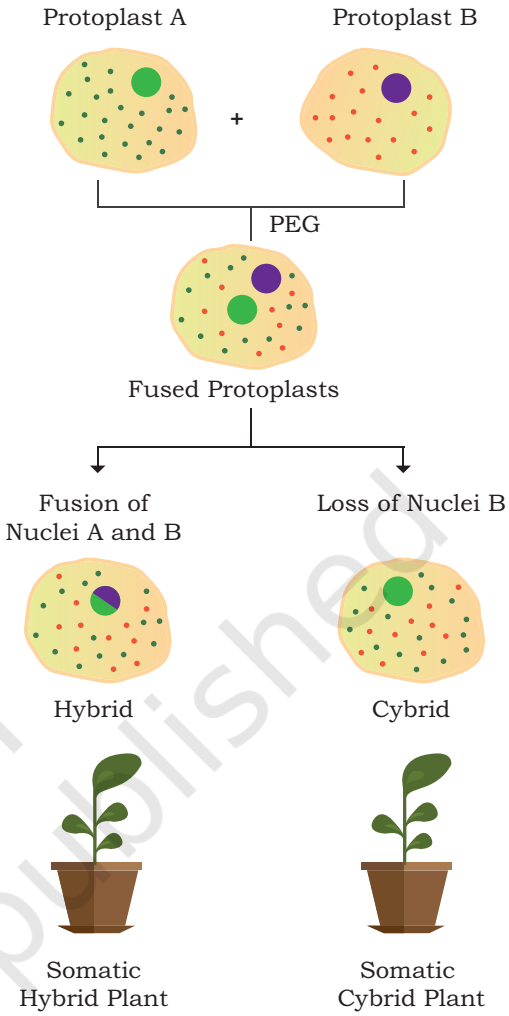


Fig. 7.4: Schematic representation of protoplast fusion and development of somatic hybrid or somatic cybrid plants. Protoplast A and B are from two different plant species

Production of virus-free plants

Most of the crop plants are infested with viruses that result in huge losses in terms of productivity and yield. For vegetatively propagated plants, virus infection is a serious problem, as the entire clonal population raised from infected stocks will also be infected with viruses. Therefore, production of virus free plants is important to maintain the yield and quality of vegetatively propagated plants. Interestingly, these virus particles have uneven distributions throughout the plant body. Generally apical or axillary meristems are free from virus particles. Therefore, very small part of apical meristems (less than 1 mm long) can be used as explant to initiate cultures for the production of virus free plants. This method is important and has been successfully used for clonally propagated crops, like sugarcane, banana and potato.

Somaclonal variations (Genetic variability)

Terminally differentiated somatic cells in plants accumulate genetic variations. As these are not associated with germline cells, these variations are not transferred to next generations. However, when these somatic tissues having genetic variations are used as explant for tissue culture, these variations are passed to and are expressed in newly regenerated plants. In addition, long-term *in vitro* callus and cell suspension cultures may introduce genetic variations, which are also expressed in newly regenerated plants. Therefore, the genetic variations observed in plants regenerated from cultured somatic tissues are called **somaclonal variations** and such regenerated plants are known as **somaclones**. These variations can be used to engineer novel traits and have been utilised for crop improvement.

The somaclonal variations can be both, a boon and a bane. If the objective of *in vitro* tissue culture is clonal multiplication of the plant with a specific trait, these variations can result in unwanted traits and compromise the commercial value of the clones. However, somaclones may be screened for novel phenotypes of agronomic or commercial advantages. Agronomically important somaclones have been identified in several crops and have been released as new cultivars. Some of the examples are disease resistant sugarcane, banana and tomato, and improved yield in wheat.

Production of secondary metabolites

Plants produce thousands of specialised compounds that are not necessary for its survival, but are important for plant's interaction with its environment. These compounds (alkaloids, flavonoids, tannins, steroids, latex, resins, etc.) are called secondary metabolites, and are often involved in plants, defense against biotic and abiotic stress conditions. These compounds are economically valuable products and have several industrial applications, such as drugs, flavours, dyes, insecticides, fragrances, etc. Plant cell or tissue-based cultures can be a potential source of specific secondary metabolite and are routinely used to synthesise metabolite of interest. Hairy root system produces high-quality secondary metabolites and is routinely used in the industry. As tissue cultures are maintained

under controlled conditions, metabolite can be produced independent of variations caused by environment, season or disease.

Many of the metabolites like alkaloids are of immense use in medicine. These compounds are produced in very small amounts in plants and therefore, overharvesting of these plants has brought them to near extinction. Further, these compounds are very expensive. It is also known that cultured cells or tissues can accumulate phytochemicals at levels, which are higher than their parent plants. Therefore, cell or tissue culture-based production of secondary metabolites of industrial importance can be a possible solution and can make it affordable for common man too. Many such compounds have been produced in cell or tissue culture-based systems and a few are listed in Box 3.

Box 3

Examples of industrially important secondary plant metabolites produced through cell and tissue cultures

S. No.	Product Name	Plant Source	Uses
1.	Artemisin	*Artemisia sp.*	Antimalarial
2.	Azadirachtin	*Azadirachta indica (Neem)*	Insecticidal
3.	Berberine	*Coptis japonica*	Antibacterial, anti-inflammatory
4.	Capsaicin	*Capsicum annum (Chilli)*	Rheumatic pain treatment
5.	Codeine	*Papaver sp.*	Analgesic
6.	Digoxin	*Digitalix lanata*	Cardiac tonic
7.	Diosgenin	*Dioscorea deltoidei*	Antifertility
8.	Scopolamine	*Datura stramonium*	Antihypertensive
9.	Quinine	*Cinchona officinalis*	Antimalarial
10.	Shikonin	*Lithospermum erythrorhizon*	Antimicrobial
11.	Taxol	*Taxus sp.*	Anticarcinogenic
12.	Vincristine	*Cathranthus roseus*	Anticarcinogenic

SUMMARY

- Plant tissue culture (PTC) refers to the cultivation of undifferentiated mass of plant cells, tissues or organs on artificial media under aseptic and controlled environmental conditions.
- Any plant organ like leaf, apical meristem, embryo, cotyledon, hypocotyl, etc., can be used as an explant and whole plants can be regenerated *in vitro.*
- Plant tissue culture media used for *in vitro* cultures is mainly composed of inorganic and organic supplements, carbon source, plant growth hormones, vitamins, gelling agents, antibiotics, etc.
- Tissue culture can be categorised as organ culture, explant culture, callus culture, cell suspension culture, protoplast culture or single cell culture.
- Plant tissue culture is routinely used for several applications in plant science, such as in micropropagation, synthetic seed formation, protoplast culture, haploid or triploid culture, virus free plants production, secondary metabolites production, etc.
- Plant growth hormones play a vital role in plant tissue culture especially, different ratios of auxin and cytokinin are employed for either root or shoot regeneration depending upon the need or objective.
- Somatic hybridisation in plant tissue culture can be exploited to produce distantly related plants as well.
- Cultured cells or tissues may accumulate a higher concentration of secondary metabolites than its parents, under optimum environmental and nutritional conditions.
- Several compounds of industrial importance have been successfully produced in tissue culture, like taxol, azadirachtin, shikonin.

EXERCISES

1. What is plant tissue culture?
2. Describe the various components of plant tissue culture media.
3. What are the general steps of plant tissue culture?
4. Describe various applications of plant tissue culture.
5. How are somatic hybrids developed?
6. What are somaclonal variations?

7. Define explant and list five most commonly used explants for plant tissue culture.
8. Describe somatic embryogenesis and their application for the development of synthetic seeds.
9. Describe briefly the role of pH in nutrient media.
10. Describe the method of somatic hybridization and its advantages.
11. What are somaclonal variations and discuss their role for improving crops.

Multiple Choice Questions

12. Which of the following tissues can be used as explant for regenerating complete plant through tissue culture?
    (a) Shoot apical meristem
    (b) Embryo
    (c) Leaf segments
    (d) All of the above
13. Which of the following explants are suitable for the production of virus free plants?
    (a) Leaf segments
    (b) Seeds
    (c) Apical meristem
    (d) Stem cuttings
14. The process of combining the nuclear genomes of one parent with the cytoplasmic genome of the other parent is called as:
    (a) Cybridization
    (b) Micropropagation
    (c) Regeneration
    (d) None of them
15. Which of the following components is not essential for Murashige and Skoog media?
    (a) Inorganic nutrients
    (b) Carbon source
    (c) Antibiotics
    (d) Organic Nutrients
16. Decrease in the pH of the media may result in:
    (a) Increase in hardness of the solidified medium.
    (b) May interfere with the solubility of media salts.
    (c) Interfere with solidification of the medium and results in poor solidification.
    (d) All of the above.

17. Somatic clonal variation can be present in which of the following plants?
    (a) Plants regenerated through tissue culture
    (b) Plant generated through seeds
    (c) Plant generated through sexual reproduction that includes fertilisation of egg with pollen nuclei.
    (d) None of the above.
18. *In vitro* tissue culture can be used for the generation of:
    (a) Virus free plants
    (b) Somatic hybrid plants
    (c) Synthetic seeds
    (d) None of the above.
19. **Assertion:** Somatic seeds are encapsulated by a layer called seed coat.

    Reason: Seed coat is the protective layer that prevents water desiccation.
    (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.
    (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.
    (c) Assertion is true but reason is false.
    (d) Both assertion and reason are false.
20. **Assertion**: Virus free plants can be produced by growing apical/axillary meristem of virus infected plants.

    Reason: Apical/axillary meristems lack vascular bundle that is required by the virus to replicate.
    (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.
    (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.
    (c) Assertion is true but reason is false.
    (d) Both assertion and reason are false.

Chapter 8 Animal Cell Culture

Animal cell culture is described as the *in vitro* maintenance and proliferation of animal cells that will continue to grow outside the living organism if supplied with appropriate nutrients and growth conditions. The process of growing cells under laboratory conditions is termed as Cell Culture. It is carried out *in vitro* as opposed to *in vivo* (within the living). It deals with the isolation of cells from animal tissue, surgical intervention for removal of tissues or organs from an animal and their placement into an environment (media) in order to enhance their survival and proliferation.

A homogenous population of cells is termed as a clone, when it is derived from a single parental cell. Therefore, all cells within a clonal population are genetically identical. The growth rate of animal cells is relatively slow and usually requires 18 to 24 hours to divide. This makes animal cell culture vulnerable to contamination, as a small number of bacteria would soon outgrow a larger population of animal cells.

Box 1

Immortal cells of Henrietta Lacks

An African–American woman named Henrietta Lacks was diagnosed with terminal cervical cancer in 1951. She was treated at John Hopkins University by a doctor named George Gey who snipped cells from her cervix without her permission. Gey discovered that Lacks' cells could not only be kept alive, but also could be grown indefinitely.

For the past many years, 'Lacks' cells have been cultured and used in various experiments ranging from determining the long-term effects of radiation to testing the live polio vaccine. Her cells were commercialised and have generated millions of dollars in profit for the medical researchers who patented her tissue.

Essential requirements for optimal growth of cells are regulated temperature, proper substrate for an attachment of cells on appropriate growth medium and an incubator that maintains the correct pH and defined osmolality. Cell culture helps to study the basis of regulation of cell proliferation, differentiation, and product formation in controlled conditions and therefore, has gained a dominant position in many branches of the life science research. This technology has now emerged as a tool in the area of molecular genetics, immunological analysis, surgery, bioengineering, and pharmaceutical industry.

8.1 Historical Perspective

The animal cell culture became a routine laboratory technique in 1950s after George Gey established the first human cell line (HeLa) from cervix cancer of the patient, Henrietta Lacks, that led to several important discoveries in medical sciences. The need for cell culture, especially at large scale, became apparent with the need for viral vaccines.

The cell culture technologies have been used in various areas, including the assessment of the efficacy and toxicity of new drugs, manufacture of vaccines and biopharmaceuticals, etc. With the development of cell culture technology, a variety of culture media have been designed. The culture medium supports cell survival and proliferation, as well as cellular functions.

Growth factors, such as nerve growth factor, epidermal growth factor, insulin-like growth factor, fibroblast

growth factor (FGF), platelet-derived growth factor, and transforming growth factor (TGF) were discovered one after another and their addition led to increased cellular proliferation. In 1976, the development of serum-free media was accelerated.

8.2 Culture Media

The most significant and critical stage in cell culture is the selection of a suitable growth medium for their proper *in vitro* culture. Appropriate media selection will depend on the kind of cells to be cultured and on the requirement for culture, such as growth, differentiation and production of desired products like pharmaceutical compounds. A typical culture medium contains a complement of vitamins, amino acids, glucose, inorganic salts, serum (as a source of growth factors) and hormones. Additionally, medium helps to maintain the pH and the osmolality. Media can be natural consisting of natural biological substances, like plasma, serum and tissue extract or artificial/synthetic composed of a basal medium with supplements such as hormones, growth factors, serum etc.

Media supplements

As you know, the culture media contains a combination of amino acids, salts, glucose, vitamins and supplemented with other nutrients. The requirements for these components is based on the cell lines that are to be cultured and thus, there are extensive number of media formulations available.

Some additional components (hormones, growth factors, and signaling substances), which are not present in the basal media and serum, are required that help in the proliferation and maintaining normal cell metabolism.

Serum is one of the most important components of cell culture media. Serum is considered a good source for amino acids, proteins, vitamins, carbohydrates, lipids, hormones, growth factors, etc. Serum provides several binding proteins, like albumin, transferrin, which can carry other molecules into the cell. In addition, serum also supplements adhesion factors that help the cells to adhere to substratum before they begin to divide. Fetal bovine serum is commonly used to support the growth of cells in culture.

Box 2 Historical perspective of animal tissue culture

Name	Year	Breakthrough
Sydney Ringer	1882	Balanced salt solution with a composition similar to that of body fluids and kept frog hearts after dissection and removal from the body
Roux	1885	Medullary plate of chick embryo in warm saline
Jolly	1903	*In vitro* cell survival and cell division of salamander leucocytes
Ross Harrison	1907	Published experiments showing frog embryo nerve fibre growth *in vitro*
Lewis and Lewis	1911	• Cultured connective tissue cells for extended periods and showed heart muscle tissue contractility over 2–3 months • The first liquid media consisted of sea water, serum, embryo extracts, salts and peptides
Alexis Carrel	1912	Aseptic techniques to tissue culture. Use of trypsin, embryo extracts/animal serum
Rous and Jones	1913	Use of antibiotics: penicillin/streptomycin
	1916	Use of laminar air-flow cabinets
	1940	Trypsinization was used to produce homogenous cell types; tissue culture media
Katherine Sanford, et al.	1940s–50s	Were the first to clone mouse L-cells. Tumor cells could give rise to continuous cell lines. Used non-malignant rodent cell culture to study the effects of carcinogens/viruses.
Margaret Gey and George Gey	1948	Observed contact inhibition among fibroblasts — the beginning of quantitative cell culture experimentation
Abercrombie and Heaysma	1952	Polio virus in human E-cells; production of polio vaccine
Enders, et al.	1954	Human cell lines for the production of vaccines — human and veterinary
Hayflick and Moorhead	1955	Described the finite lifespan of normal human diploid cells.
	1961	Published the methods for maintaining differentiated cells (of tumor origin)
Harry Eagle	1962	Developed defined media
	1970	Described attachment factors and feeder layers
Buonassisi, et al.	1962	Studied the differentiation of normal myoblasts *in vitro*
Littlefield	1964	HAT selection
David Yaffe	1968	Human foetal lung fibroblasts
Kohler and Milstein	1975	First hybridoma capable of screening a monoclonal antibody

Box 3 Various types of media

Category	Definition	Type	Description	Disadvantages/ Advantages
Natural media	Consisting of natural biological substances, such as plasma, serum, and embryo extract	Coagulant or clots	Plasma separated from heparinised blood, serum and fibrinogen	The greatest disadvantage of natural media is poor reproducibility and reduced uniformity because the exact composition of these natural media are not known.
		Tissue extracts	Extracts of chicken embryos, liver, spleen, and bone marrow	
		Biological fluids	Plasma, serum, lymph, amniotic fluid, and pleural fluid	
Synthetic media or Artificial media	Composed of a basal medium and supplements, such as serum, growth factors, and hormones	Serum-containing media	Human, bovine, equine, or other serum is used as a supplement	The quality of serum varies from batch to batch and deteriorates within one year. Therefore, every batch of serum needs fresh testing.
		Serum-free media	Crude protein fractions, such as bovine serum albumin or α- or β-globulin, are used as supplements	It has the ability to make a medium selective for a particular cell type, since each cell type appears to require a different recipe.
		Xeno-free media	Human-source components, such as human serum albumin, are used as supplements but animal components are not allowed as supplements	
		Protein-free media	Undefined components, such as peptide fractions (protein hydrolysates), are used as supplements	
		Chemically defined media	**Basal media** Phosphate buffered saline (PBS), Dulbecco's phosphate buffered saline (DPBS), Hank's balanced salt solution (HBSS), Earle's balanced salt solution (EBSS)	Balanced salt solution (BSS) is composed of inorganic salts that maintain the physiological pH and osmotic pressure. The physiological role played by the inorganic ions is to maintain the membrane potential.

			Complex media • Eagle's Minimum Essential Medium (EMEM) • Dulbecco's Minimal Essential Medium, • Roswell Park Memorial Institute (RPMI-1640), • Ham's Nutrient Mixtures (Hams's F-12)	They also work as cofactors in enzyme reaction and in cell attachment. Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, $S0_2^{4-}$, SO_4^{-2} PO_4^{-3} and HCO_3^- are the inorganic molecules involved. To maintain the osmolality, concentration of NaCl is generally adjusted.

However, there are certain disadvantages of serum in media:

- Serum contains insufficient amount of cell specific growth factors and thus, cannot be used as stand alone for culture.
- It may also contain cytotoxic compounds and some growth inhibiting factors that will cause the inhibition of the cultured cell growth and proliferation.
- Serum may contain high risk of contaminants, like various virus, fungi and mycoplasma.
- The serum in the culture media may interfere with the purification and isolation of cell culture products including pharmaceutical compounds and thus, some additional approaches are applied for the isolation of cell culture products.

Even though not essential for the growth of cells, use of antibiotics such as penicillin and streptomycin, is common in culture medium to control the growth of contaminants such as bacteria and fungus.

8.3 Physical Environment for Culturing Animal Cells

Animal cell culturing under *in vitro* conditions involves simulating suitable micro-environments, nutritional, physical, and hormonal conditions to enable optimal growth of cells. Therefore, controlling the temperature, osmolality, pH, gaseous requirements, a supporting surface and giving protection to the cells from physical, chemical and mechanical stresses are needed.

Temperature

CO_2 incubators are used to grow mammalian cells at 37°C, as this temperature simulates the body temperature of *Homo sapiens*. Moreover, most of the cells have been derived from the warm-blooded animals that will grow optimally at this temperature.

Osmolality

Osmolality of the medium has substantial effect on cell growth and function. It maintains the integrity of the cell membranes. If the outside osmotic pressure becomes lower or higher than the pressure that is maintained inside the cell, it will swell or shrink accordingly. The osmolality of the medium used is determined by the formulation of media. Both, glucose and salt are the major donors to the osmolality of the medium, even though amino acids also contribute significantly. Generally, all the commercial media are formulated so as to have a final osmolality of approximately 300 mOsmol. Osmolality can be measured by an osmometer.

Buffering systems

For optimum culture conditions, the regulation of pH is critical and is achieved by one of the following buffering systems:

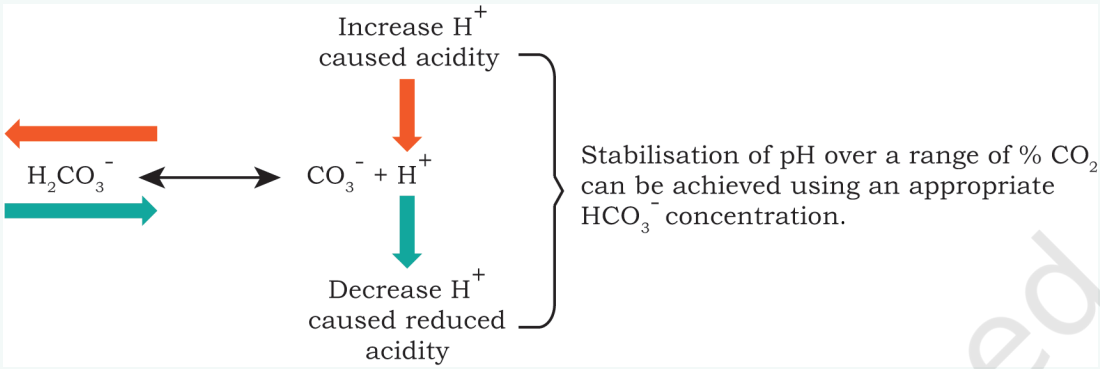
(A) Natural buffering system

In a natural buffering system, gaseous CO_2 balances with the CO_2/HCO_3^- content of the culture medium. The concentrations of CO_2, HCO_3^-, and pH are interrelated. By increasing the external/exogenous CO_2, the pH will be reduced making the medium acidic.

The culture media with a natural buffering system need to be maintained in air with 5 –10 percent CO_2, which is usually maintained by a CO_2 incubator. Natural buffering system is low cost and non-toxic. The presence of pyruvate in the medium results in the increased endogenous production of CO_2 by the cells. Thus, the use of pyruvate is advantageous because of less dependency on the exogenous supply of CO_2.

Box 4

Bicarbonate buffering follows Le Chatelier's principle. Increased acidity in the medium is established by an increase in Hydrogen (H^+) ions; free bicarbonate ions then react with the extra H^+ ions to form carbonic acid 'shifting the reaction to the left', stabilising pH. Similarly, a decrease in H^+ ions will result in a 'shift to the right'.



(B) Chemical buffering system

In the chemical buffering system, a zwitter ion, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), is used that has a superior buffering capacity in the pH range 7.2–7.4, and with HEPES, a controlled gaseous atmosphere is not required. It is relatively expensive and toxic at a higher concentration for some cell types.

Phenol red

Phenol red is generally used in commercial media as a pH indicator. This is effectively used to monitor the pH change owing to the metabolic activities during cell growth. Due to the presence of phenol red, the medium changes its colour as pH changes. At acidic pH levels, phenol red turns the medium yellow, while at alkaline pH levels, the medium turns pink. Medium is bright red at optimal pH—7.4. (Fig. 8.1)

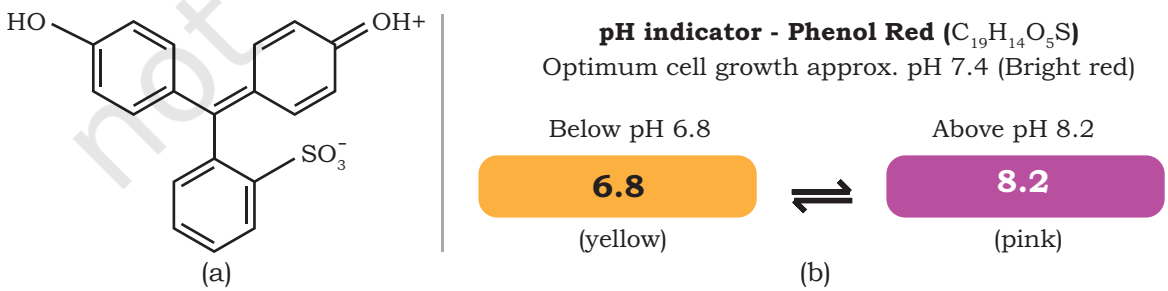


Fig. 8.1: (a) Structure of phenol red, (b) Phenol red as pH indicator

8.4 Equipment Used for Cell Culture

Cultures should be maintained and examined daily for the colour of the medium to understand their morphology and density of cells that are grown in culture flasks, Petri dishes or multi-well plates of several sizes and shapes (Fig. 8.2) at an appropriate temperature, humidity and gas mixture (typically, 37°C, 95% and 5% CO_2 for mammalian cells) in an incubator respectively. Culture conditions differ extensively for each cell type, and these variations in conditions for any particular cell type may result in the expression of altered phenotypes.

(a) (b)

Fig. 8.2: (a) Culture flask, microplates and Petri dishes, (b) Cells scraper

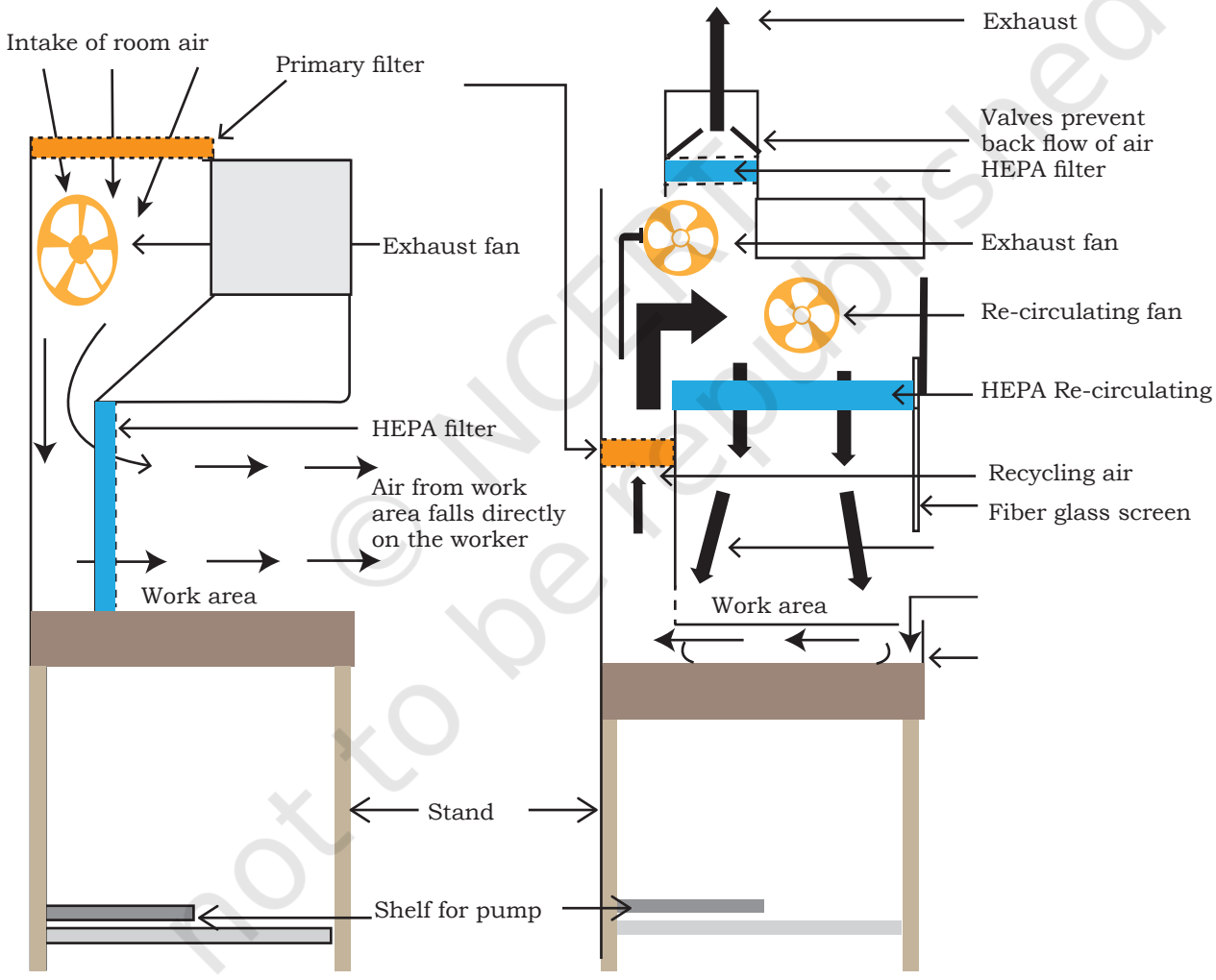
An ideal animal cell culture laboratory should have equipment like laminar hood, CO_2 incubator, an inverted microscope, autoclave, centrifuge, etc. They are discussed in the following sections.

Laminar Flow Hood or biosafety cabinets

The entire work of animal tissue or cell culture should be done aseptically in the laminar hood. A laminar flow cabinet provides controlled filtered air in which the levels of particulates, microbes and contamination of all kinds are regulated and kept to a minimum by constant air filtration with industrial-grade HEPA (High Efficiency Particulate Air) filters. Laminar hoods operate by drawing air under negative pressure, into the top of the unit. This air initially passes through a pre-filter, which traps fungi, bacteria and dust particles. Air passed through this pre-filter is continuously fed by fans/ blowers through HEPA

filters into the enclosed cabinet, creating a sterile work area for the operator. The laminar flow chamber is usually enclosed on both sides and kept under constant positive pressure to prevent the inflow of contaminated room air.

In Vertical laminar flow Fig. 8.3(b), the sterilised air is passed vertically down on the work area - vertical (down flow). This is considered to be safer as the air does not blow the directly towards the person carrying out the experiments. In the horizontal flow hood, air is directed towards the person doing the experiments [Fig. 8.3(a)]. Hence, hence horizontal (crossflow) are not popular as



(a) Horizontal laminar flow (b) Vertical laminar flow

Fig.8.3: Laminar Flow Hoods (a) Horizontal (b) Vertical

they offer no protection to the operator from the potential hazards of some of the cultured cells.

CO_2 Incubators

Incubators for cell culture work depend on an external supply of CO_2 so as to maintain a fixed level of CO_2 in the incubator. To maintain sterile handling area and cleanliness, the CO_2 cylinder should be kept outside the laboratory and the gas should be piped through. The essential functions of the incubator are to maintain—the sterility of the chamber, a constant temperature, an atmosphere with a 5–10% level of CO_2, and high relative humidity (~95%) (Fig. 8.4). The medium is further buffered with sodium bicarbonate/carbonic acid and the pH must be strictly maintained.

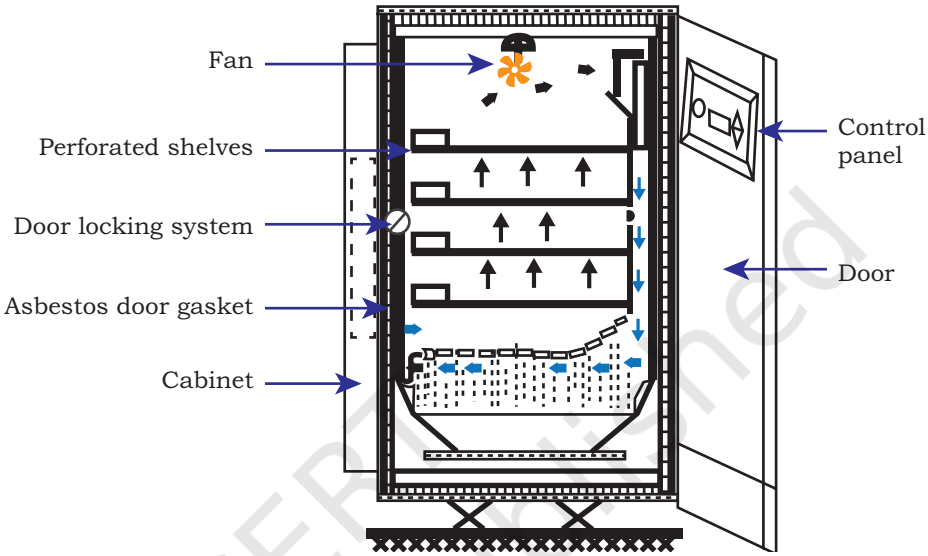


Fig. 8.4: CO_2 incubator

Inverted microscope

A cell culture laboratory essentially requires a good quality inverted microscope with phase-contrast optics and preferably a photographic facility (Fig. 8.5). The morphology of cell, granularity, degree of spreading, membrane blebbing, the proportion of multi-nucleates, vacuolation, and so on, can be regularly monitored for understanding the signs of stress in cells. Cell morphology is sensitive to the alterations in culture conditions hence, problems can be easily detected. Early indications of a microbial contamination can also be spotted with a good phase-contrast microscope. Regular checking of cultures under the microscope can help to avoid the loss of irreplaceable material by allowing a problem to be noticed at an early stage. Also, experiments done on unhealthy cells may give

variable or erroneous results. When choosing a microscope, select the long or extra-long working distance condenser so that the flasks and even roller bottles can be viewed. Normally a 20x magnification objective is sufficient; their depth of field is often too low to obtain a sharp image of all but the most flattened. A good, low-power, wide-field objective is extremely useful for scanning culture colonies.

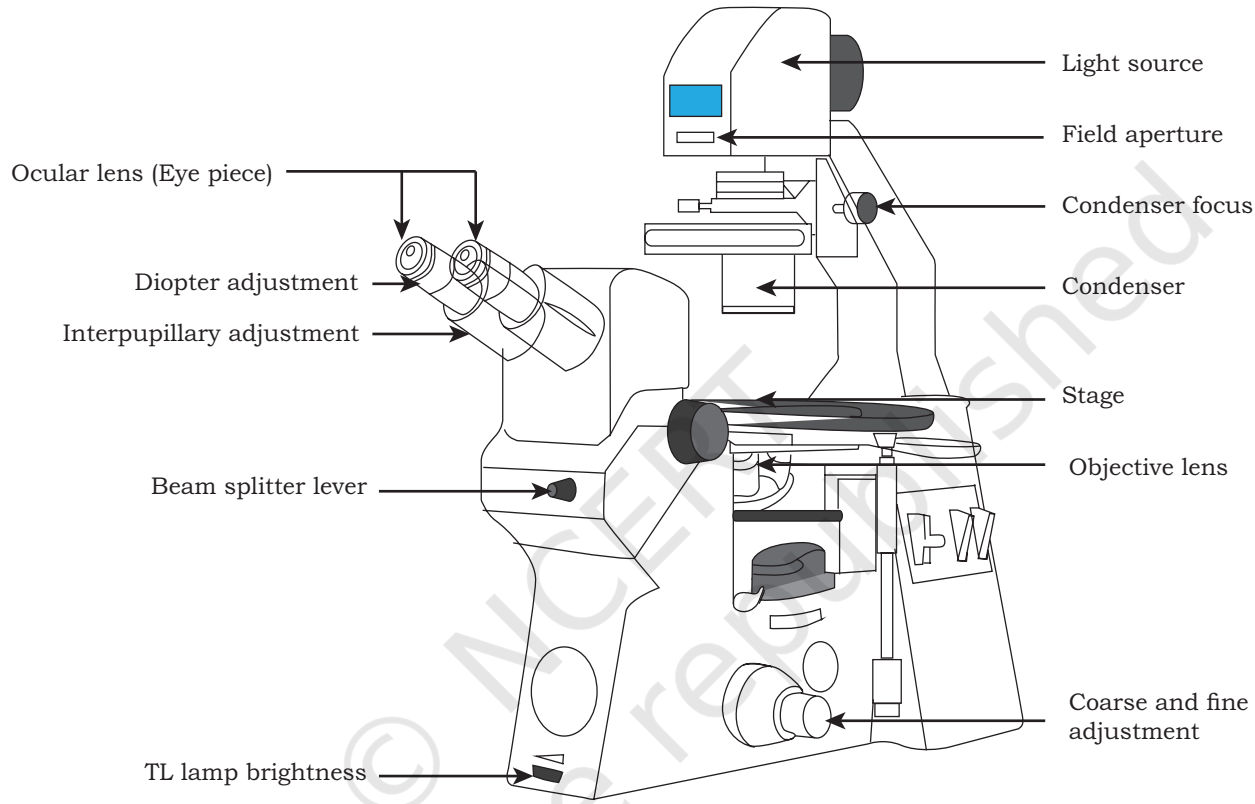


Fig: 8.5: Inverted microscope

8.5 Types of Animal Cell Cultures and Cell Lines

The animal cell cultures and cell lines are broadly classified as primary and secondary cell cultures and cell lines.

(i) Primary cell cultures

Primary cell culture is obtained directly from the host tissue. The cells dissociated from the parental or host tissues are grown in a suitable culture media in a container and thus, obtained culture is called primary cell culture.

This type of culture contains mostly heterogeneous cells and these cells are similar to their parents and is called primary cell lines. For the preparation of primary cultures and cell lines, a tissue is dissected in sterile conditions followed by mechanical or enzymatic disaggregation. The tissue can simply be chopped and the pieces attached to a dish. Now cells will grow out from the fragment and may be used directly for culture. The fragment of tissue is called explant that may be transferred to a fresh dish. Primary cultures can also be made by disaggregating tissue with the help of enzymes, such as trypsin or collagenase, etc., and the cell suspension are allowed to adhere, and spread out on the substrate. Cells may grow as adherent cultures or may remain in suspension.

(ii) Secondary cell culture

When a primary culture is sub-cultured or passaged, the resulting culture is called secondary cell culture. During sub-culturing, a portion of cells are transferred to a new vessel having fresh growth medium for continual growth of cells. Sub-culturing of primary cells leads to the generation of secondary cell lines. During the passage, the cells with highest growth capacity predominate, leading to genotypic and phenotypic uniformity in the population. As the cells are sub-cultured at regular intervals, they may become different from the original cell.

On the basis of the culture life span, the cell lines are categorised into two types:

(a) Finite cell lines

The cell lines with a limited number of cell division and having a limited life span are called finite cell lines. As the cells pass several times, they lose their ability to proliferate (i.e., **senescence)**. Cell lines derived from primary cultures of normal cells are finite cell lines.

(b) Continuous cell lines

Some cells of the finite cell line undergo transformation and resume the ability to divide indefinitely, and become a continuous cell line. This type of transformation or mutation can occur spontaneously or can be chemically or virally induced. Cell cultures prepared in this way can be sub-cultured and grown indefinitely as permanent cell lines

and are immortal and tumorigenic. The continnous cell lines are less adherent and can also grow in suspension; they become fast growing, less demanding for nutrients and are able to grow up to higher cell density.

Adherent cells

These cells are anchorage dependent and proliferate as a monolayer and they require the attachment to a solid or semi-solid substrate for proliferation. These cells adhere to the culture vessel with the help of extracellular matrix, usually derived from tissues that are immobile and present in the network of connective tissue, (e.g., fibroblasts and epithelial cell types). As the bottom of the culture vessel is covered with a continuous layer of cells with one cell in thickness, known as monolayer cultures. A majority of continuous cell lines grow as monolayers. As they are single layers, such cells can be transferred directly to a coverslip to be examined under microscope.

Suspension cells

Suspension cells, also called anchorage-independent or non-adherent cells, are not attached to the surface of the culture vessels and float in the culture medium. Hematopoietic stem cells (derived from blood, spleen and bone marrow) and tumor cells are the examples of suspension cells. These cells grow faster, as they do not require frequent replacement of the culture medium and are homogeneous in nature.

Subculture

After the isolation of cells and subsequent culture, the cells can proliferate under appropriate conditions and these adhere to the available substrate to reach confluence. After a few days, the cells form a monolayer and overgrow becoming crowded, and this can be unfavourable to their growth, leading to cell death. At this point, the cells need sub-culturing or passaging, which is the process of using enzyme, e.g. trypsin, to enable the cells to detach from the old medium. It is then removed and the cells are transferred to a fresh flask after centrifugation and addition of fresh media. This is commonly termed as 'splitting' as the cells of one flask are equally split into two flasks for culturing.

A passage number refers specifically to the number of times a cell line has been sub-cultured. For the passaging of adherent cells, first all the cells are detached/dissociated from the surface of the culture vessel by enzymatic activity (trypsin, trypsin + EDTA, etc.) or mechanical means (using cell scraper, shaking, vigorous pipetting, etc.) and after that they are transferred into fresh media.

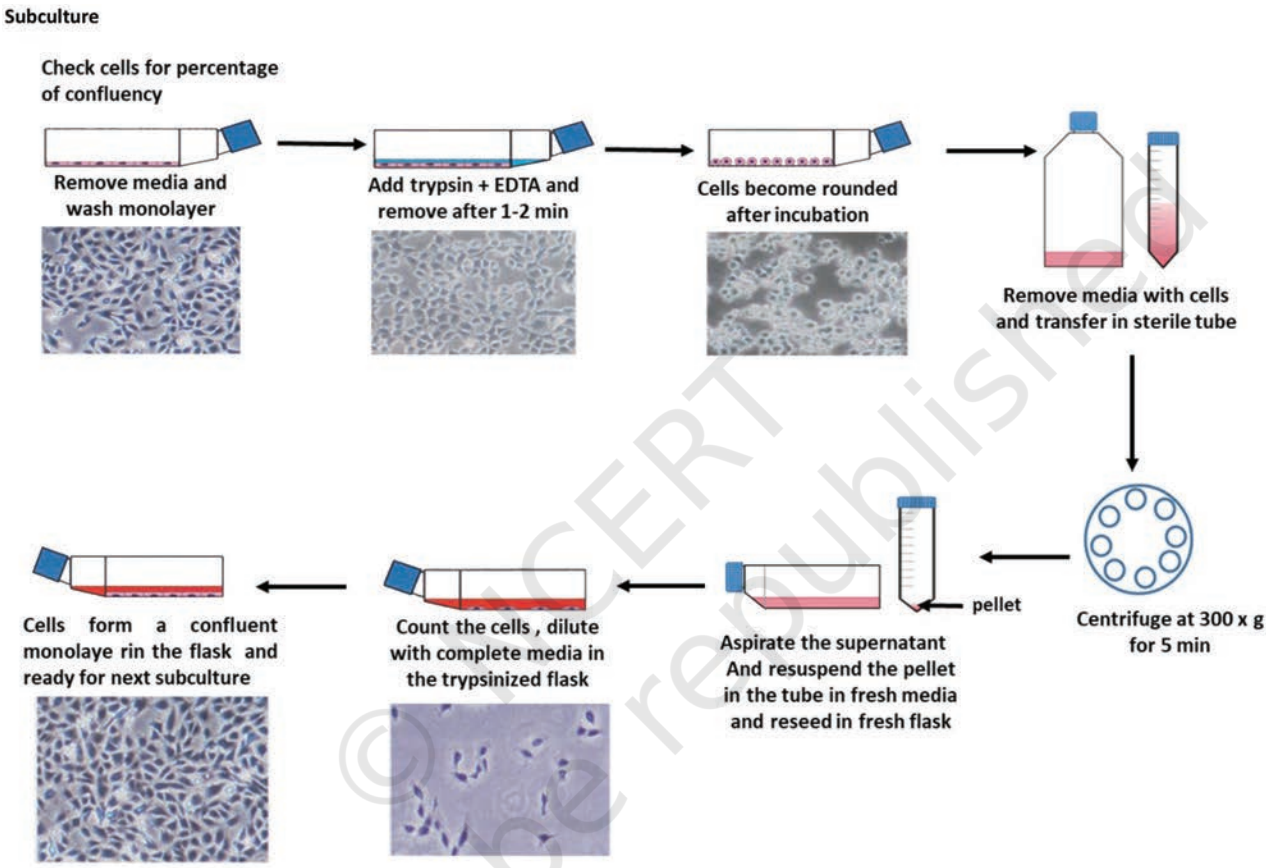


Fig: 8.6: Step involved in Subculture/Passage

Cryopreservation

Very low temperatures (–180 to –196°C) are required to store cells. Liquid nitrogen is used for freezing cells at low temperatures as the formation of ice crystals is reduced below –130°C. Freezing can cause cell mortality due to the damage caused by the formation of ice crystals, alterations in electrolyte concentration, dehydration, and pH changes. To nullify the effects of freezing, glycerol or Dimethyl

sulfoxide (DMSO) as cryoprotective agents are added. Typically, the freezing medium contains 90% serum and 10 percent DMSO. Preferably, healthy cells growing in log phase should be used and the medium should be replaced 24 h before freezing. Also, the cells should be slowly cooled from room temperature to –80°C to allow the water to move out of the cells before they freeze. Frozen cells should be thawed as rapidly as possible by placing the vial containing them into a 37°C water bath with moderate shaking. This is to minimise ice and crystal formation, which may damage cells. After thawing, the cells can be transferred directly into a tissue culture vessel containing suitable media for further growth.

8.6 Cell Viability Determination

The measurement of cell viability, i.e., the determination of living, plays a vital role in cell culture. The cell viability measurements can be applied to evaluate the live status of culture cells. This is required to determine the effectiveness of a pesticide or insecticide, to evaluate the damage due to toxins as well as to evaluate the potential of a drug, etc. Usually, the cell viability assay or test should be performed at regular intervals to check whether the cells are alive or dead. Following are the two types of viability assay:

(a) Dye exclusion viability assays

In this process, a dye or stain is used that enters the cell and usually intercalates with the DNA in the nucleus. The internalisation of the dye into the cell indicates loss of cell membrane integrity that causes cell death. In other words, live cells exclude the dye, while dead cells allow the dye to enter hence, it is called dye-exclusion assay. Some examples of dye or stains are trypan blue (Fig. 8.7), propidium iodide, 7-aminoactinomycin D (7-AAD), acridine orange, etc.

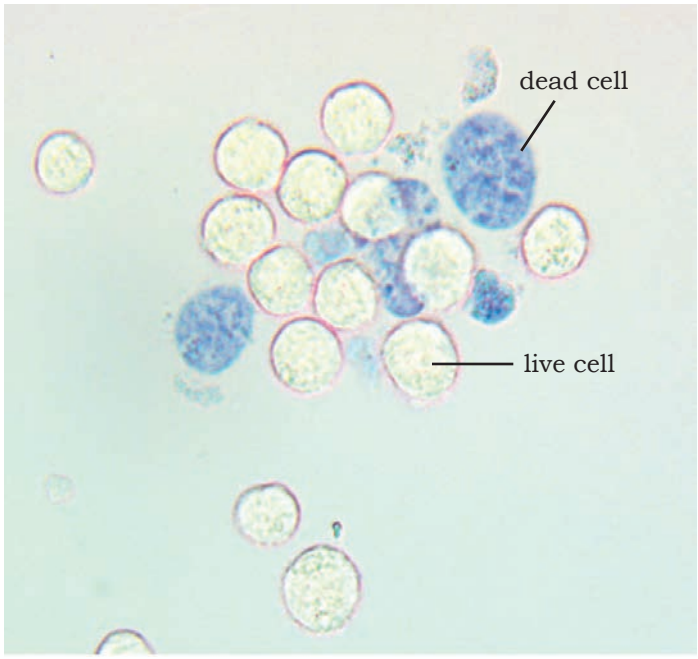


Fig.8.7: Cell viability by trypan blue exclusion assay (20X magnification)

(b) Metabolic viability assays

Metabolic viability assays rely on the ability of cells to execute a specific biochemical reaction that can be measured by various means including absorbance, fluorescene or luminescence methods (Fig. 8.8). These methods are developed to estimate the cell number based on the cellular content of enzyme or substrate and subsequent extraction of the dye one such method is MTT assay in which a yellow-coloured water-soluble salt 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide [MTT] reacts with the mitochondrial dehydrogenase enzymes of live cells, which reduces them to purple-coloured insoluble formazan crystals, which are precipitated and dissolved in DMSO. Dehydrogenase content is consistent among the cells of a specific type and the amount of formazan reduced is proportional to the cell number.

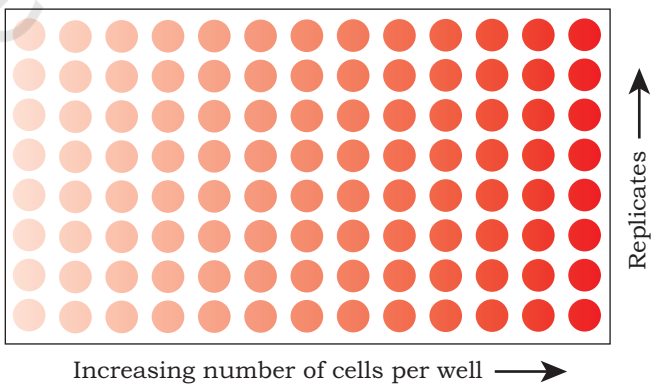


Fig. 8.8: Result of metabolic viability assays. Colour is directly proportional to the number of cells

8.7 Advantages of Animal Cell Culture

The advantages of animal cell culture are:

- Can be grown under controlled physico-chemical environment.
- Can proliferate into a homogenous genetic population.
- Available in adequate numbers to conduct chemical studies.
- Easy production of biopharmaceuticals.
- Ethical clearance not required.

However, there are a few limitations that are as follows:

- It is a highly sensitive technique, and even small changes can reduce the productivity.
- May not be a holistic representation of *in vivo* phenotype/genotype.

8.8 Applications of Animal Cell Culture

Animal cell culture has diverse applications in various areas. Some are listed below:

- As a model system for the study of interactions between cells and diseases causing agents as well as their drugs.
- As a convenient and economic tool to study virus research
- As a useful technique for vaccine production on a large scale
- As a production house for various medically important protein pharmacueticals. The important ones are listed in Table 8.3.

Table 8.3: Pharmaceutical proteins and their therapeutic use

Proteins	Animal cells used	Applications
Follicle Stimulating Hormone (FSH)	CHO cells	Infertility
Human Growth Hormone (HGH)	CHO cells	GH deficiency
Erythropoietin (EPO)	CHO cells	Anemia
Factor VIII	CHO cells	Hemophilia A
Factor IX	CHO cells	Hemophilia B
Interleukin 2 (L-2)	CHO cells	Cancer therapy
Tissue Plasminogen Activator (tPA)	CHO cells	Stroke
Monoclonal antibodies (mAbs)	Hybridoma cells	Cancer therapy & Autoimmune diseases
* CHO — Chinese Hamster Ovary		

Erythropoietin (EPO) is a glycoprotein hormone secreted by kidney under hypoxic (shortage of oxygen) or anoxia (lack of oxygen) conditions caused by anaemia that induces RBC production (erythropoiesis) and wound healing. EPO stimulates the bone marrow to produce more red cells and thereby increase the oxygen-carrying capacity of the blood. EPO is used for treating certain types of anaemia caused by chemotherapy in cancer patients, treatment of AIDS, and even in chronic renal failure. Recombinant human EPO (r-HuEPO) has been produced using Chinese Hamster Ovary (CHO) cell line. The use of r-HuEPO is advantageous over blood transfusion as it does not require donors or transfusion facilities, and the risk of transfusion-associated disease is minimised.

Factor VIII for Haemophilia A, is a common inherited genetic disorder in which Factor VIII required for blood clotting is not produced in the body. Like EPO, Factor VIII is also a glycoprotein and may be produced in CHO cells.

Factor IX for Hemophilia B or Christmas disease is a bleeding disorder caused due to the deficiency of factor IX. Recombinant Factor IX produced in CHO cells is used to treat haemophilia B.

Tissue Plasminogen Activator (tPA) is a serine protease that catalyses the conversion of plasminogen to plasmin which is responsible for dissolving blood clots. It is approved for use in certain patients having a heart attack or stroke. tPA was the first drug to be produced through mammalian cell culture. Production and mode of action of tPA is given in Fig. 8.9.

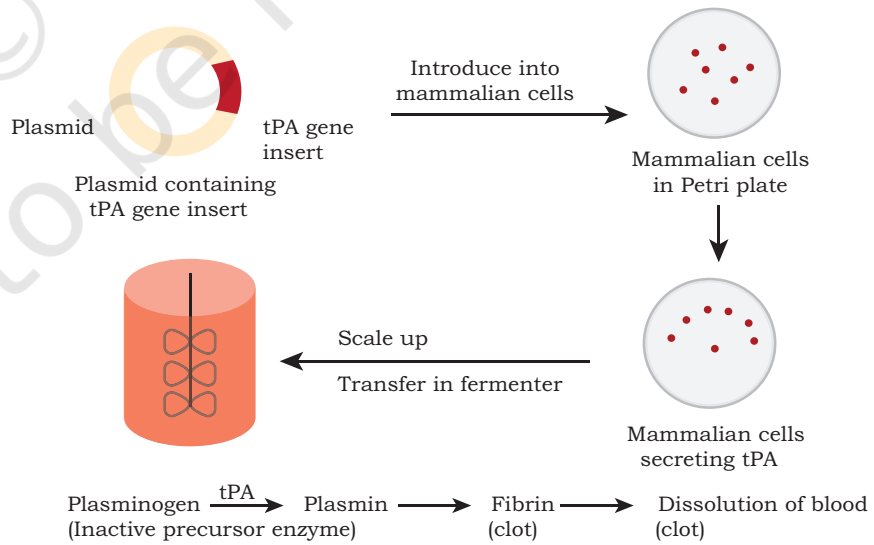


Fig. 8.9: Production and mechanism of action of tPA

Therapeutic MAB – OKT3

Muromonab-CD3 (OKT-3) is a murine monoclonal antibody directed against CD3 receptor. It is used as an immunosuppressant drug that is given intravenously to reverse acute rejection of transplanted organs, such as kidney, heart and liver. When OKT–3 is bound to CD3, the T-cell receptor (TCR) undergoes endocytosis resulting in an inert T-cell and acts by blocking the function of T cells which play a major role in acute graft rejection (Fig. 8.10). This prevents subsequent recognition. T cells are then eliminated by phagocytosis. After OKT–3 therapy is over, T cell function usually returns to normal within a week. OKT–3 was the first monoclonal antibody to be approved for the treatment of acute rejection in clinics.

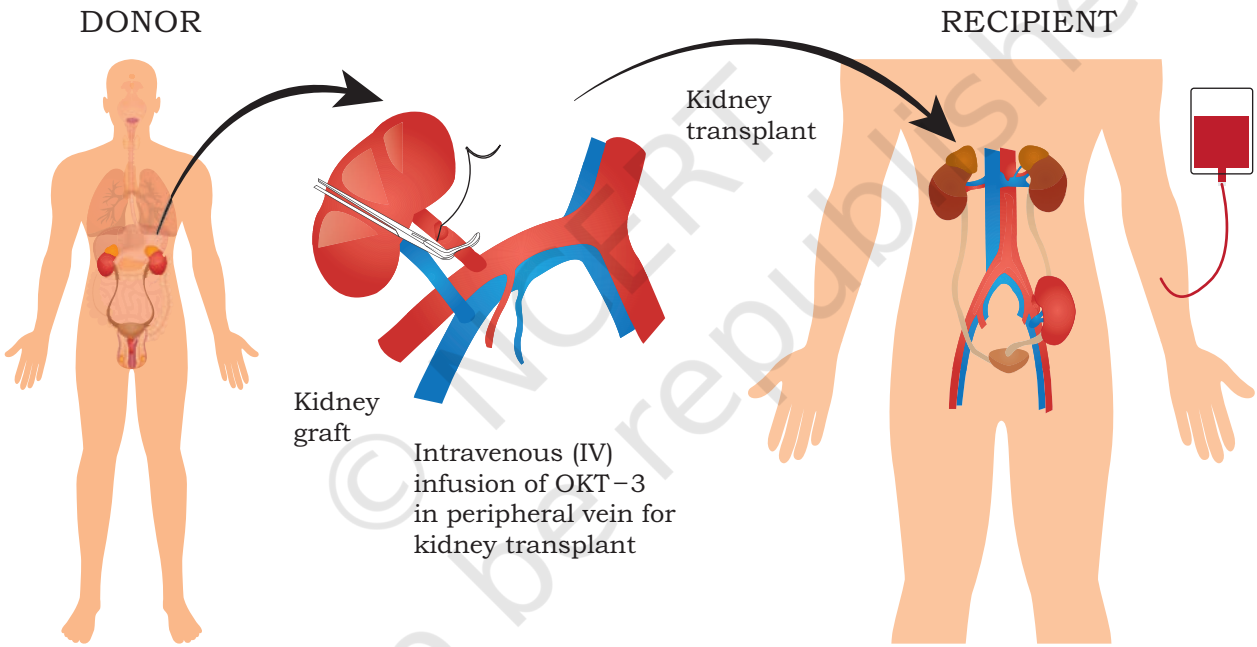


Fig. 8.10: Use of OKT–3 during kidney transplantation (KT)

Trastuzumab (brand name is Herception) is a monoclonal antibody approved for therapy of early-stage breast cancer that is Human Epidermal growth factor Receptor 2-positive ($HER2^+$). Normal cells also express HER2 but cancer cells overexpress $HER2^+$ along with dysregulation of receptor activation. These cell surface receptors receive signals that stimulate the cells to

proliferate. Trastuzumab works by attaching itself to HER2 receptors and blocking them from receiving growth signals. The result is impaired growth of breast cancer.

SUMMARY

- Animal cell culture is *in vitro* maintenance and proliferation of animal cells using an appropriate nutrient media.
- The basic requirement for optimal growth of animal cells include temperature, pH and appropriate growth medium.
- Animal cell media can be categorised into two major categories, namely natural media and artificial or synthetic media.
- Natural media consists of naturally occurring biological fluids, such as plasma, tissue extract, etc., and it is suitable for the culture of a wide range of animal cells.
- Artificial or synthetic media is made up of various nutrients (both organic and inorganic), vitamins, salts, O_2 and CO_2, serum, carbohydrates, cofactors, etc., and it can be modified according to purpose and it is divided into four categories namely, serum containing, serum free, chemically defined, and protein-free media.
- The cell culture can be classified as primary cell culture and secondary cell culture (cell line).
- Primary cells can grow either as an adherent monolayer or in a suspension.
- Secondary cell lines are derived from primary cells after sub culturing.
- On the basis of the life span of culture, the cell lines are categorised into finite and continuous cell lines.
- The cell viability measurement, i.e., the determination of living or dead cells, is very significant in cell culture and is done *via* dye exclusion or viability assays and metabolic viability assays.
- Cell culture technology has applications in various areas, such as molecular genetics, immunological analyses, gene therapy, bioengineering, pharmaceutical industry, etc.
- Animal cell culture plays an important role in research and development of drug and also helps to improve the health and quality of life of patients suffering from various diseases, such as cancer, genetic disorders, etc.

EXERCISES

1. What is animal cell culture?
2. Describe animal cell culture media and their types.
3. Write the advantages and disadvantages of serum in the culture media.
4. Describe any two chemically synthesised media.
5. What is primary cell culture? Also discuss as to how it is developed.
6. What is subculture or passaging of cells?
7. Differentiate between finite and continuous cell lines.
8. How is cell viability measurement performed?
9. Write a detailed account of application of cell culture.
10. The example of animal cell culture media is:
    (a) DMEM
    (b) MS media
    (c) LB Media
    (d) All of the above
11. Name the type of culture that is prepared by inoculating directly from the tissue of an organism to culture media.
    (a) Primary cell culture
    (b) Secondary cell culture
    (c) Cell lines
    (d) Transformed cell culture
12. Sodium bicarbonate is added to animal cell culture media to
    (a) keep cells stuck to the plastic
    (b) promote the uptake of CO_2 into animal cells
    (c) maintain the correct pH when CO_2 is present
    (d) keep iron soluble
13. Which of the following is NOT present in growth medium for animal cell culture?
    (a) Inorganic salts
    (b) Bicarbonate
    (c) Carbon source
    (d) Starch
14. Disaggregating of cells can be performed by:
    (a) Physical disruption
    (b) Enzymatic digestion

(c) Treating with chelating agents

(d) All of the above

15. The approach in which genes are transferred into animals to obtain a large scale production of the proteins encoded by these genes in the milk, blood, etc., is called

(a) *In situ* culture

(b) Molecular pharming

(c) Gene therapy

(d) Hybridoma technology

16. Which of the following is a protein-free animal cell culture media?

(a) RPMI-1640 Media

(b) MS media

(c) LB media

(d) None of the above

17. MTT assay is used for

(a) Cell viability test

(b) Monitoring of variation in pH of culture media

(c) Transformation screening

(d) Cell dissociation from substratum

18. Passaging of animal cells in animal cell culture is

(a) Sub-culturing of the cells

(b) Isolation of cells

(c) Passing the cells from culture tube to Petri dishes

(d) Counting of cells

19. Which of the following is NOT the major function of serum?

(a) Enhance cell attachment

(b) Stimulate cell growth

(c) Promotion of tuber and bulb formation

(d) Provide transport proteins

20. **Assertion:** Serum is the most important component of culture media.

Reason: Serum is a good source of nutrients and also helps in cell proliferation and cell-matrix attachment.

(a) Both assertion and reason are true and the reason is the correct explanation of the assertion.

(b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.

(c) Assertion is true but reason is false.

(d) Both assertion and reason are false.

21. **Assertion:** Cell lines derived from the primary culture of normal cells are finite cell line.

Reason: Some cells of the finite cell line undergoes transformation and retain the ability to divide indefinitely.

(a) Both assertion and reason are true and the reason is the correct explanation of the assertion.

(b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.

(c) Assertion is true but reason is false.

(d) Both assertion and reason are false.

Chapter 9

Stem Cell Culture and Organ Culture

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Stem cell study forms an exciting and attractive area of contemporary biomedical research that has enormous potential for both basic and translational research. Stem cells can be used for alleviating suffering from many diseases that currently have no effective therapy. The field has progressed to the clinic and it is very important that the basics of stem cells be reinforced by excellent science and rigorous standards of clinical research. Besides this, organ culture is another modern approach that involves developing a part or the whole organ using tissue culture techniques. In this chapter, we are going to study in detail about stem cell culture and organ culture along with their applications.

9.1 Stem Cell Culture

Life forms are characterised by the ability to reproduce itself. One of the key features of sexual reproduction is the formation of zygote through the process of fertilisation resulting from the fusion of the male gamete (sperm) and female gamete (oocyte). Formation of embryo from the zygote involves cell division resulting into the formation of two

celled, four celled, eight celled organism so on. Ultimately, these daughter cells differentiate into a variety of cells, such as muscle cells, skin cells, liver cells, cardiovascular cells, epithelial cells, etc. However, during the process of differentiation, cells vary in their potential to make final cells such that some cells become 'mature' while some remain 'immature'. Such 'immature' cells which have the potential to differentiate into a wide range of specialised cell type, are called **stem cells**. These stem cells have the potential for *self-renewal via* mitotic cell division and then differentiate into a wide range of specialised cell types.

Historical perspective

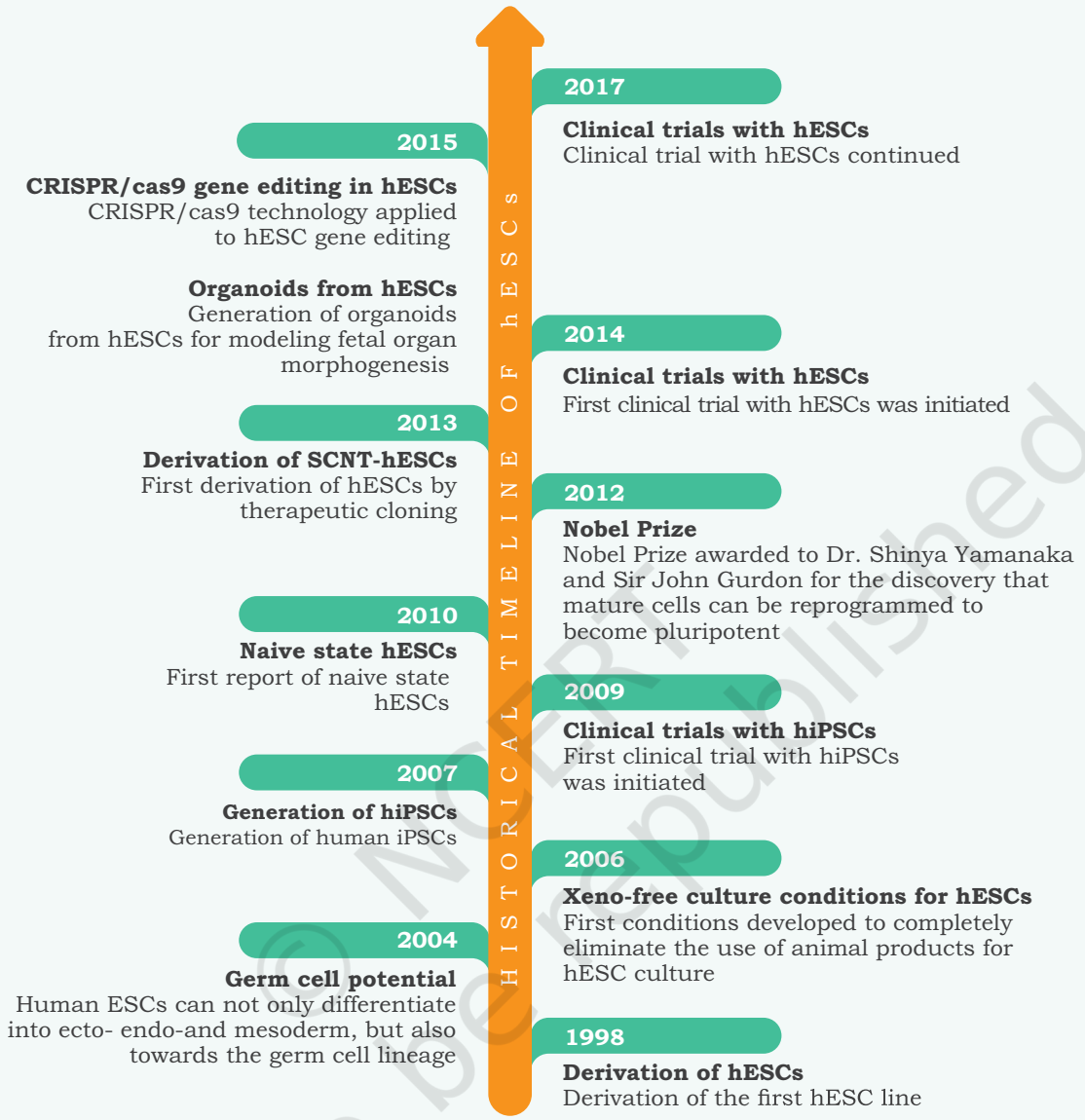
Stem cells have been known to procreate more interest, inspection and debate than any other area of scientific study. The first stem cells were isolated from blood cells. At present, scientists all around the world are working on various types of stem cells to revolutionise the area of regenerative medicine by using the potential of stem cells to regenerate the tissue or organ. The story of this amazing journey has been detailed in the form of a flow chart to reveal its most salient moments through the voice of some of the pioneers in this exciting field (Box 1).

Stem cells are non-specialised cells with an inherent property of self-renewal and potency, i.e., they have the potential for self-renewal via mitotic cell division and then differentiate into a wide range of specialised cell types. Stem cells are present in most of the multi-cellular organisms and are able to endure adverse conditions for long-time periods. In humans, stem cells are found in umbilical cord, placenta, inner cell mass of the early embryo, few tissues of foetus and in some adult organs.

Due to the virtue of differentiation, stem cells can be useful for treating and understanding the diseases and can be used to:

- develop new cells *in vitro* to replace the damaged tissues or organs.
- study the cause of genetic defects in cells as well as to study the cause of diseases and their treatments.
- test new lead molecules as drugs.

Box 1



Historical Timeline of human embryonic stem cells (hESCs). [human Induced pluripotent stem cells (hiPSCs); Somatic Cell Nuclear Transfer (SCNT)]

9.1.1 Stem cell classification

Stem cells may be classified depending upon the source and potency (Fig. 9.1).

Classification based on source

On the basis of the source, stem cells have been categorised into three major groups— the embryonic stem cells, the

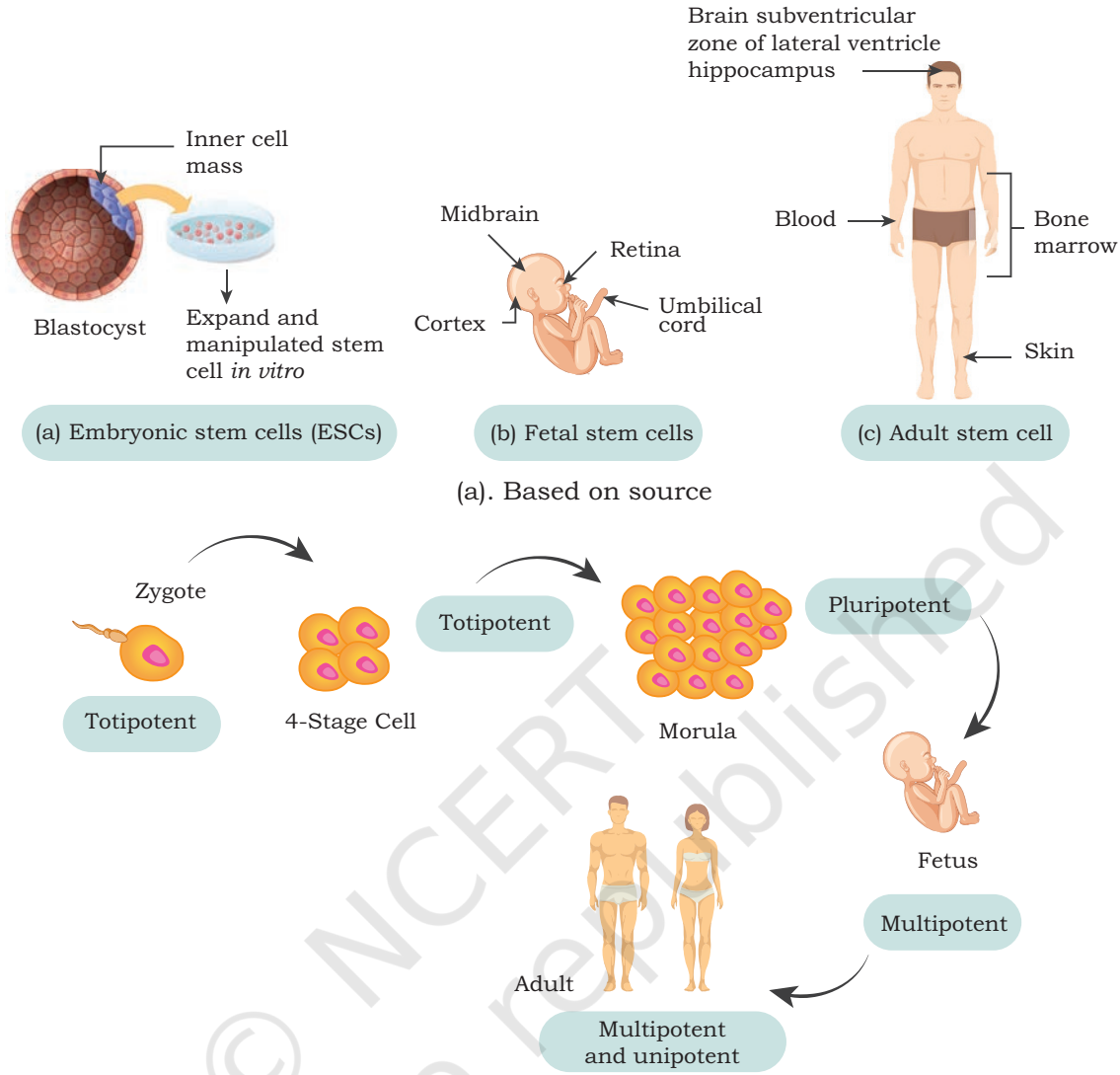


Fig 9.1: Various type of stem cells

fetel stem cell and adult stem cells. There is one more group called fetal stem cells. Embryonic stem cells are also known as early stem cells and are present in the inner cell mass of blastocyst after around five days of development. Adult stem cells or mature stem cells are present in the umbilical cord, placenta after birth and in mature body tissues. As the embryonic stem cells have the property to differentiate into any tissue of the body, they are more promising for clinical applications than adult stem cells. However, the use of embryonic stem cells in humans is limited because of technical safety and some ethical dilemma. On the

contrary, there are no controversies regarding the safety and ethical issues associated with adult stem cells. Adult stem cells were thought to be irreversible, dedicated to a specific lineage. However, now they have been shown to exhibit plasticity, i.e., stem cells from one tissue can differentiate and produce cells of a completely different tissue.

(a) Embryonic stem cells

Embryonic stem cells are classified as self-reproducing pluripotent cells and are possibly immortal. The human embryonic stem cells are derived from the embryo that is blastocyst. The distinctive features of embryonic stem cells are as follows:

1. These cells can be isolated from the inner cell mass or epiblast of the blastocyst.
2. These cells are pluripotent in nature and have the ability to give rise to all the three germ layers, i.e., ectoderm, mesoderm and endoderm.
3. They have the capability of self-renewal and exhibit stable diploid chromosome.
4. They retain the characteristics of embryo founder cells even after extensive manipulation.
5. Depending on the conditions of the *in vitro* environment, they can self-renew or differentiate into multiple tissue types.

(b) Fetal stem cells

Cells derived from a fetus that retains the ability to divide, proliferate and provide progenitor cells and can differentiate into specialized are called fetal stem cell. The embryo is referred to as a fetus after the eighth week of development. Once a fetal stem cell has been harvested, it has the potential to live indefinitely in the laboratory. Fetal stem cells can be isolated from fetal blood and bone marrow as well as from other fetal tissues, including liver and kidney. Fetal blood is a rich source of haemopoietic stem cells (HSGs), which proliferate more rapidly than those in cord blood or adult bone marrow.

(c) Adult stem cells

Adult stem cells are precisely called **somatic stem cells**, because they can come from children or umbilical cord and not from adults only. They are undifferentiated multipotent or totipotent stem cells. The adult stem cells are present all over the body after embryonic development and replace and regenerate the damaged tissues or dying cells by cell division. These cells have two basic characteristics: ability to self-renewal without compromising with stemness and ability to differentiate into mature morphologically and functionally dissimilar tissue specific cells. The main role of adult stem cells in an organism is to preserve and repair the tissue in which they reside. Therefore, these cells maintain cellular homeostasis throughout the lifetime of mammals. The highest frequency of adult stem cells is present in the bone marrow.

The embryonic stem cells are defined by their origin but the origin of adult stem cells in some tissues is still under examination. The adult stem cells can either be hematopoietic or mesodermal in origin.

(i) Hematopoietic stem cells (HSCs)

Hematopoiesis, the process of formation of the different types of blood cells occur in bone marrow. Bone marrow contains hematopoietic and non-hematopoietic stem cells. Hematopoietic stem cells (HSCs) give rise to different types of blood cells - RBCs, monocytes, neutrophils, acidophils, basophils, lymphocytes and platelets. Non-hematopoietic stem cells are originated from common mesodermal precursor cells.

(ii) Mesenchymal stem cells

MSCs have the ability to self-renew, exhibit multi-lineage differentiation and are extremely proliferative. These cells can be isolated from a variety of tissues, such as bone marrow, adipose tissue, umbilical cord etc. The major advantages of MSCs include their genetic stability, avoid allo-rejection, compatible in tissue engineering applications and are capable of repairing many vital tissues. They are thought to be responsible for wound healing, growth and replacing cells that are lost through daily wear and tear and pathological conditions. MSCs are important for making

and repairing skeletal tissues, such as cartilage, bone and fat found in the bone marrow. Because of these functions, they have been shown to be effective in the treatment of tissue injury and degenerative diseases.

Classification based on differentiation potency of stem cells

On the basis of the ability of self-replication and differentiation into various tissues, stem cells have been categorised into totipotent, pluripotent, multipotent and unipotent. Each of them has been described in the following sections.

Totipotent stem cells

Totipotent (toti = whole, potent = able to) stem cells are the most versatile form and have the utmost potential for differentiation that permits the cells to produce both embryo and extra-embryonic membranes, and all the post-embryonic tissues and organs that form an entire functional organism. For example, zygote has the potential to develop into a complete organism.

All cells of the early embryo are totipotent until the eight-cell stage, after that they begin to specialise and form blastocyst. After nearly four days, the inner cell mass of blastocyst becomes pluripotent.

Pluripotent stem cells

Pluripotent (pluri = several, potent = able to) stem cells have the potential to differentiate into almost all cell types of the embryo except the cells of the extra embryonic support tissues, (placenta and yolk sac). These cells can give rise to all the germ layers but are not able to form extra-embryonic tissues, such as placenta, as a consequence, they cannot give rise to a complete organism like totipotent stem cells. The inner cell mass of blastocyst gives rise to tissues of the adult organism while the outer layer trophectoderm forms the placenta. The pluripotent stem cells go on dividing, until they begin to specialise in the gastrulation stage. Examples comprise embryonic stem cells and cells that are derived from the ectoderm, mesoderm and endoderm germ layers that are formed in the beginning stages of embryonic stem cell differentiation.

Multipotent stem cells

The multipotent cells (multi = many, potent = able to) have the ability to differentiate into a closely related family of cells. These are the plastic and more differentiated stem cells. These multipotent stem cells can specialise into various types of cells within the specific cell lineage. For example, multipotent hematopoietic (adult) stem cells can develop into various types of blood cells, such as red blood cells, white blood cells and platelets. Similarly, neural stem cells can give rise to neurons, oligodendrocytes and astrocytes. After differentiation, the abilities of these cells are restricted to the cells of its specific lineage.

Unipotent stem cells

It is an undifferentiated cell present in the differentiated tissue with the property of dividing repeatedly. Unipotent stem cells have the ability to only produce cells of their own type and also have the property of self-renewal needed to be labelled as stem cell. They can self-renew and differentiate into specialised cells of the tissue, such as epidermal stem cells, muscles and endothelial, etc. The repetitive division in unipotent stem cells make them a promising candidate for therapeutic use in regenerative medicine.

It should be remembered that totipotent and pluripotent stem cells belong to the embryonic life of the organism, whereas multipotent and unipotent stem cells are found in the adult life. In general, multipotent and unipotent stem cells are termed as adult stem cells or somatic stem cells.

9.1.2 Characteristics of stem cells

Over the past several years, a great deal of attention has been paid on the differentiating ability of the adult stem cells. **Plasticity** of adult stem cells is the presumed ability of tissue specific stem cells to attain the fate of cell types distinct from the tissue of origin. Differentiation of one cell type against its fate into other cell type requires massive molecular rearrangement within the cells, for which the competence of the responding cells and micro-environmental cues are most important. For example, all the types of bone marrow cells are not competent for differentiating into neurons or hepatocytes. Similarly, the competent bone marrow cells do not

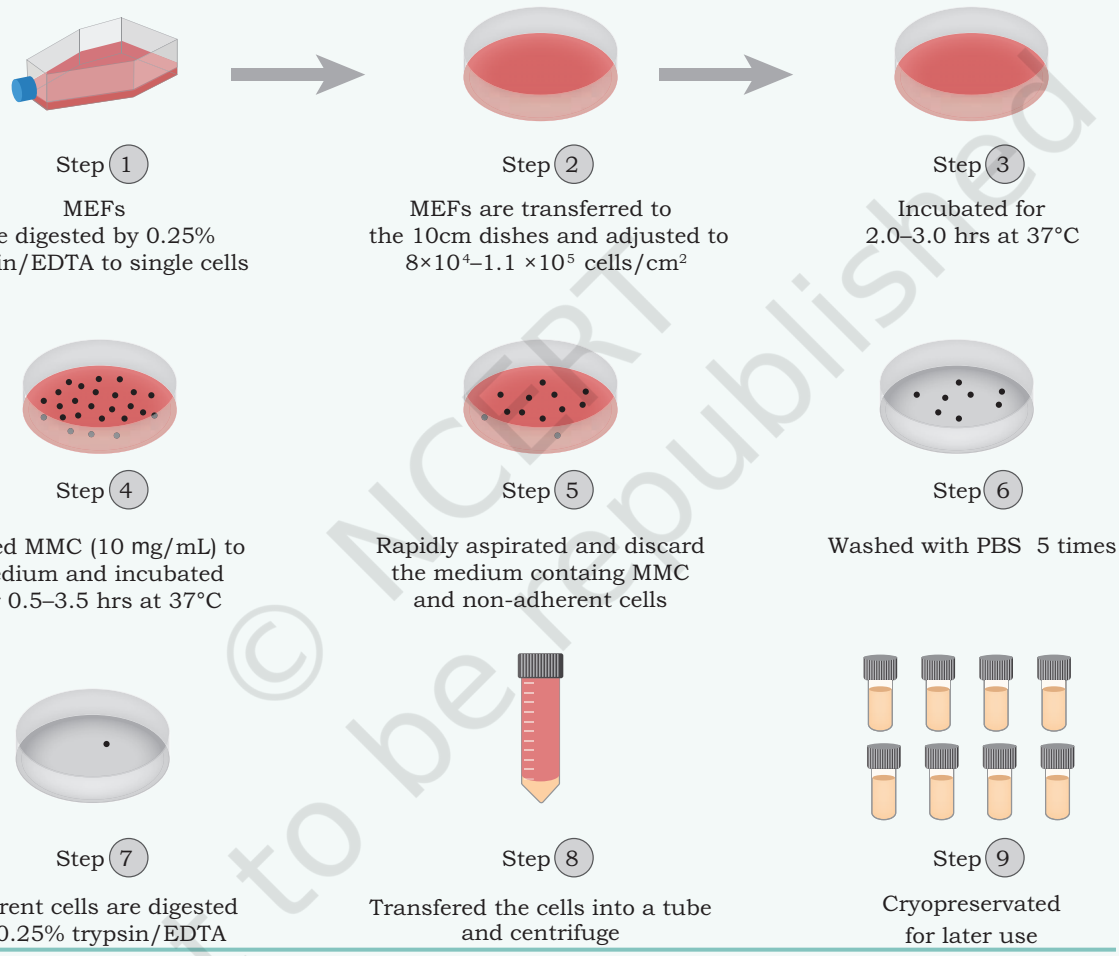
undertake differentiation program against fate, unless they are exposed to inductive microenvironment. Some of the alternative fate changing pathways by somatic cells to switch lineages are trans-determination, trans-differentiation, dedifferentiation, heterogeneity, versus gene level pleiotropy and fusion. The first is **trans-determination**, in which a stem cell that is programmed to produce certain lineages, switches to another stem cell and gives rise to the cell types of that precursor, i.e., its potential is redirected. The second pathway is **trans-differentiation**, in this process a differentiated cell can achieve the phenotype of another differentiated cell. **Dedifferentiation** is common in lower vertebrates but rare in mammals. By dedifferentiation process, adult stem cells revert back to master cells from which they originate and the master cells can differentiate into other cell types. Heterogeneity is the most acceptable explanation for the plastic nature of adult stem cells. One of the gold standards for plasticity of adult stem cells is its clonal level purity. Normally, the purity of stem cells is determined by the presence and absence of the specific surface markers. When plasticity between two cells originated from the same germ layer can be explained by clonal level impurity, it is known as **pleiotropic**. **Fusion** is an alternative mechanism proposed for the plasticity in adult stem cells.

9.1.3 Applications of stem cells

The goal of any stem cell therapy is to repair a damaged tissue that cannot heal itself. Ongoing research on stem cell treatments give hope and confidence to the patients who would generally not get treatment to cure their diseases but just to improve the symptoms of their chronic illness. Stem cell therapies involve more than simply transplanting cells into the body and directing them to grow new, healthy tissue. It may also be possible to persuade the stem cells already in the body to work overtime and produce new tissue. There are a number of stem cell therapeutics developed, but most of them are at experimental stages and costly, with the notable exception of bone marrow transplantation. Many medical researchers predict that soon the embryonic and adult stem cells will be able to treat muscle damage, cancer, Huntington's disease, Type 1 diabetes mellitus, Parkinson's

disease, cardiac failure, celiac disease, neurological disorders and many more. Before the application of stem cell therapeutics in clinical medicine, further research is required to understand the stem cell behaviour upon transplantation and the mechanisms of stem cell interaction with the injured or diseased micro-environment. Some clinical applications of stem cell therapies are detailed as follows.

Box 2



Maintenance of cell culture (Mouse Embryonic Fibroblasts: MEFs, Phosphate Buffer Saline: PBS, Macromolecular Crowding: MMC, Ethylenediamine tetraacetic acid: EDTA)

Neurological diseases

The stem cells may become a good alternative therapeutic candidate for the treatment of neurological diseases. For example, the Amyotrophic Lateral Sclerosis (ALS) disease is a progressive neuromuscular disease, which is characterised by the loss of motor neurons in the brain and spinal cord. The investigation for the treatment of ALS using bone marrow stem cells and induced pluripotent stem cells (iPS cell or iPSCs) is under clinical trials.

During the spinal cord injury cases, the nerve fiber bundles are damaged, which lead to paralysis. The investigation using adult stem cells to regenerate new nerve cells and trigger the growth of severed nerve fibers is being carried out.

Stem cells therapies are being used for the eye disease treatments. In one of such cases an improved vision was observed with the transplantation of retinal pigment epithelial cells derived from the embryonic stem cells and retinal stem cells. This observation brings a hope for new therapeutics for eye diseases.

Wound healing or skin replacement

The skin (keratinocyte) stem cells are present in the hair follicle and these stem cells are removed after plucking hair and are cultured. After culture of these cells, they can be used as equivalent to the patients, own skin which has less problem of rejection. Thus, the stem cell become a possibility to develop skin from a patient's plucked hair. Stem cells are a better alternative for faster healing of the skin injuries, genetic disorders, and burnt wounds with lower inflammation levels as compared to the conventional skin grafts that fail to restore the complete composition of the dermis lost.

Cardiovascular Diseases

The cardiovascular diseases are basically characterised by ischemia and heart muscle injury leading to hypertrophy and congestive heart failures. The traditional and standard treatments for this are surgical procedures that repair the blocked arteries, medications that reduce fluid retention and lifestyle changes. With the advent of stem cells, a better therapy is under investigation that aim to re-establish the lost function of heart tissue and blood vessels.

Auto-immune Disorders

An auto-immune disorder occurs when the immune system of the body becomes over reactive and considers its own healthy tissues as foreign and attacks them.

Type 1 Diabetes

Type I diabetes is caused by the degradation of insulin-producing pancreatic beta cells by body's own immune system. For treatment, insulin administration is an effective strategy but optimisation of doses of insulin is one of the major limitations, because the amount of insulin depends on the glucose level. Thus, its treatment with the cells derived from haematopoietic stem cells is being explored.

Multiple Sclerosis

Multiple sclerosis is a chronic inflammatory disease that occurs in brain or spinal cord. This disease is due to over-activation of body's own immune system, in which the myelin sheath of the neurons get damaged. For the treatment, the bone marrow stem cells and neural stem cells are being investigated to regenerate the neurons with a proper myelin sheath.

Arthritis

In rheumatoid arthritis, the symptoms are chronic pain, inflammation of the joints, which are mainly due to the destruction of cartilage by the immune system. The currently used treatment strategies involve drugs that reduce the pain and inflammation. But the stem cells can be differentiated into cartilage making cells, mainly chondrocytes, thus the use of stem cells could be a better alternative.

In pharmaceutical industries, the stem cells have evolved as a suitable model to investigate the potential or mode of action of drugs and have become an improved alternative against the limitation of the ethical challenges of animal models. In the current scenario, pharmaceutical industries emphasise the use of stem cells for drug screening. Furthermore, the cardiomyocytes originated from the human embryonic stem cells are used as a model for cardiac diseases. The neuronal cells originated

from stem cells are used as working model for neuronal disorders.

Stem Cell Research Challenges

In the current research scenario, the stem cell research is an emerging area with various applications, but it has some challenges yet to be resolved. One of the major challenges is immunological rejection, in which the stem cell transplants are rejected by the recipient's immune system. Thus, to resolve it, immunosuppressive treatments are given to recipients, that make them prone to microbial infections. However, the use of inducing pluripotent stem cells (derived directly from the recipient's own cells) may be one strategy to resolve the above limitation of rejection to some extent. The second major challenge is the behaviour of stem cells. The normal characteristics of a stem cell may have limitations for certain applications. For example, the embryonic stem cells, which divide indefinitely, might induce tumour growth. The safety of stem cells during application is also one of the challenges, as stem cells are prone to microbial infection that leads to various infectious diseases in the recipients.

9.2 Organ Culture

So far, you have studied the different aspects of cell culture which deals with the *in vitro* maintenance and proliferation of isolated cells using appropriate nutrient media. In cell culture, although cell-to-cell interaction is possible, but the *in vivo* structural complexity is lacking which is the main limitation of cell culture. Conventional human cell cultures that are generally used to establish differences in species, are also constrained in their depiction of the *in vivo* responses owing to the lack of a suitable microenvironmental framework of the responding cell types. Preclinical trials on animals had partial success in predicting human pathology, physiology and therapeutics. Modern culture approaches, such as **three-dimensional (3D) cultures, organs-on-a-chip or organoids** have attempted to partially mimic the tissue microenvironment.

Development of a part of an organ or the whole organ itself from tissue culture techniques for research is termed

as organ culture. In this, the parts of an organ are explanted and grown in vitro such that the anatomical relationship and physiological functions of several tissue components are preserved in the culture on artificial medium, resulting in simulating its parent tissue.

For success in any organ culture technique, it is crucial that the tissues must be handled with extreme care and should not be damaged or disrupted during handling. Tissues should be transported to the laboratory as fast as possible to minimise deterioration, ideally within minutes of collection. Further, analysis of the organ cultures can be done by immunochemistry, autoradiography and histology.

9.2.1 Characteristics of organ culture

Primarily, the structural design has to be preserved in organ culture of the organ or tissue and direct it towards normal development. The important characteristics of organ culture are as follows.

Structural integrity

A major limitation in organ culture is the lack of a vascular system in tissues, restricting the size (by diffusion) and possibly the polarity of the cells within the organ culture. Overall, in the whole organ culture, some proliferation may occur on the outer cell layers as in organs. These cells are unified as a single unit, while the isolated cells are discrete. Also, in organ culture, the cell-to-cell communication and association are greatly preserved. Therefore, owing to the retention of structural integrity of the native tissue, the associated cells can interchange signals *via* cell interaction or adhesion.

Nutrient and gas exchange

Organ cultures are devoid of vascular system and this condition results in restricted gas exchange and nutrient supply to the cells. When the cells are cultured as a solid mass of tissue, nutrient exchange and diffusion of gases ensues from the periphery and this diffusion rate restricts the size of the tissue. As a result, at the central part of the tissue or organ, some amount of necrosis may happen.

Most of the time, organ cultures are exposed to high O_2 concentration. However, the use of high O_2 concentration is accompanied with the O_2 prompted toxicity risk in organs.

To deal with this difficulty in organ cultures, tissues are maintained at a gas–liquid phase to facilitate the exchange of gases, while maintaining the access to adequate nutrients. Anchorage of the tissue to a solid substrate can lead to the development of an outgrowth of cells from the tissue and resultant alterations in geometry, though this effect can be minimised by using a hydrophobic surface. The significance of maintaining organ cultures at the interface of gaseous and liquid phase is that if the liquid is retained at optimal level then the tissue maintains a spherical geometry. However, if the level of liquid is too superficial, then the tissue will outgrow and flatten as a result of surface tension, whereas, if the level is too deep, the exchange of gases is compromised. To enhance the permeation of oxygen in organ cultures, hyperbaric oxygen or enhanced pure oxygen content is used.

9.2.2 Growth and differentiation

Growth refers to increase in the number of cells is a culture whereas, differentiation refers to change in the function of the differentiated cell. Growth and differentiation are related to each other such that the differentiated cells may not be able to proliferate further. There is a possibility that irrespective of cell density, termination of growth may promote the induction of differentiation. Most of the organ cultures do not grow, instead they proliferate only on the outer layers of cells as a consequence of physical limitations executed by the structure and shape. The organ culture is tolerant to the suitable cellular communications and differentiations and also to maintain the appropriate environment. Soluble growth factors are supplied to facilitate differentiation.

Whole embryo culture

Spratt (1950s) explained the effect of metabolic inhibitors on the developing embryo *in vitro*. To culture the embryo, a suitable medium has to be prepared and poured onto a watchglass, which was then placed on a small piece of moist absorbent cotton wool pad in a

petridish. To observe the chick embryo in culture, the eggs were incubated at 38°C for 40–42 hours so that a dozen of embryos could be produced. The egg shell was sterilised with 70 percent ethanol, then broken into pieces and kept in 50 ml of balanced salt solution (BSS). The vitelline membrane covering the blastoderm was removed and kept in BSS. The adherent vitelline membrane was removed with the help of a forcep. The embryo was observed under a microscope to study the developmental stage of blastoderm. The blastoderm was then carefully placed on the sterile absorbent cotton wool pad in the watchglass in the petridish and the embryo culture of chick incubated at 37.5°C for further development.

9.2.3 Types of organ culture

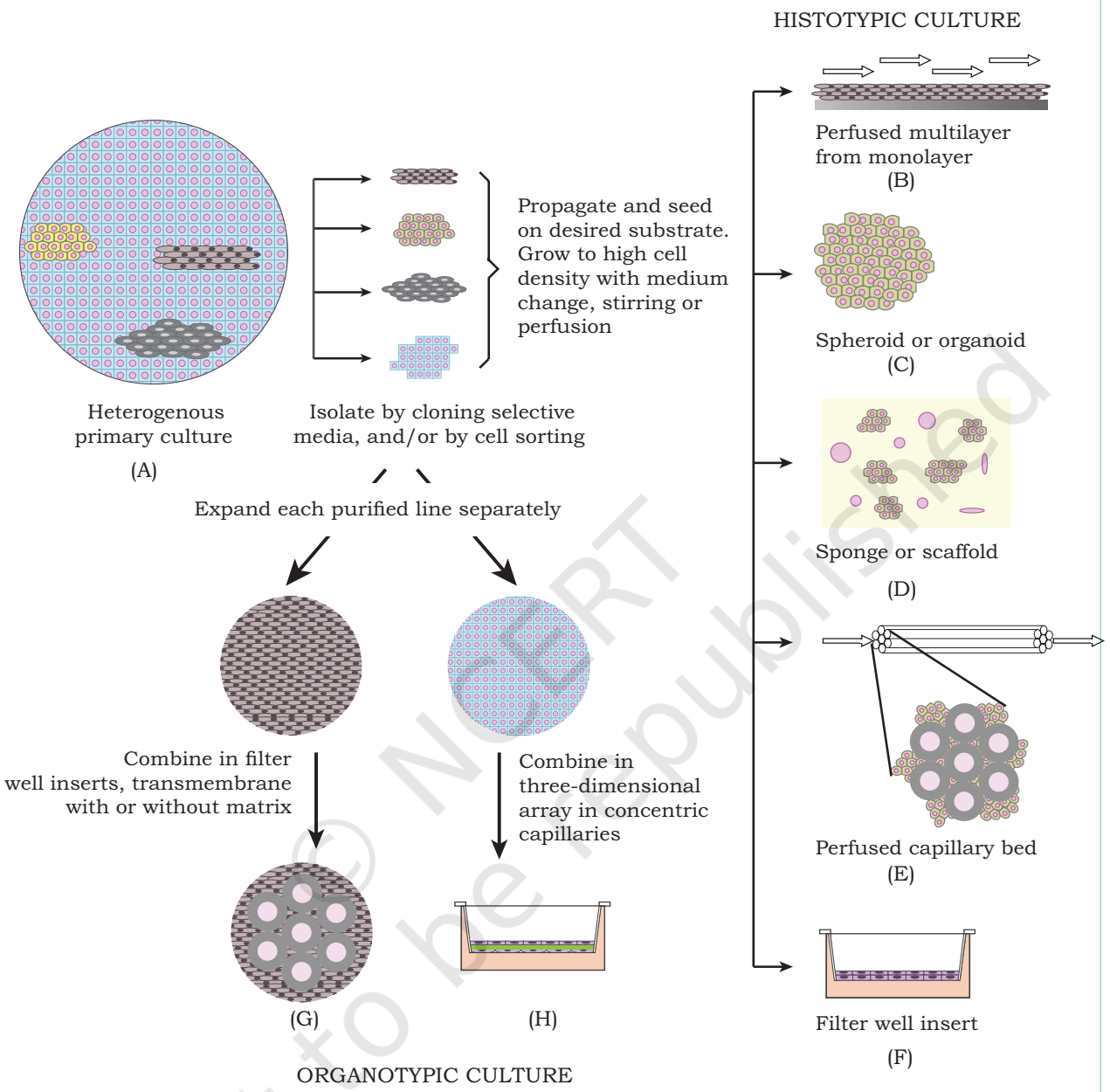
Histotypic culture

When any characterised cell line is proliferated or cultured at a high density in the presence of suitable soluble factors and extracellular matrix, then such a culture is termed as histotypic. Vascular endothelial cells can form capillary tubules in the presence of appropriate soluble factors when grown in a collagen matrix. Cellulose sponges coated with extracellular matrix components such as collagen is another example. Alternatively, cells may infiltrate the sponge and form glandular structures.

Organotypic culture

The limitation in a histotypic culture is the inability to evaluate the heterologous cell interactions. When cells of different lineages are co-cultured to create a tissue-like structure, then it is termed as organotypic culture, which is the easiest way to maintain a co-culture of two cell types. For instance, co-culture of fibroblast and epithelial cell clones derived from the mammary gland allows the epithelial cells to differentiate functionally. This has been demonstrated by their ability to produce milk proteins in optimal hormonal environment. The formation of characteristic structures is further validated by functional differentiation, such as the three-dimensional cords in which the fibroblast cells reorganise themselves into

Box 3: Histotypic and Organotypic Culture



Histotypic and organotypic culture. Heterogeneity of a primary culture (A), how to purify defined cell populations, that can be expanded and seeded into appropriate conditions can give high-density cultures of one cell type in perfused multilayers (B), spheroids or organoids in stirred suspension (C), sponge or scaffold (D), three-dimensional multilayers in perfused capillaries (E), monolayers or multilayers in filter well inserts (F). Expansion of purified populations and recombination can generate organotypic cultures, in filter well inserts (G) or on concentric microcapillaries (H).

bundles that are further enveloped by the epithelial cells. 'Organoids' are self organised 3D tissue culture derived from stem cells. Thus the organoid is made from a combination of cells, growth factors, collagen etc. Example: artificial liver surgically implanted into the peritoneal cavity of a rat. These organoids have the potential to replace any diseased or non-functional organ, or deliver genetically altered cells into a patient's body.

9.2.4 Applications of organ culture

Cultured organs can be used as a substitute for organs. This may be beneficial as the accessibility of transplantable organs is decreasing. One more significance of organ culture is that the organs are produced by using patient's own stem cells, that would permit transplantation of organs without the use of immunosuppressive drugs by patients.

The organ culture helps to study the actual behaviour of a tissue in an *in vitro* system as well as to understand the biochemical and functional characteristics of an organ or tissue and their comparison with that of the similar organs *in vivo* in an easier way. The organ culture is also suitable for the study of hormones and their effects as individual, or combinations with other hormones. For example, mammary gland is one of the most commonly cultured organ of the mouse.

9.2.5 Limitations of organ culture

There are some limitations of organ culture. Fundamentally, the organ cultures depend on histological techniques and not much on biochemical and molecular analyses. The biochemical analyses is based on the reproducibility that cannot be achieved in organ culture. Furthermore, the organ cultures exhibited more difficulty in the preparation of replicates than that of the cell culture. The organ culture exhibited high variations and low reproducibility. Thus, in organ culture, a fresh organ from a donor is needed for each experiment.

Various limitations of organ culture are as follows:

(a) Since the organ cultures cannot proliferate, therefore fresh organs are required for every experiment.

(b) In organ culture, reproducibility is less and disparities are high. This can be because of a slight variation

in geometry and handling, sampling difference in organising an organ culture and differences in the proportions of types of cell among cultures.

(c) Organ cultures are very challenging to prepare and are also very expensive.

(d) By this technique, the behaviour of integrated tissue only can be examined and not of isolated cells.

9.2.6 Future Prospects

One of the future challenges is to adapt the cell-based assay protocols developed for the analysis of 2D cell culture to the altered conditions of cells growing in 3D structures. 3D cell cultures include multicellular spheroids, scaffold hydrogels, organoids, organs-on-chips, hanging drop, microfluidics, magnetic levitation, microtissues and 3D bioprinting.

SUMMARY

- Stem cells are unspecialised cells and have the ability to renew themselves and differentiate into a diverse range of cell types.
- On the basis of the potency, the stem cells can be classified into totipotent, pluripotent, multipotent or unipotent cells.
- Totipotent stem cells have the ability to differentiate into all cell types of an organism.
- Pluripotent stem cells have the potential to differentiate into almost all cell types of the embryo except the cells of the extra embryonic support tissues.
- The multipotent cells have ability to differentiate into a closely related family of cells.
- On the basis of the sources, the stem cells are divided into early (or embryonic) stem cells and mature (or adult) stem cells.
- The early stem cells (embryonic stem cells) are present in the inner cell mass of a blastocyst.
- Adult stem cells are undifferentiated totipotent or multipotent cells and are found in specific mature body tissues as well as the umbilical cord and placenta after birth.
- Stem cells have the potential to treat a number of diseases including cancer, Type 1 diabetes mellitus, Parkinson's disease, cardiac diseases, neurological disorders, etc.

- Development of a part of an organ or the whole organ itself from tissue culture techniques are termed as organ culture.
- The most important advantage of organ culture is that the whole three-dimensional structure of the organ can be recreated.
- The important characteristics of organ culture are — structural integrity, nutrient and gas exchange, growth and differentiation.
- The types of organ culture are — whole embryo culture, histotypic culture and organotypic culture.
- The organ culture helps to study the actual behaviour of a tissue in an *in vitro* system as well as to understand the biochemical and functional characteristics of an organ or tissue and their comparison with that of the similar organs, *in vivo,* in an easier way.
- Organ culture helps in understanding the developmental biology and interaction in tissues.

EXERCISES

1. Briefly describe the stem cells and their properties.
2. Differentiate totipotent, pluripotent and multipotent stem cells.
3. What are embryonic stem cells and how do they differ from adult stem cells?
4. Describe some applications of stem cells.
5. What parameters should be monitored during stem cell culture?
6. What is organ culture?
7. Describe the main characteristics of organ culture.
8. Discuss the various types of organ culture.
9. What are the advantages of organ culture over cell culture?
10. Describe the applications of organ culture.
11. Describe the various support systems used in organ culture.
12. Stem cells are present in:
    (a) unicellular organisms
    (b) multicellular organisms
    (c) non-living things
    (d) viruses

13. Differentiation potential of stem cells specifies:
    (a) Stochastic differentiation
    (b) Asymmetric replication
    (c) Potency
    (d) Self-renewal
14. Which of the following cells is a multipotent cell?
    (a) T-cell
    (b) B-cell
    (c) HSC
    (d) Monocytes
15. A stem cell is:
    (a) a cell out of which the stem of the tree is made up of
    (b) a part of the tissue that forms the outer layer of the skin in human beings.
    (c) it is a cell that can divide and give rise to specialised cells.
    (d) a type of specialised cell
16. _____ can be cured with stem cells.
    (a) Spinal cord injuries
    (b) Type 1 diabetes
    (c) Both (a) and (b)
    (d) None of these
17. The stem cells may be obtained from sources such as:
    (a) Bone marrow
    (b) Umbilical cord blood
    (c) Adipose tissue
    (d) All of these
18. **Assertion:** Embryonic stem cells can give rise to different cell types.

    Reason: Embryonic stem cells are pluripotent.
    (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.
    (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.
    (c) Assertion is true but reason is false.
    (d) Both assertion and reason are false.
19. **Assertion:** Stem cells are undifferentiated and found in multicellular organisms, and undergo numerous mitotic cycles.

Reason: Stem cells have 'self-renewal' feature and do not exhibit 'cellular potency'.

(a) Both assertion and reason are true and the reason is the correct explanation of the assertion.

(b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.

(c) Assertion is true but reason is false.

(d) Both assertion and reason are false.

Chapter 10 Bioprocessing and Biomanufacturing

Students are already aware that living organisms especially microbes and biological processes therein are used for making various household products (curd/yoghurt, idli, kinema, etc.) and industrial products (ethanol). We have already learnt in the previous class that living organisms are endowed with a variety of metabolic processes which lead to the formation of chemical compounds, called metabolites, that are broadly classified into primary and secondary metabolites. Primary metabolites are the compounds produced directly out of primary metabolic pathways associated with essential cellular functions such as growth and development. On the contrary, secondary metabolites are intermediates or indirect products, elaborated by entirely different metabolic pathways called secondary metabolic pathways. Secondary metabolites take part in a variety of functions. For example, they are used in defence for protection against pathogens, phytoplanktons and herbivores, to improve tolerance to abiotic stresses; as attractants for insects and animals for fertilisation, seed dispersal in plants or to contribute in causing the displeasure to the unwanted feeders.

Nowadays, most of these secondary metabolites have a variety of applications in the form of pharmaceuticals, dyes, food additives, enzymes, vitamins, etc. In view of the variety of applications, production of these compounds (biochemicals) at commercial level requires their production in bulk quantities, as quantities produced naturally are not sufficient. These compounds are usually produced at commercial level in a purified form through a series of steps covered under bioprocessing. For large scale production, where large volume (on an average 100–10,000 litres) of culture can be processed, the development of bioreactors was required.

10.1 Historical Perspective

After the breakthrough discovery of Penicillin and its role in killing bacteria by Alexander Fleming in 1928, the significance of products from biological systems was well understood. Now, the challenge before the scientific/ research community was to enhance the production of penicillin. Production in larger amount for its use in treatment would obviously require a systematic process using the culture of biological entity in question, i.e., *Penicillium* species. The need of involvement of microbial physiologist and other life scientists and technologists was identified. This followed the identification of a number of products of living organisms and processes especially from microbes for application in bioprocessing. Many companies and government laboratories, assisted by different universities and institutions came together to take up this challenge and efforts were made to increase the production of penicillin. All this paved a way for the emergence of a new area of biological applications, which is now known as **bioprocessing**. Thus, bioprocessing involves biological or living systems or their components (e.g., enzymes, chloroplasts, etc.) and chemical engineering processes to obtain the desired products at commercial level as depicted in Fig. 10.4.

At industrial or commercial stage, all bioprocesses are carried out in vessels called **fermenter** or **bioreactor**. We are also aware that after the advent of rDNA technology,

microbes are extensively employed for the production of a number of biological material for the welfare of mankind.

Box 1

Discovery of Penicillin

It was in 1928 when Alexander Fleming at St. Mary's Hospital in London, while trying to isolate boils causing bacterium, *Staphylococcus aureus,* found that one of the Petridishes was contaminated inadvertently with a foreign entity. Instead of discarding the Petri plate for disinfection, Fleming made an important observation in the unwashed contaminated plate that no bacteria grew near the invading entity. The observation surprised Fleming's intellect and he soon realised that this chance observation may be a meaningful arena of interest.

Later this antibacterial foreign entity was identified as a common mould, the *Penicillium notatum*, and the metabolite secreted has powerful antibacterial properties called penicillin. Its full potential as an effective antibiotic was established by Ernest Chain and Howard Florey. This antibiotic was extensively used to treat the American soldiers wounded in World War II. Fleming, Chain and Florey were awarded the Nobel Prize in 1945 for this discovery.

10.2 Instrumentation in Bioprocessing: Bioreactor and Fermenter Design

Bioreactor is an engineered vessel made up of glass or steel that supports a biologically active environment, where cells can be cultivated under aseptic conditions with appropriate nutritional and environmental requirements. In a bioreactor, the biochemical processes involve the cultivation of microbial, plant and animal cells or biochemically active substances derived from such cell cultures or organisms. Commonly, bioreactors are cylindrical and vary in size. The design and components of a typical bioreactor are shown in Fig. 10.1.

A bioreactor should fulfil the following requirements:

1. a **sterile environment**, so that a pure culture may be grown without contamination
2. **adequate supply of air** for cellular respiration in culture
3. **uniform mixing of nutrients**, cells and air throughout the bioreactor vessel without causing any shear stress to the cultured cells

4. a system for **maintenance of optimum temperature** conducive for the growth and product formation in a desired culture
5. a system for **monitoring the environmental process** parameters, such as pH, dissolved oxygen, etc.

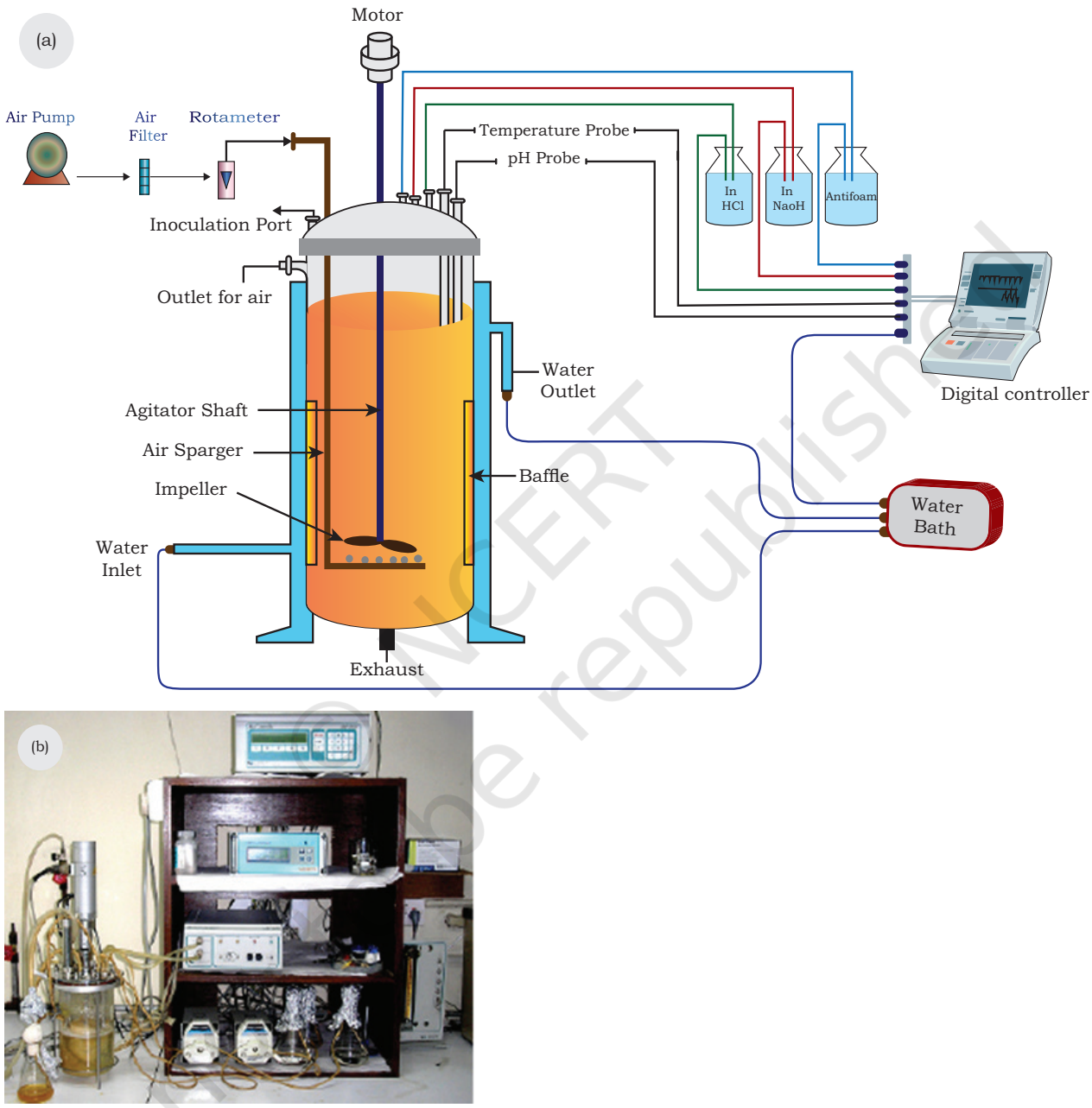


Fig. 10.1: (a) Diagrammatic representation of the design and components of a typical bioreactor (b) Photograph of a laboratory bio-reactor

Thus, in order to fulfil these requirements a typical bioreactor consists of the following:

- **Agitator shaft:** It helps in mixing contents of the bioreactor and keeps the cells in perfect homogenous conditions that provide better transport of nutrient and oxygen throughout the running bioprocess. An impeller is fixed at the bottom of an agitator shaft.
- **Sparger**: It helps in providing an adequate and continuous supply of sterilised air (oxygen) using microfilters for growing cells submerged in the liquid media inside the bioreactor system.
- **Baffle**: It helps in breaking the vortex formation, which is highly undesirable as it changes the centre of gravity of the system making it consume extra power to run the system.
- **Jacket**: It provides area for the circulation of water at the given temperature for maintenance of optimum temperature inside the device required for the growth of cultivated cells and product formation.
- **Sensitivity probe for temperature and pH:** These are the probes to sense temperature and hydrogen ion concentration of a bioprocess.
- **Digital controller for controlling process parameters:** Digital controller is connected to bioreactor through probes and its one separate unit is connected to a water bath that pumps water of desired temperature in and out of the jacket present around the bioreactor unit for the maintenance of temperature throughout. It is also connected to pH probes and bottles containing 1M NaOH and 1N HCl. As the probe senses acidity or alkalinity, digital controller commands any of the two bottles for addition of either dilute NaOH or HCl for the maintenance of the desired pH. All process parameters including temperature, pH, speed of stirring (*rpm*), etc., are exhibited over the display of the controller.

10.2.1 Types of bioreactors

On the basis of the design or configuration, important types of bioreactors are discussed below:

- **Stirred tank reactors** are the most conventional bioreactors. In these reactors, agitator facilitates the

mixing of nutrients, oxygen and growing cells. The reactor is characterised by the presence of agitator shaft. Design, shape and size of the impeller differ for various bioprocesses [Fig. 10.2(a)].

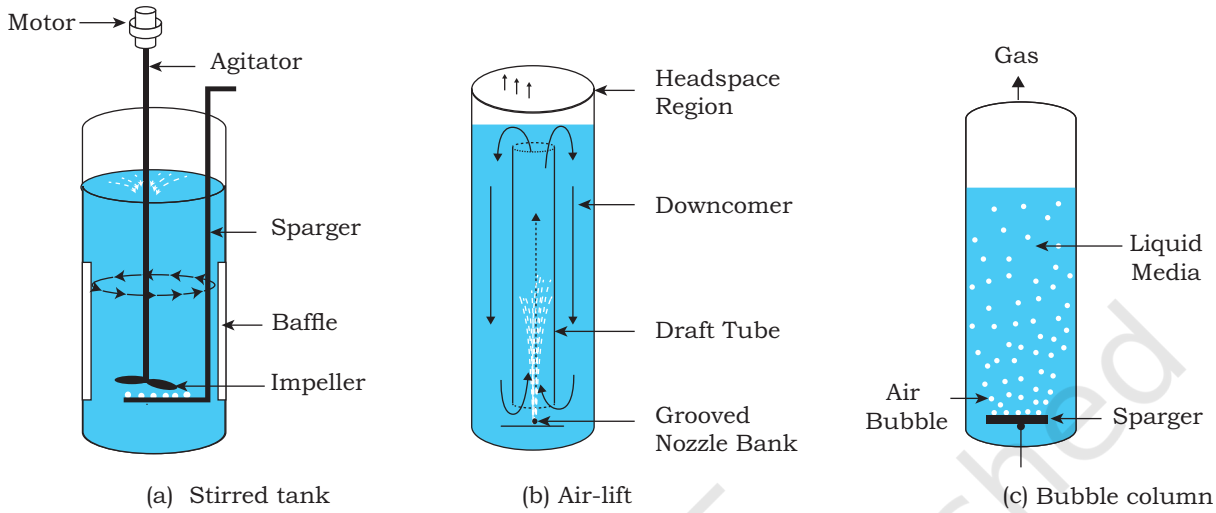


Fig. 10.2: Various types of bioreactors

- In **Air-lift reactors**, a motion of air is created using a draft tube. The mixing of nutrients and oxygen is maintained by creating the air current that lifts the fluid broth and cells up and down, inside-out or vice-versa through a draft tube inside the reactor vessel [Fig. 10.2(b)].
- In **Bubble column** reactor, the mixing of nutrients and oxygen is maintained with the help of air bubbles produced through sparger jet. These reactors provide low shear environment, which may be a critical consideration for some cells and high oxygen transferred per unit of power input [Fig. 10.2(c)].

10.3 Operational Stages of Bioprocess

A bioprocess is composed mainly of two stages for converting raw material into the final product namely, upstream and downstream processing (Fig. 10.3).

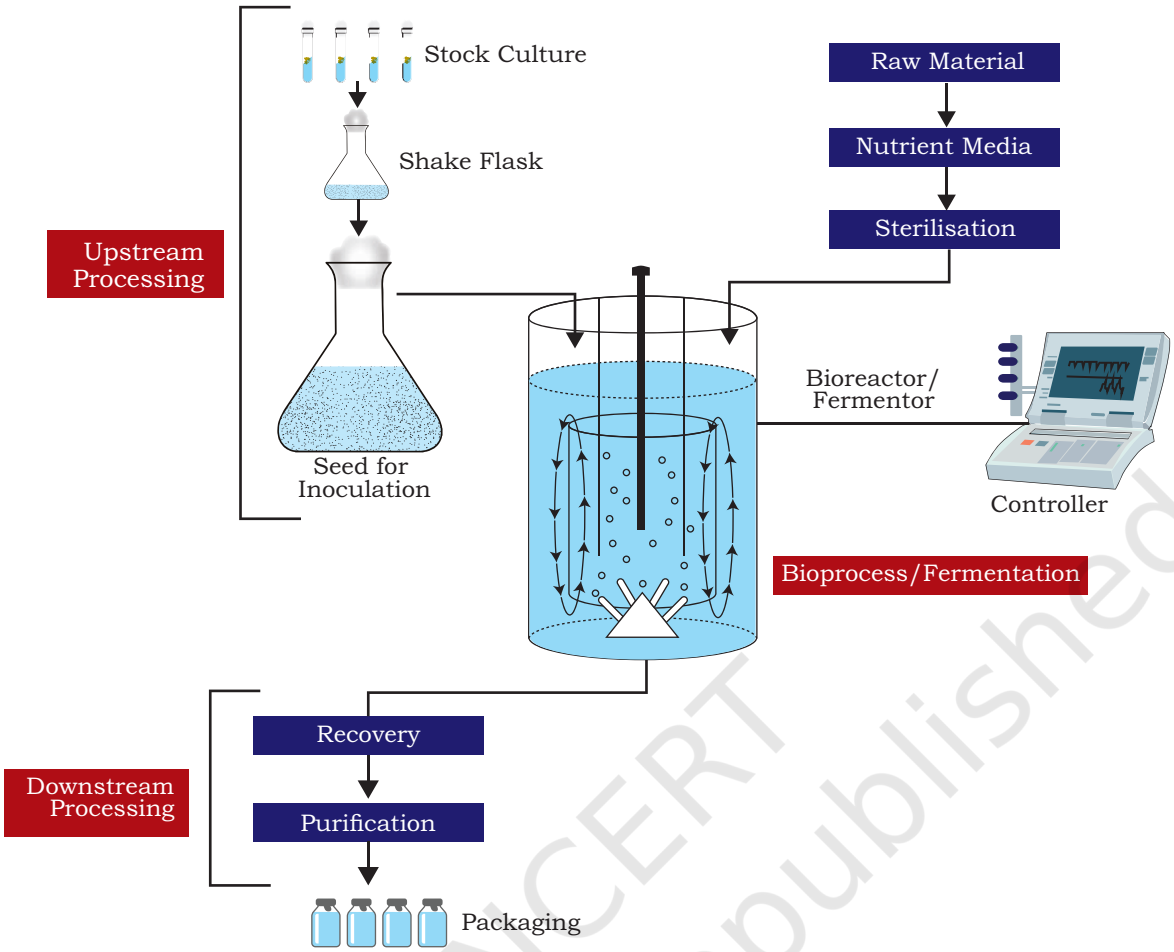


Fig. 10.3: Different stages of upstream and downstream processing

The upstream bioprocessing consists of four components as detailed below:

1. optimisation of nutritional conditions in artificial media and formulation for culturing the living organisms, cells or its components
2. sterilisation of media, bioreactor and other additional tools and equipment
3. production of pure, active and healthy inoculums in sufficient quantity
4. optimisation of environmental conditions for growth and product formation

On the contrary, downstream processing consists of two steps as detailed below:

1. extraction, recovery and purification of product
2. the disposal of effluents produced by the process

Upstream and downstream processing are described in detail in the following sections.

10.3.1 Upstream processing

A typical upstream bioprocess involves raw materials such as the biomass of microbial, plant or animal cells to be usually treated and mixed with other ingredients that are required for the cells to grow well. The raw material to be used in bioprocessing is first converted to a suitable fermentable form. Preparation of liquid or solid nutrient medium, sterilisation, aeration, agitation and shear sensitivity besides many other preparatory operations and scaling up for high product formation are described in the following sections.

Nutrient media or culture media required for maximum growth and product formation of a particular culture is formulated using chemicals and nutrients, etc. Different media formulations are prescribed for microbial, plant and animal cell culture (Chapter 6–8).

Requirement of medium constituents varies with the species of an organism for biosynthesis and cell maintenance. The following equation based on stoichiometry may be considered for growth and product formation:

Carbon and energy source + Nitrogen source + other requirements → Cell biomass + products + Carbon dioxide + Water + heat

The equation is important for economical designing of the media by minimising wastage of media components. For example, under aerobic conditions, carbon requirement of a particular culture may be estimated by determining the cellular yield coefficient (Y) as:

$$Y = \frac{\text{Quantity of cell dry weight produced by the culture}}{\text{Quantity of carbon substrate utilised}}$$

Similarly, in a bioprocess other media components may also be determined for their minimal wastage and optimum productivity of the culture. Thus, media formulation is very important for a bioprocess.

Inoculum of viable, healthy, fast growing and high producing living cells, organ or organism is very much required for good growth and technologically viable and

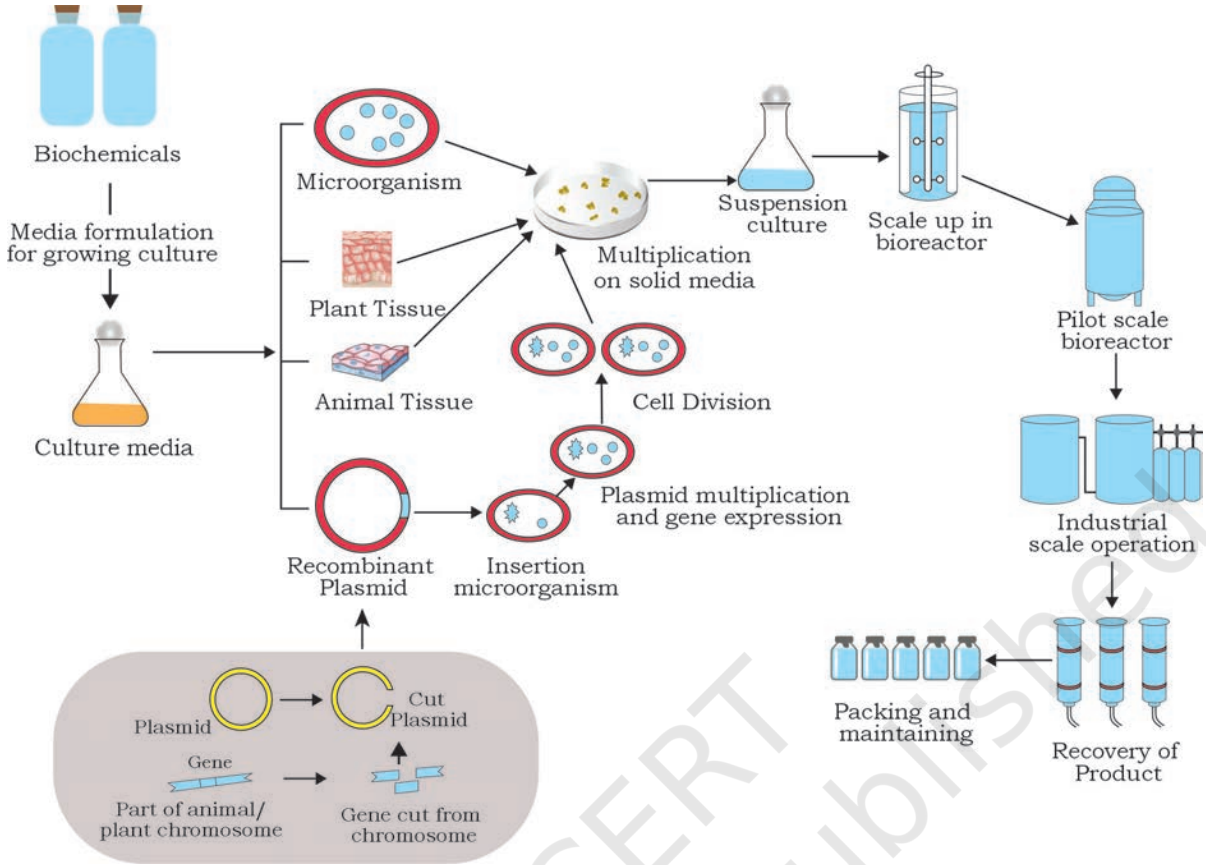


Fig. 10.4: Bioprocess Development

economically efficient production. Inoculum may be developed on solid culture or liquid culture. Liquid culture may usually be developed in shake flask and called suspension culture. The culture to be used as inoculum must fulfil the following criteria:

1. inoculum should be healthy and in log or exponential phase of the growth cycle (i.e., actively dividing).
2. in subsequent transfer, inoculum should not exhibit long lag phase.
3. it should be available in sufficient amount to provide an inoculum of optimum size.
4. it should be available in a suitable morphological form.
5. it should be free of contamination.
6. it should retain its product forming capability.

Aeration is required to fulfil oxygen requirement of submerged suspension culture in shake flask and bioreactors. Ideally, maximum amount of dissolved oxygen in pure water is approximately 8 g/L, which is available for

growing submerged culture in liquid media. In bioreactor, pure sterilised air is sent through sparger to maintain the oxygen levels in the media for growth of the culture.

A mild **agitation** is required for uniform distribution of oxygen and other nutrients. Some cells such as animal cells are more shear sensitive than plant cells. Plant cells are more sensitive to shear than microbial cells. Thus, in shake flask, shear stress or sensitivity of cells to shear can be controlled by increasing or decreasing the speed of shaker, i.e., revolution per minute (*rpm*) of the shaking condition. The same is maintained in bioreactors by increasing or decreasing the speed of agitator.

Temperature required for maximum growth and production has to be maintained. Temperature requirement for different cultures is different. There may be an optimum temperature for the formation of a desired product. The optimum temperature may be similar or different for culture growth and product formation.

Hydrogen ion concentration (pH) is another parameter that affects growth and product formation. Optimum pH for growth and product formation has to be maintained for an efficient bioprocess.

Thus, before a bioprocess is run, all processing parameters, such as nutrient media formulations, temperature, hydrogen ion concentration, etc., are optimised separately.

Sterilisation is an essential requirement for a successful bioprocess. It requires sterilisation of tools, glassware, media, air or in-site sterilisation of bioreactor, etc., and even the maintenance of aseptic conditions to perform processes and scale up in the bioreactor. Thus, contamination in a bioprocess may be avoided by the following:

1. sterilisation of nutrient media
2. sterilising the bioreactor vessel
3. sterilising all the materials to be added to the bioreactor vessel during the process.
4. maintenance of aseptic conditions during the fermentation.
5. using a pure culture as inoculum.

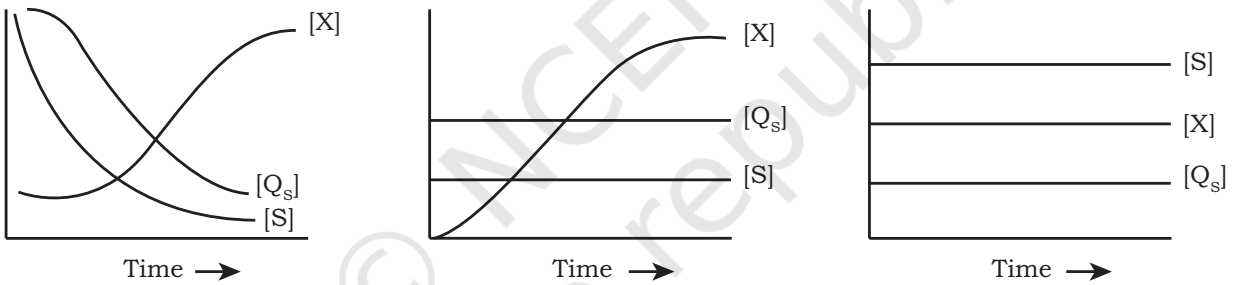
- A typical bio process development stage are summarised in fig. 10.4

Modes of Bioprocess Operation

One of the important decisions in the development of a bioprocess is that the mode of bioprocess operations is to be applied to a particular bioprocess. Mainly there are three different modes under which a bioprocess may be operated:

1. Batch
2. Fed-batch
3. Continuous

1. Batch mode: It is a closed culture system with an initial fixed amount of nutrients. An inoculated batch culture passes through a number of phases of growth cycle. When cells are grown in a nutrient medium, cells start growing in number and size to some extent. In a suitable nutrient medium, the cells utilise nutrients from the medium for growth and energy production and convert the material to be bioprocessed (fermented) into the product. Fig. 10.5(a) is showing cell biomass [X], rate of substrate consumption [Qs] and substrate concentration [S] in a batch culture.



(a) Batch mode (b) Fed-batch mode (c) Continuous culture mode

S = Substrate conc, X = Microbial cells, Q_s = Rate of utilisation of substrate

Fig. 10.5: Graphical representation of growth of cell culture in (a) Batch mode (b) Fed batch mode and (c) Continous culture mode.

2. Fed-batch mode: In a batch culture if the growth of cells become limited due to the concentration of one or more substrate components, the same are fed intermittently or continuously to the growing culture as per the requirement from time to time. In such culture, volume in the fermenter increases due to extra feed added and no removal of any volume of culture. This is known as fed-batch mode of culture, which is advantageous for maintaining the residual substrate concentration at very low levels and

thereby avoiding their toxic effects. In this culture system, substrate concentration's' and rate of substrate utilisation [Q_s] remains constant and cell biomass/product keeps on increasing Fig. 10.5(b).

3. Continuous mode: In this mode, the design of the bioprocess (reactor) is such that the fresh lot of nutrient media is added and the used media is removed in such a way so that continous supply of the desired product is to be ensured. Similarly, a fresh lot of inoculum could also be added. Thus, in the continuous mode, a steady state is maintained that is, the formation of new biomass by the culture is balanced by the loss of cells from the reactors vessel Fig 10.5(c). Thus, during steady state rate of growth and product formation, substrate concentration and rate of substrate utilisation remains constant.

Downstream processing

In the process, the desired product is recovered in an efficient way which involves efficient separation and purification technique. Thus, the product of bioprocess may be cell biomass, extracellular component of liquid media (broth) or intracellular product of cell. The major process of downstream processing is shown in Fig. 10.6. The extraction and purification of product in the culture fluid may be difficult and costly. High quality efficient recovery requires the following considerations:

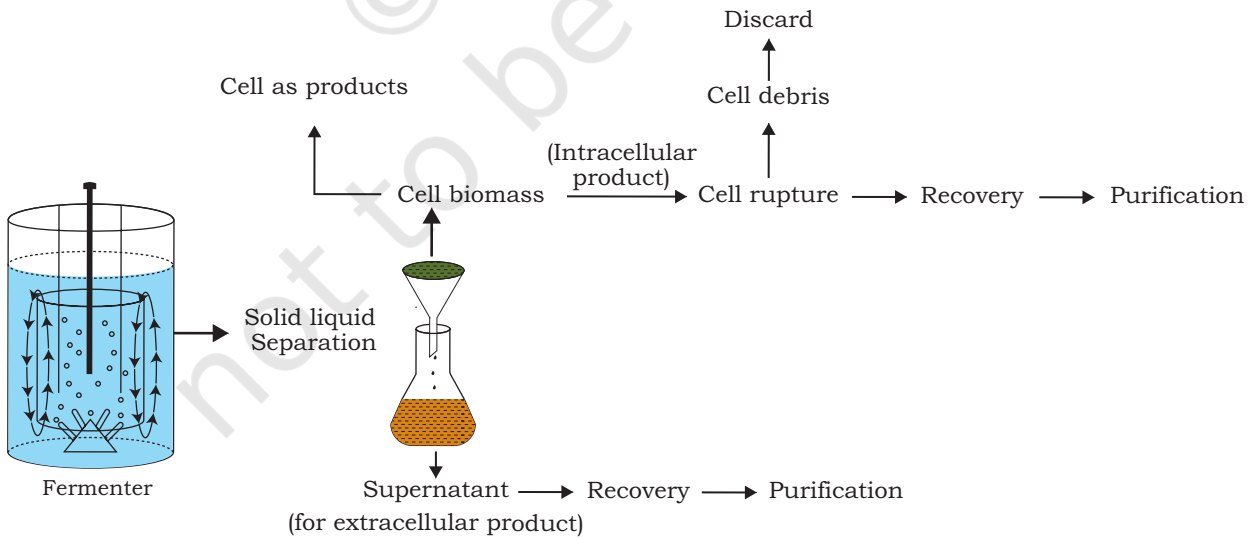


Fig. 10.6: Major steps in downstream processing

1. the process chosen must be quick.
2. the chosen process must have minimum investment and operated at minimum cost.

The downstream processing mainly consists of physical separation as well as purification operations, which includes the separation of particulate, dialysis, reverse osmosis, solid–liquid separations, adsorption, liquid-liquid extraction, distillation, drying, etc.

Solid–Liquid Separation: The first step in product recovery is separation of solids, such as biomass, insoluble particles and macromolecules from culture fluid or fermentation broth. In some cases, the culture broth or fermentation fluid needs pre-treatment, such as heating or pH adjustment or treating with coagulating and flocculating agents for the separation of insoluble products from the fluid or broth. Major methods used for the separation of cell biomass are filtration or centrifugation.

Filtration is the most common cost-effective method to be used for separation of large particles and cell biomass from the culture fluid. The conventional filtration involves the separation of large particles (pore diameter dp>10 mm) by using canvas, synthetic fabrics or glass fibre as filter medium. Continuous rotary filters are most widely used filters in the industry. Ultra-filtration or microporous filtration is also used for the separation of cell biomass.

Centrifugation is used for the separation of particle size between 100 μ – and 0.1 μ – from liquid in centrifuge and ultracentrifuge.

Cell disruption: if the product is intracellular, it may be recovered by cell rupture. Cell rupture techniques may be powerful but mild enough so that it should not damage the desired product. Disruption of cell may be achieved by physical, chemical and biological methods.

Physical methods include the mechanical means of cell disruption by milling in high-speed bead mills, homogenisation by creating very high shear rates using high pressure homogeniser and ultrasonic vibrations through sound waves in ultrasonicator. Cell disruption using ultrasonicator is very effective with most of the cell suspension culture.

Non-mechanical methods include the treatment of cells with either chemicals, such as surfactants, alkalis, organic

solvents, or by osmotic shocks besides biological methods such as enzymatic degradation of the cell wall.

Recovery

After solid and liquid are separated, a dilute aqueous solution is obtained from which the product has to be recovered and purified. Extraction and adsorption are the processes that are exclusively categorised as techniques for recovery of the product.

Choice of recovery process is based on the following criteria:

1. intracellular or extracellular location of the product.
2. concentration of product in the culture fluid.
3. physical and chemical properties of the desired product.
4. minimal acceptable standard of purity.
5. impurities in the culture fluid.

The separation of a component from a liquid mixture by treatment with a solvent in which the desired component is preferentially soluble is known as **liquid–liquid extraction**. The specific requirement is obtaining a high percentage extraction of product but concentrated in a smaller volume of solvent. Efficient extraction requires choosing a suitable solvent for extraction and optimising conditions of temperature, pH, light, etc. After complete extraction, the solute rich phase is called the extract and the residual liquid from which solute has been removed is called raffinate.

Purification: Purification techniques include precipitation, chromatography, electrophoresis, membrane separation, dialysis, reverse osmosis, ultra-filtration, etc., some of these technique are also used for the recovery of products. In Class XI, you have already learnt about chromatography and electrophoresis. Some of the other purification techniques are discussed below:

- **Precipitation** is a technique widely used for the recovery of proteins and antibiotics. It can be induced by the addition of salts, organic solvents and ultrafiltration.

Membrane Separation process can be classified into three categories: microfiltration, ultrafiltration and reverse osmosis. These are all pressure driven membrane separation processes in which separation is achieved through very small pore sizes. In microfiltration, the pore

size from 0.1 to 10 μm, while in ultrafiltration, it ranges 0.01 – 0.1 μm.

Table 10.1: Different types of filtration processes

Process	Size Cutoff	Molecular Wt. Cutoff	Pressure Drop (psi)	Material Retained
Microfiltration	0.1–10 μm	<1000,000 Da	10	Suspended material (bacteria, etc.)
Ultrafiltration	0.01–0.1 μm	300-300,000 Da	10–100	Biological, colloids, macromolecules
Reverse osmosis	<0.001 μm	<300 Da	100–800	All suspended and dissolved materials

Protein products are under the range of molecular cut-off for ultrafiltration.

If saline water is separated from pure water by a semipermeable membrane, osmosis occurs, i.e., water molecules move from pure water phase to saline water phase [Fig. 10.7(a)]. As the water moves to saline phase, its pressure increases. This pressure is called osmotic pressure [Fig. 10.7(b)]. In **reverse osmosis** (RO), pressure is applied onto a salt containing phase, which drives water molecules in reverse direction, that is, from salt containing phase to pure water phase [Fig. 10.7(c)]. The pressure required for the movement of water in reverse direction is slightly larger than the osmotic pressure as the solvent flux takes place in the direction against the concentration gradient [Fig. 10.7(c)].

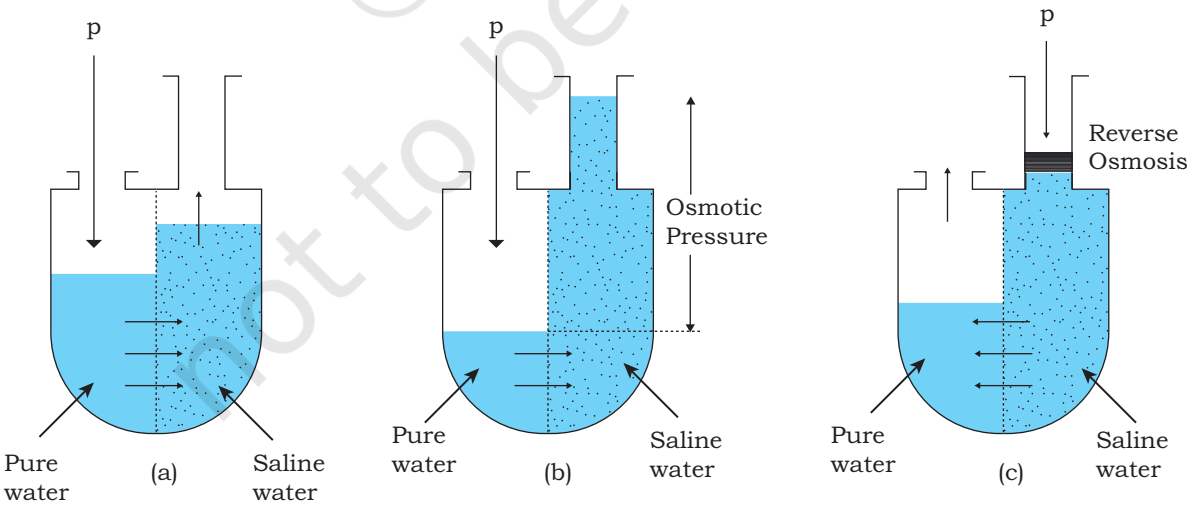


Fig. 10.7: Reverse Osmosis: Pressure driven membrane separation processes

Dialysis is an operation used for the removal of low-molecular weight (MW) solutes, such as organic acids (MW = 100–500 Da) and inorganic ions (molecular weight = 10–100 Da) from a solution through a selectively permeable membrane. A well-known example is the use of dialysis membranes to remove urea (MW = 60) from urine in artificial kidney (dialysis) devices.

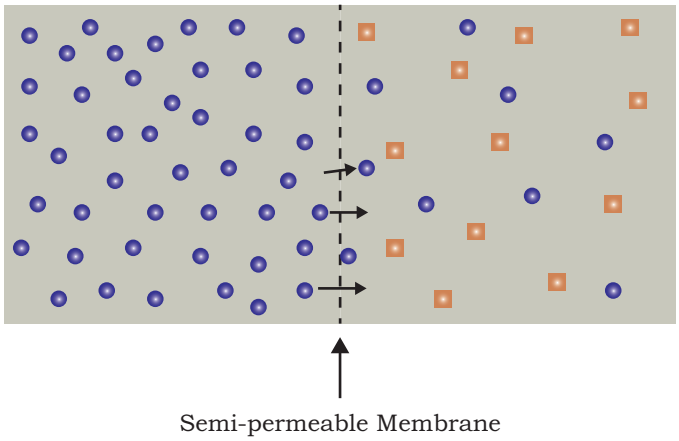


Fig. 10.8: Dialysis

As depicted in Fig. 10.8, the dialysis membrane separates two phases containing low and high molecular weight solutions. Since, the cut-off size of pores of a dialysis membrane is very small, only low molecular weight molecules move from a high to low concentration region. At equilibrium, the chemical potentials of diffusing compounds on both sides of a membrane are equal.

10.4 Bioprocessing and Biomanufacturing of Desired Products

These days bioprocessing industries have successfully provided a number of valuable products from primary (amino acids and organic acids) as well as secondary metabolites (antibiotics) using microorganisms, animal and plant cells as well as their constituents. Some of the examples are: production of alcohols, antibiotics, amino acids, organic acids, enzymes, vitamins, vaccines, recombinant proteins, pigments, plant alkaloids, etc., (Table 10.2). These products have now become an integral part of our day-to-day life. Some of these are described in Table 10.3.

Since time immemorial, yeasts have been used for the production of ethanol. A common species of yeast *Saccharomyces cerevisiae*, commonly called brewer's or baker's yeast, is used in the fermentation of malted cereals and fruit juices for ethanol production. Depending upon the type of raw material and the processes, a variety of alcoholic drinks are obtained. Wine and beer are produced without distillation, whereas whisky, brandy and rum are produced by distillation of the fermented broth.

Table 10.2: Examples of variety of bioprocessing products

Types	Products
Cell biomass	Baker's yeast, single cell protein
Extracellular	Alcohols, organic acids, amino acids, enzymes, antibiotics
Intracellular	Recombinant DNA protein

Table 10.3: Some of the major products of bioprocesses at commercial level

Products	Category	Microorganisms or Plants or Animals cells used
Ethanol	Alcohol	*Saccharomyces cerevisiae*
L-glutamic acid	Amino acids	*Corynebacterium glutamicum*
Lactic acid	Organic acid	*Lactobacillus delbrueckii*
Proteases	Enzymes	*Bacillus spp.*
Pectinase	Enzymes	*Aspergillus niger*
Penicillin	Antibiotics	*Penicillium chrysogenum*
B_{12}	Vitamins	*Propionibacterium shermaniior, Pseudomonas denitrificans*
Diphtheria vaccine	Vaccines	*Corynebacterium diphtheriae*
Insulin	Recombinant Proteins	Recombinant *Escherichia coli*
Shikonin	Pigments (quinone derivatives or naphthaquinone)	*Lithospermum erythrorhizon*
Taxol	Plant alkaloids	*Taxus brevifolia*

Antibiotics production is another significant contribution of bioprocessing and biomanufacturing towards the welfare of human society. It has already been discussed earlier in this chapter as to how the penicillin, the first antibiotic, was discovered which in fact was a chance discovery. After penicillin, other antibiotics were also purified from other microbes.

Amino acids, such as lysine and glutamic acid, are useful in food industry as nutritional supplements and flavour enhancing compounds, respectively. Production of amino acids is typically carried out by mutants that have reduced the capability to synthesise a specific amino acid or a key intermediate. Mutants of *Corynebacterium glutamicum* are used for the commercial production of glutamic acid and lysine. Certain chemicals, such as organic acids, enzymes and several other bioactive molecules, are produced commercially in industry through bioprocessing. Several species of microorganisms are used in the bioprocessing of a number of organic acids. A fungal species *Aspergillus niger* was used for citric acid production, and species of bacteria *Acetobacter aceti* was used for the production of acetic acid; *Clostridium butylicum* for the production of butyric acid and *Lactobacillus* sp. for the production of lactic acid.

Enzymes are also produced commercially through bioprocessing. Lipases are used in detergent formulations and are helpful in removing oily stains from the laundry. You must have noticed that bottled fruit juices bought from the market are clearer as compared to home-made ones. This is because the bottled juices are clarified by the use of pectinases and proteases. Proteases are also used in leather industry. Bioprocessing of these enzymes at commercial level uses a variety of fungal genera including the species of *Aspergillus*, *Bacillus*, *Mucor*, *Trichoderma,* etc. Streptokinase, produced using *Streptococcus* species and modified by genetic engineering, is used as a 'clot buster' for removing clots from the blood vessels of patients who have undergone myocardial infarction leading to heart attack. Another bioactive molecule *cyclosporin A*, used as an immunosuppressive agent in organ-transplant patients, is produced using the fungus *Trichoderma polysporum*. Statins produced by the yeast *Monascus purpureus* have been commercialised as blood-cholesterol lowering agents. It acts by competitively inhibiting the enzyme responsible for the synthesis of cholesterol.

Several vitamins specifically B_{12} and riboflavin are commercially produced by fermentation using microorganisms. Vitamin B_{12} was first obtained as a

by-product in the production of various antibiotics—streptomycin, chloramphenicol, or neomycin, using the fermentation of bacterial genera *Streptomyces*. Later, high-yielding *strains of Propionibacterium freudenreichii, Pseudomonas denitrificans, Bacillus megaterium* and *Streptomyces olivaceus* were developed for the production of vitamin B_{12}.

Riboflavin is commercially produced through biotransformation as well as fermentation. In biotransformation, glucose is first converted to D-ribose by mutant strains of *Bacillus pumilus*. The D-ribose so produced is converted to riboflavin by chemical reactions. In acetone-butanol fermentation using *Clostridium acetobutylicum* and *Clostridium butylicum*, riboflavin is formed as a byproduct. Commercial production of riboflavin is predominantly carried out by direct fermentation using the ascomycetes. High-yielding strains of *Ashbya gossypii* is preferred due to its high producing capability of riboflavin.

Vaccines, the single most important health weapon, have also been produced through bioprocess using various cell or microbial cultures. *Corynebacterium diphtheria* is used in the process for the production of diphtheria toxin, which in turn is processed as diphtheria toxoid and then to diphtheria toxoid vaccine. The cell-based vaccine manufacturing process uses cells from mammals to culture the influenza virus for vaccine production. Various pharmaceutical companies use different sources of mammalian cell cultures for the vaccine manufacturing process.

Plant cell and tissue culture have long been used in bioprocess for commercial production of a variety of biochemicals, such as pigments, quinone derivatives, plant alkaloids, etc., which have been used in a variety of tasks, such as dying, clothes, food additives, pharmaceutics, etc. The bioprocess for the production of dye shikonin was commercially developed for the first time using cell culture of plant species *Lithospermum erythrorhizon*. Successful commercial production of berberine, ginseng, saponin and taxol are the examples of bioprocessing that uses plant cell and tissue culture of *Coptis japonica*, *Panax ginseng* and *Taxus brevifolia*.

After having cloned the gene of interest and having optimised the conditions to induce the expression of the target protein, one has to consider producing it on a large scale. A protein encoding gene expressed in a heterologous host leads to the synthesis of desired biological product, which is a **recombinant protein**. Cells harbouring cloned genes of interest may be grown on a small scale in a laboratory. The culture may be used for extracting the desired protein and then purifying it by using different separation techniques. The recombinant DNA method is used for large scale production of human insulin. Two-phase cultivation process is followed for the production of human insulin; a glycerol batch and a continuous methanol fed-batch.

This way in industrial applications, a bioprocess is run with optimised process parameters for high production of desired compounds. Many compounds are in pipeline and the researches are going on all over the world for commercial production of the desired bioactive compounds from living organisms.

SUMMARY

- There are various metabolic processes in living systems, which are responsible for the synthesis of many metabolites, which can be classified into primary and secondary.
- Primary metabolites are essential for the growth and development of living organisms whereas, secondary metabolites have diverse functions in defense system, tolerance to abiotic stress, etc.
- Secondary metabolites are used in many industries, such as pharmaceuticals, cosmetics, drugs, food additives, etc. However, these compounds are synthesized in very small amounts in the natural system. Therefore, efforts are being made to scale up the production of these beneficial metabolites using bioprocess engineering.
- Bioreactor or fermenter is an engineered vessel, which may provide optimum conditions for the product formation and based on the requirement , different types of bioreactors may be configured in bioprocessing.
- Bioprocessing can be operated through two stages: upstream processing and downstream processing.

- In upstream processing, formulation and sterilisation of the media and equipment take place along with the production of pure, healthy and active culture for inoculation.
- The growth of organisms under optimum conditions for desired product formation takes place in a bioreactor or a fermenter.
- There are three modes of operations in bioprocessing: (i) batch (closed vessel system), (ii) (fed-batch) (growth limiting substrate is fed intermittently or continuously) and (iii) continuous (growth limiting substrate is fed continuously).
- In downstream processing, the product is recovered and purified using various techniques, such as reverse osmosis, distillation, drying, etc.
- Many desired products of animals, plants and microbial origin have been commercialised till date.

EXERCISES

1. Differentiate between primary and secondary metabolites based on their functions with example.
2. Explain the challenges encountered during the development of a bioprocess.
3. Describe briefly the design and components of a typical bioreactor and their applications.
4. Explain the basic operational stages of a bioprocess using concept map.
5. Describe briefly the following:
   (a) upstream processing
   (b) downstream processing
6. Explain the recovery and purification process of an intracellular product with the help of a flow diagram.
7. Write short notes on the following:
   (a) reverse osmosis
   (b) dialysis
8. Match the following:

(a)	Agitator	(i)	Breaking the vortex formation
(b)	Sparger	(ii)	Provides area for circulation of water of desired temperature
(c)	Baffle	(iii)	Helps in mixing the contents
(d)	Jacket	(iv)	Provides adequate and continuous supply of air

9. A culture in a closed vessel to which no additional medium is added is called _____ culture.
   (a) Continuous
   (b) Batch
   (c) Fed-batch
   (d) Semi continuous
10. **Assertion:** Secondary metabolites are used in defense against pathogens, phytoplanktons, improving tolerance to abiotic, etc.

    Reason: Secondary metabolites are intermediate or indirect products.
   (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.
   (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.
   (c) Assertion is true but reason is false.
   (d) Both assertion and reason are false.

UNIT IV
Bioremediation

Chapter 11: Bioremediation

Ananda Mohan Chakrabarty
(4 April 1938 – 10 July 2020)

Ananda M. Chakrabarty is an Indian American microbiologist, who is known for his work in developing genetically engineered organisms using plasmid transfer. In 1965, he earned his Ph.D. from the University of Calcutta and moved to the University of Illinois in United States for higher studies. He started to work with environmental microbiology with an aim to develop microbes for biodegradation. He specifically worked with *Pseudomonas sp.* and in 1971, developed a genetically engineered *Pseudomonas* strain that was capable of using oil as nutrient for its growth. He called these microbes as "multi-plasmid hydrocarbon-degrading *Pseudomonas*", which could digest most of the hydrocarbons found in oil spills. He was the first to get a patent for a recombinant microbe. Patenting living organisms was not available at that time and his efforts to patent a recombinant microbe landed in the Supreme Court in 1980. This famous trial "Diamond v. Chakrabarty" made it possible to patent microbes or higher organisms. Later, his research group has done pioneer work with cupredoxin (proteins) from *Pseudomonas* and its role for cancer biology.

Prof. Chakrabarty served as an expert on legal issues related to patents or intellectual property rights. In 2007, Indian government recognised his efforts and he was awarded with the Padma Shri.

Chapter 11 Bioremediation

Human activities at domestic, agricultural and industrial level have resulted in the entry of a large number of pollutants in air, water and soil resulting in an alarming situation in many areas across the world. Many of these untreated chemical toxicants and excess of fertilisers and pesticides used in agriculture are drained to various water bodies and poses threat to the ecosystem and its flora and fauna including human beings. It is very much desirable that such pollutants must be eliminated from the ecosystem. Recently, a good number of microorganisms have been reported to be capable of modifying and degrading such synthetic agrochemicals. In this chapter, we will discuss the management of wastes and pollutants by conventional approach as well as by using microorganisms and plants. Since living organisms (bios) are employed to remediate or solve the problem of damage caused by chemical toxicants, the process is often referred to as bioremediation.

11.1 Waste Water Treatment

Waste water from a home, community or industry is collectively called sewage. Such wastes are very difficult

to treat due to the presence of antiseptic, chemicals and its high oxygen demand. Almost all the industries (dairy, tannery, cannery, distillery, oil refinery, textile, coal and coke, synthetic rubber, steel, etc.) produce their own characteristic sewage. Some are readily treated while others are practically unamendable through biological treatment. An average individual in India produces 0.8 kg waste per day.

11.1.1 Composition of Sewage

Sewage consists of human excreta, wash waters, industrial waste, agricultural wastes and wastes from livestock, i.e., poultry, cattle, horse, etc. The bulk municipal sewage consists of approximately 99 percent water and 1% inorganic and organic matter in suspended and soluble forms. Lignocellulose, cellulose, proteins, fats and various inorganic particulate matter exist in suspended state, whereas sugars, fatty acids, alcohols, amino acids and inorganic ions constitute the soluble forms. The organic content of sewage is measured in terms of its oxygen equivalence by means of the biochemical or biological oxygen demand (BOD) test. BOD may be defined as the quantity of oxygen required during the stabilisation of

Table 11.1: Composition of untreated and treated domestic sewage

Constituent	Concentration (mg/L) before treatment	Concentration (mg/L) after treatment
Suspended Solids (SS)	100–750	Upto 35
Total Nitrogen	20–80	Upto 15
Total Phosphorus	05–20	Upto 5
Chlorides	230–2700	<250
Grease and oil	50–100	<10
Biochemical oxygen demand (BOD)	100–300	Upto 25
Chemical oxygen demand (COD)	600–900	75–100
pH	05–7.5	6.5–8.5
Total coliform	10^7–10^9	Not detectable

decomposable organic matter and oxidisable inorganic matter by aerobic (oxidative) biological action. The efficiency of mechanical, chemical and biological treatment of sewage is based on the amount of BOD reduction. Table 11.1 shows the composition of an untreated and treated domestic sewage.

Box 1: Biochemical Oxygen Demand (BOD)

Biochemical Oxygen Demand is a measure that signifies the amount of oxygen dissolved in water required by different microorganisms present in it. BOD is, in a way, measurement of the oxidisable organic matter present in sewage. Higher BOD value signifies higher amount of oxidisable organic matter present and such a sewage is called strong, whereas weak sewage has comparatively less oxidisable matter. Entry of strong sewage in a water body leads to a quick consumption of dissolved oxygen. Fishes and other aquatic fauna die due to very low level of dissolved oxygen, which ultimately decompose and increased level of organic matter ultimately makes water unsuitable for recreational purpose and unfit for drinking.

Measurement of BOD is done by dilution of a measured amount of sewage with water already saturated with oxygen followed by incubation of sewage and water mixture at 20°C simultaneously with a control, which is diluted water. After five days of incubation, the residual oxygen present in both the sewage sample and the control is measured. Difference in the level of oxygen is the expression of the capacity to consume oxygen by the sewage, which is expressed in parts per million (ppm). However, for the purpose of estimation of BOD of a sewage sample (in which the organic load may be too high and dissolved oxygen is too low to be measured), the same is diluted with double distilled water. One litre of BOD free water (double distilled water) in a bottle is aerated for one hour by an aerator followed by adjusting the pH to 7.0 using buffer. BOD is calculated as follows:

$$\text{BOD } (O_2 \text{ mg/L}) = \frac{D_1 - D_2 \times 100}{\% \text{ dilution}} \quad \text{or } (D_1 - D_2) \times \text{Dilution factor}$$

D_1 = Dissolved oxygen (DO) of sample before incubation

D_2 = Dissolved oxygen (DO) of sample after incubation

Calculation of Dilution factor

S.No.	Volume of Sample (mL)	Volume of Dilution Water added (mL)	Dilution Factor
1	1000	NIL	1
2	500	500	2
3	200	800	5
4	100	900	10
5	50	950	20
6	20	980	50

Wastes disposed from the industries consist of detergents, antibiotics, paints, biocides, etc. The pulp and paper industries discharge cellulosic and inorganic chemicals. Therefore, sewage composition differs with the type of industrial effluents discharged into sewage systems.

A variety of microorganisms are present in sewage, for example, bacteria, fungi, protozoa, algae, nematodes, amoebae and viruses. The microbial population per millilitre of sewage may vary from a few lakhs to several millions. Bacteria occurring in sewage are mainly intestinal and soil inhabiting and their common types are *Coliforms*, *Streptococci*, *Clostridia*, *Micrococci*, *Broteus*, *Pseudomonas*, and *Lactobacilli*.

Box 2: Common pathogenic organisms found in water (domestic waste water) and the respective diseases

Organism	Disease
Bacteria	
Escherichia coli	Gastroenteritis
Salmonella typhi	Typhoid
Vibrio cholerae	Cholera
Shigela sp.	Shigellosis
Viruses	
Adenovirus	Respiratory diseases
Enteroviruses, Rotavirus	Gastroenteritis
Hepatitis A	Infectious hepatitis
Protozoa	
Entamoeba histolytica	Amoebic dysentery
Giardia lamblia	Giardiasis
Balantidium coli	Balantidiasis
Helminths	
Ascaris lumbricoides	Ascariasis
Schistosoma sp.	Schistosomiasis
Fasciola hepatica	Fascioliasis
Taenia saginata	Taeniasis

11.1.2 Waste water Disposal Plant

Treatment of sewage water is essential before its disposal. If raw domestic or sanitary sewage is discharged without its biological and/or chemical treatment directly into water bodies, the water becomes a vector of diseases, available oxygen and other hydrogen acceptors in the water are soon used by the microorganisms living in the sewage and then foul smelling anaerobic processes start creating nuisance. Survival of fishes and other forms of higher aquatic life is also negatively affected leading to economic losses. Further, the water that receives the sewage becomes unfit for drinking or recreation purposes. The objective behind the sewage treatment is to kill pathogenic microorganisms, prevent anoxia, raise the pH to alkaline side, increase photosynthetic rate, reduce organic content, etc. For this, waste water is first routed to sewage disposal plants for treatment before it is discharged into the water bodies. The wastewater treatment plants are designed to carry out various steps namely, primary (physical or mechanical) treatment, secondary (biological) treatment, and tertiary (advanced) treatment. The steps involved in all types of treatments are shown in Fig. 11.1.

11.1.3 Primary or Physical Treatment

In primary treatment, sewage is first subjected to physical (or mechanical) methods such as screening, passage of wastewater into grit chamber and sedimentation to remove its coarse solid materials. In screening, bulky foreign matter such as bottles, paper, wooden boxes, etc., are removed. The sewage is passed through grit chamber having filters of graded openings and then allowed to flow through sedimentation tanks or sedimentation basins. The sewage is held in these tanks usually from 2 to 10 hours. Coarse solid materials are concentrated in these tanks and collected from it. The particulate materials are now collectively called primary sludge, which is removed for further processing. At this point, treatment efficiency ranges from 30 to 40 percent BOD removal. Following sedimentation, the sludge and liquid effluent are processed separately during secondary treatment.

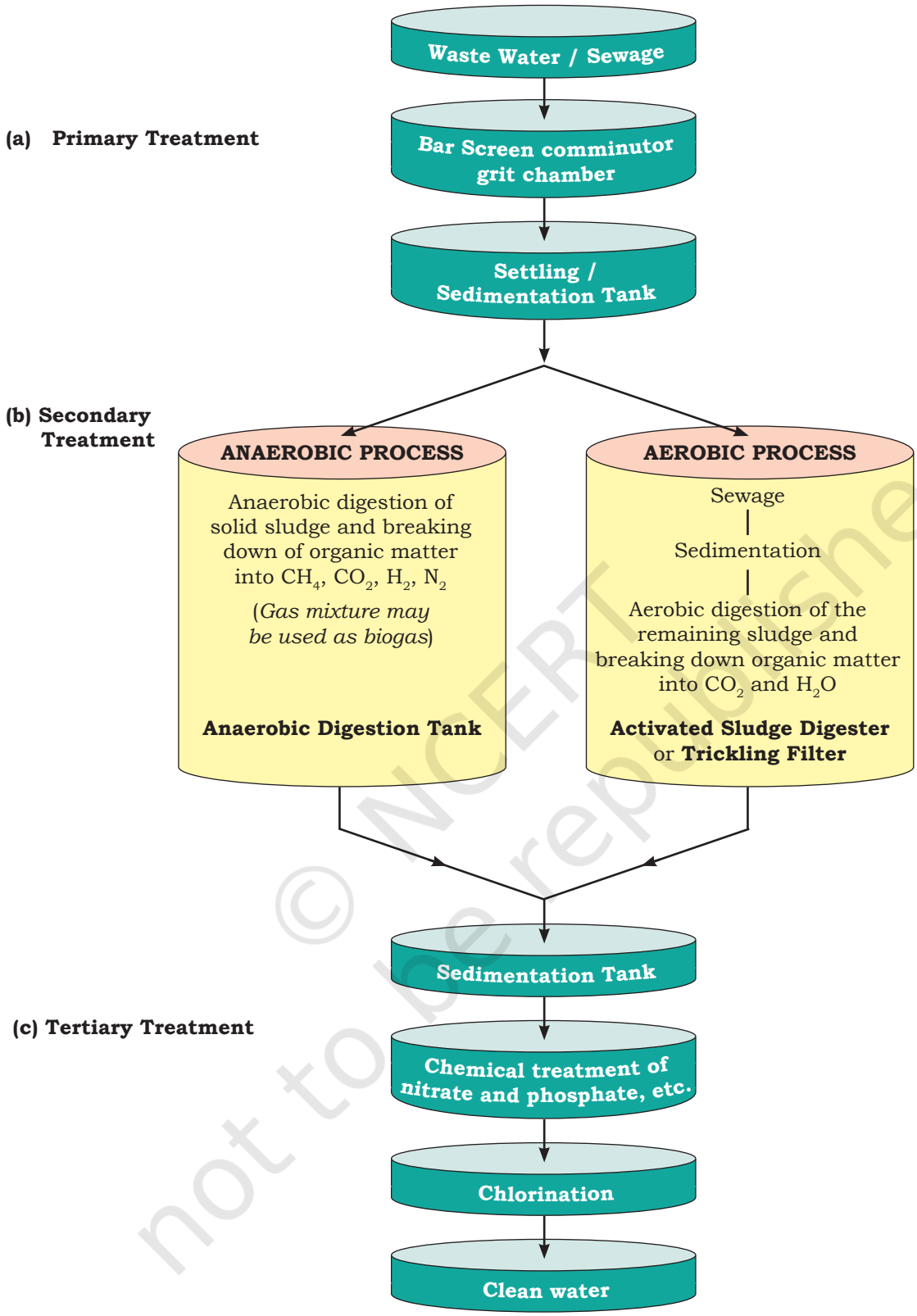


Fig. 11.1: Schematic representation of a modern municipal sewage treatment plant. (a) Primary (mechanical) treatment; (b) Secondary (biological) treatment; and (c) Tertiary (final) treatment

11.1.4 Secondary or Biological Treatment

The main function of secondary treatment is to remove much of the suspended organic material that remain in the effluent after primary treatment through the following steps. All biological waste treatment depends on microbiological degradation activities (hydrolysis, oxidation, reduction) on decomposable nutrients in sewage. Decomposition and stabilisation of wastes may be accomplished either anaerobically or aerobically, depending on the choice of the engineer, and both methods depend on the characteristics and amount of waste being treated.

The aerobic secondary treatment includes activated sludge process, aerobic digestion of sludge by various filters (e.g., trickling filters) and oxidation ponds, while anaerobic secondary treatment is represented by anaerobic digestion of sludge. However, regardless of the process being, waste stabilisation occurs in a series of more or less discrete stages. During activated sludge process a large volume of compressed air is passed through sewage to maintain aerobic condition. Aerobic microbes, during the activated sludge process, break down the organic carbon, nitrogen and phosphorus into minerals. In aerobic phase all of the complex, decomposable and organic matters in sewage are eventually changed largely into oxidised inorganic materials, such as sulphates, phosphates, nitrates, CO_2 and H_2O, i.e., they are mineralised.

Activated sludge process

Figure 11.2 explains the sludge process in which there is an aeration tank and a settling tank. The sludge that settles at the bottom of the second settling tank is called activated sludge because it bears a high density of bacteria that have become conditioned to the unique environment of the system. A part of activated sludge is piped to the sludge digester. The remaining part is recycled back to the aeration tank and subsequently to the settling tank, where it in turn activates the inflowing deactivated effluent (Fig.11.2). In this process, plenty of oxygen is pumped into sewage tank and oxidation of sewage effluent is brought about by aerobic microorganisms, which break down organic matter to CO_2 and H_2O. Now, the effluent is passed

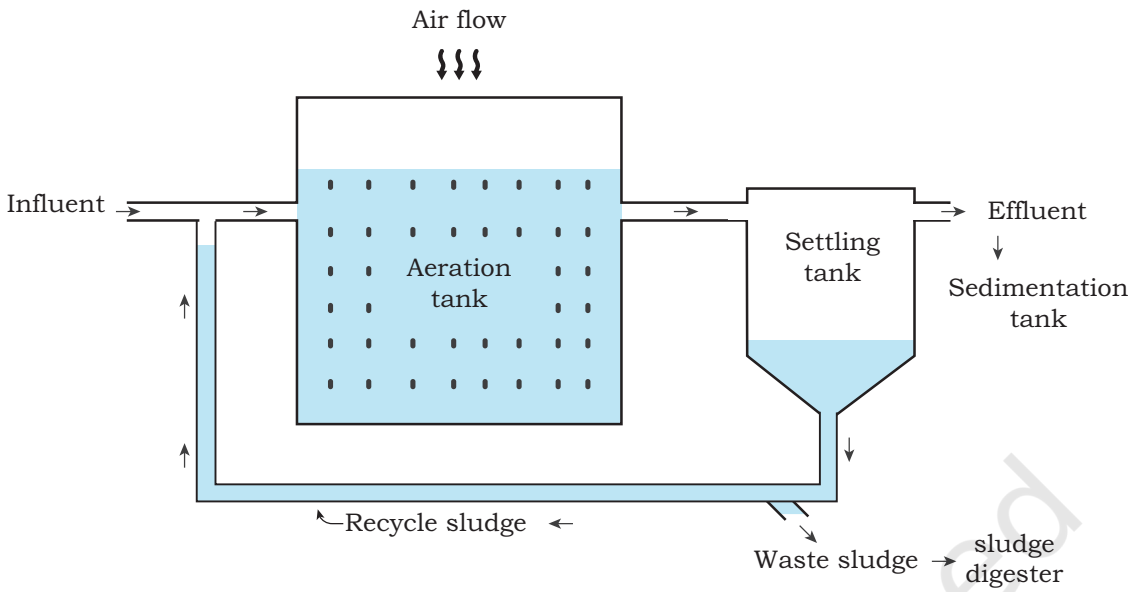


Fig. 11.2: Activated sludge process under aerobic conditions

through a sedimentation tank. Though about 90 percent of the organic matter of the effluent is digested *via* this process, the effluent still contains a considerable amount of nitrate and phosphate, etc. It is, therefore, not safe to discharge effluent at this stage into water bodies as both nitrate and phosphate can cause eutrophication. Now the effluent, which looks clear at this stage, is subjected to tertiary (final) treatment for further purification.

Trickling filter

Another form of secondary treatment is trickling filter process in which the sewage, after primary treatment, is sprayed by the arms of a rotating sprinkler into a filter bed of graded mixture of crushed stones, gravel, clinker and slag (Fig. 11.3). Spraying saturates the effluent with oxygen. The bed surface and stones are coated with slimy film of microbes (bacteria, fungi, protozoa and algae) that have accumulated during the operation of filter. As the effluent seeps over, the aerobic microbes degrade the organic matter. However, the treated effluent collected at the bottom of the tank is passed to sedimentation tank and, like activated sludge process, the effluent subjected

Box 3: Eutrophication

Excessive nutrients including nitrogenous and phosphorous compounds in water bodies due to sewage or run-off from agricultural fields favours the growth of photosynthetic microorganisms especially algae and phytoplankton. A rapid growth of algae in water bodies is called algal bloom. Such a phenomenon of excessive growth of photosynthetic microorganisms is referred to as Eutrophication. Eutrophication may occur either naturally or due to artificial means. Artificial or Anthropogenic eutrophication is caused by human activities, such as discharge of nitrate or phosphate containing industrial wastes, detergents, fertilisers, or sewage, into the water bodies. This excessive photosynthetic microorganisms growth on water surface prevents the entry of light and the submerged plants ultimately die. Algal population also releases toxins, which are harmful for the aquatic flora. In a situation of exhaustion of nutrients and due to the presence of toxic substances, algal growth is drastically stunted leading to decomposition of algal biomass by heterotrophic microbes. Dissolved oxygen also gets depleted leading to the death of aquatic fauna including fishes.

Natural eutrophication on the contrary, refers to excessive enrichment of water bodies as a result of merging of nitrogen and phosphorus rich streams into the lake. As explained above, organic debris and silt gradually pile up making the lake shallower and warmer. The warm-water organisms suppress the growth of those who thrive in a cooler environment. The shallower lake basin is overgrown by marshy plants, followed by the crowding of large masses of floating plants. Finally, the lake may convert into land. The natural eutrophication process is very slow, whereas eutrophication due to anthropogenic activities is faster.

to tertiary treatment. Primary treatment combined with secondary treatment can reduce BOD upto 90 percent, total nitrogen to about 50 per cent, total phosphorus to 30 percent and suspended matter to 90 percent.

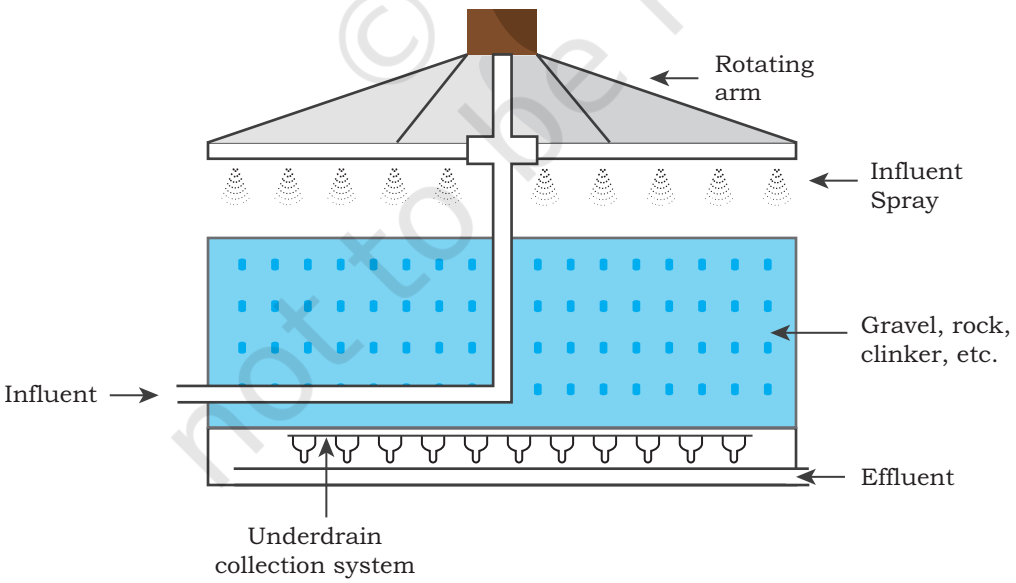


Fig. 11.3: Sketch of trickling filter process

Box 4: Sewage Oxidation Ponds or Lagoons

Oxidation ponds (or lagoons) are up to 5 feet deep shallow ponds designated to allow direct wind action and algal growth on the sewage effluent. The lagoon treatment process is usually recommended for only small communities in rural areas, where large acreages of land are available. Oxygenation of the wastewater is accomplished by natural means (e.g., wind action and photosynthetic action of algae) or by mechanical aeration. Efficient and properly operated lagoons are capable of reducing BOD by 75 to 95 percent or greater. Oxygen supplied from air and produced as a result of algal photosynthesis fulfils the BOD of sewage effluent and thus helps in maintaining aerobic condition in sewage effluent. In such condition, the aerobic microbes grow rapidly and digest organic matter. In view of conservation of ground water for domestic, industrial, irrigation and recreational purposes, oxidation ponds are receiving considerable attention in many arid areas.

Anaerobic Sludge Digestion

Anaerobic waste treatment represents the use of anaerobic microorganisms to process waste material in the absence of oxygen. These microorganisms digest the complex organic material to grow and reproduce. The sludge collected after primary treatment of sewage is subjected to anaerobic (in absence of oxygen) digestion in a separate tank designed especially for the purpose. Since anaerobic condition prevails in this tank, the anaerobic microbes digest the organic matter by degrading them to soluble substances and gaseous products, such as methane (60–70%), CO_2 (20–30%), H_2S and small amounts of H_2 and N_2. This gas mixture can be used for operating power for the sewage plant or as a fuel. Anaerobic sludge digestion requires 30 to 40 days for complete digestion. Remaining sludge, after digestion, undergoes dewatering by air-drying on sand filter sludge beds, vacuum filtration, centrifugation and wet air oxidation prior to the final disposal. After dewatering, the sludge is pulverized, incinerated or sold as a fertiliser. The heating kills all pathogens. Milorganite is a good example of such fertiliser. Important constituents are various soluble compounds of nitrogen, phosphorus and potassium—essential for plant growth.

11.1.5 Tertiary or Advanced Treatment

Since the sewage-effluent treated during secondary treatment process still contains non-biodegradable organic

pollutants (if the sewage contains industrial wastes) and mineral nutrients, particularly nitrogen and phosphorus salts, it is subjected to tertiary (or final) treatment for their removal. If not done, the sewage effluents containing nitrogen and phosphorus salts can cause serious eutrophication in aquatic ecosystems. Non-biodegradable organic pollutants are normally removed by using activated carbon filters, whereas phosphorus and nitrogen salts are removed by chemical treatment. Phosphorus salts are precipitated by liming. Lime reacts with phosphorus inorganic compounds in the effluent to form insoluble calcium phosphate ($Ca_3(PO_4)_2$), which then settles to the bottom of the settling tank and is removed.

Nitrogen is present mainly as ammonia and it is removed by volatilisation (vigorous aeration at elevated temperature) at high pH. Ammonia containing waste water is directed into a metal tower. As this water slowly flows downwards over a series of small plastic baffle plates, air is forced upwards through the effluent and removes the ammonia gas from the water. Carbon absorption is done to remove the dissolved organic compounds from the effluent. This is done by passing water through a tower packed with small particles of carbon. Dissolved organic matter sticks to the carbon particle, a process called carbon polishing.

These treatments result in a high-quality effluent that does not cause eutrophication. The final step of tertiary treatment is chlorination by using either sodium or calcium hypochlorite (NaOCl or $CaOCl_2$), respectively or chlorine to disinfect the effluent. Now the effluent is a clean water but still it cannot be used as drinking water.

11.2 Solid Waste Management

All non-liquid waste materials generated from households, streets, industrial, commercial and agricultural activities are called solid wastes.

Sources and types of solid wastes

Major sources of solid waste are households, agricultural fields, industries and mining, hotels and catering, roads and railways, hospitals and educational institutions, cultural centers and places of recreation and tourism, etc. (Table 11.2).

Table 11.2: Major sources and types of solid wastes

Source	Typical waste generators	Type of solid waste
Residential	Single and multifamily dwellings	Food waste, paper, cardboard, plastics, glass, leather, yard waste, wood, glass, metals, ashes, special waste (e.g., bulky items, consumer electronics, white good, batteries, oil, tires and household hazardous waste)
Industrial	Light and heavy manufacturing, fabrication, construction sites, power and chemical plants	Housekeeping waste, packaging, food waste, construction and demolition materials, hazardous waste, ashes, special waste
Commercial	Stores, hotels, restaurants, markets, office, buildings, etc.	Paper, cardboard, plastics, wood, food waste, glass, metals, special waste, hazardous waste
Industrial	School, hospital, prisons, government offices	Same as commercial
Construction and demolition	New construction site, road repair, renovation site, demolition of buildings	Wood, steel, concrete, dirt, etc.
Municipal services	Street cleaning, landscaping, parks, beaches, other recreational areas, water and waste water treatment plant	Street sweeping landscape and tree trimming, general wastes from park, beaches and other recreational areas, sludge.
Process (manufacturing etc.)	Heavy and light manufacturing refineries, chemical plants, power plants, mineral extraction and processing	Industrial process wastes, scrap materials, off specification products, slay, tailings
Agriculture	Crop, orchards, vineyards, dairies, food lots, farm	Spoiled food wastes, agricultural waste, hazardous wastes (e.g., pesticides)

11.2.1 Risks of solid waste

Solid waste provides breeding sites for mosquitoes and flies which are carriers of many life-threatening diseases. For example, mosquitoes of *Aedes* genus spreads dengue and yellow fever; *Anopheles* genus of mosquitoes transmits malaria; and mosquitoes of *Culex* genus transmit microfilariasis.

Burning of some of these solid wastes produce carcinogens and other toxins. Poor management of collection and disposal of solid waste may lead to leachate pollution of surface and ground water. This may cause a significant

problem if the waste contains toxic substances and disposed near the source of drinking supplies. Unhygienic and polluted environment affect the health of people.

Box 5

1. Spread of plague episode in Surat (Gujarat) has been associated with the mishandling of solid waste disposal at city level.
2. Spread of dengue fever in Delhi is linked with the widespread stagnant water in different forms— water cooler, open sewage, badly kept drains, etc.

11.2.2 Solid waste management

Solid waste management refers to collecting, treating, and disposing off solid material that is discarded or is no longer useful. Solid waste management is an important aspect of urban area management. Improper disposal of municipal solid waste can create unsanitary conditions, which can lead to environmental pollution and the outbreak of vector-borne diseases. It is the discipline associated with the control of generation, collection, storage, transfer and transport, processing and disposal of solid wastes in a manner that is in accord with the best principles of public health, economics, engineering, conservation, aesthetics and other environmental considerations.

The task of solid waste management presents complex technical challenges. They also pose various economic, administrative and social problems, which need urgent attention.

Strategies for solid waste management

Strategies for solid waste management can be presented broadly as shown in Fig. 11.4. The handling and managing of solid waste involves two approaches: (i) dumping the waste and (ii) recycling the waste. These two steps are of significance in managing the overall burden of the solid waste.

In order to establish effective solid waste management in the affected area, the given process should be used:

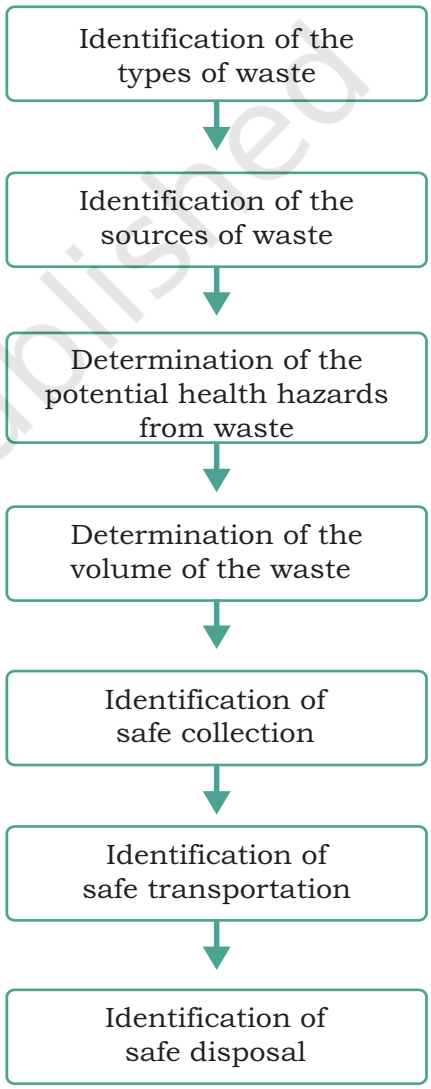


Fig.11.4: Stages of solid waste management

Sorting and management of solid waste

To maximise the role of recycling of solid wastes, it is essential to sort the waste at the waste generating point in different categories to make recycling practice easy. Recycling of solid waste can be achieved under the following heads. Organic waste can be recycled through the process of composting. Fruits and vegetables waste, animal dung, fallen leaves from plants form excellent soil conditioner and fertiliser (compost). Implantation to make compost at home level can be achieved by using a suitable container. The contents of the container after a few weeks can be used as fertiliser. Vegetable waste and dried weeds can be chopped and compressed into small bricks and sun dried. Like dried cow dung, they can be used to replace charcoal or wood as cooking fuel. Glass and plastic waste can be reused in plastic and glass industries to reproduce wares for reuse. The task of collecting these waste items under different heads makes the recycling task more efficient and convenient.

Construction debris and other waste building material can also be reused. Often the construction of new house or building involves razing old structures. The debris produced in this manner can be very effectively used as filling material under floors.

Used tyres can be recycled by establishing appropriate recycling units. In the absence of this facility, it is advisable to bury them to avoid collection of water during rainy season, which encourages mosquito breeding. Burning of tyres has to be compulsorily avoided as the process leads to the emission of toxic fumes.

Solid waste management at community level

Some household items cannot be degraded at home level and their storage can cause space problem. Plastics, bones, metals, used batteries, broken glass, etc., falls in this class. A common facility made at community makes the subsequent processing easier, once the solid waste is sorted.

Community refuse pit dug near the community dwelling units can be used to fill the refuse, which cannot be handled at home level. The site chosen to perform this function should be located away from water source to avoid any leaching of waste into the water. The surroundings of the

pit should be fenced to prevent animals from intruding into the refuse pit. Collection of waste at community level can be organised by employing the service of trained persons who are aware to take adequate precautions while handling waste. The waste collected at community collection site should be taken to a designated city disposal site as and when the need is felt.

Managing special solid wastes

Some solid waste needs expert handling by using special equipment to protect the handler. Solid waste under this class falls in the following categories.

Medical solid waste

Healthcare solid waste is generated both at medical facilities and some routine activities at home and hospitals. The waste often contains infectious pathogens. Also, use of medical solid wastes (like masks, gloves, PPE kits, syringes and needles, etc.) increase significantly during pandemic/ epidemic. Therefore, high risk medical solid wastes must be separated as per guidelines and incinerated. The handler of the waste needs to take all precautions (wearing mask and gloves) while handling. In the absence of incinerator (such as at home), the waste should be treated with strong disinfectant. Sharp needles and glassware used at health facility should be disinfected. Used needles should be destroyed to prevent their reuse.

Slaughterhouse waste

Slaughterhouse waste consists of decaying animal carcasses, blood, fecal matter, hair and bones. Collection of such wastes should be handled by trained staff at specific sites meant to handle this kind of waste. The functioning and maintenance of the facility should comply with health standards and the community should ensure regular inspection of the sites by the authorities concerned for a proper disposal.

Industrial solid waste

Industrial solid waste can be of different kinds and the main concern associated with industrial waste is the proper disposal of chemical, biological and toxic metals. In general, the processing of waste from industrial units is handled by the respective industry itself as per the environmental rules and regulations.

Disposal of toxic chemicals

Disposal of toxic chemicals should be done according to the manufacturer's guidance, in case of doubt as to how to dispose specific toxic chemicals, local health and environment officials should be contacted.

Composting, vermiculture and methane production

Composting is the most powerful biological, chemical and physical activity, available in natural environment, where organisms under suitable conditions carry out biological degradation of material, considered waste from humans point of view. Organisms including microorganisms break down organic waste and produce carbon dioxide, water, heat and humus. The process stands out as the most powerful environmental tool by which a large variety of complex molecules, after degradation, can be recycled and reutilised. Composting concept has been successfully exploited by humans to degrade the toxic and persistent compounds present in water/soil.

Mechanism and procedure of composting

The waste material subsequent to its proper sorting of degradable material, after grinding, is mixed with water and sewage sludge containing organisms. To maintain the efficient digestion of waste material, suitable environment (such as optimum moisture content 45–65%) for optimum growth and activity of organisms is needed. Composting methods are either aerobic or anaerobic. Aerobic process requires enough oxygen availability within the compost heap or compost pit. The design and size of the composting pit ensures the maximum efficiency of degradation. Under optimal conditions, composting proceeds through three phases: (i) the **mesophilic**, or moderate-temperature phase, which lasts for a couple of days, (ii) the **thermophilic**, or high-temperature phase, which can last from a few days to several months, and finally (iii) a several-months cooling and **maturation phase** (Fig.11.5).

As composting proceeds, temperature changes, correspond to the dominance of specific communities of microorganisms at work. Mesophilic phase involves rapid breakdown of the soluble, readily degradable compounds

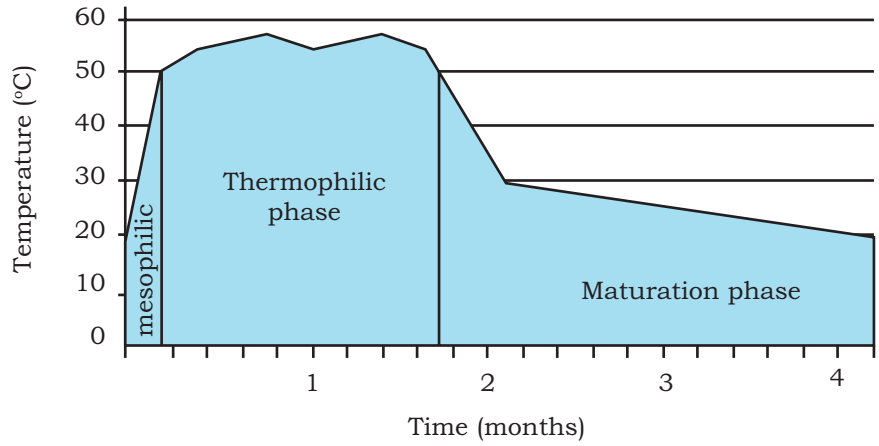


Fig. 11.5: Temperature changes during composting

by mesophilic class of microorganisms. Degradation activity accompanies heat production and rapid rise in the compost temperature. As temperature rises above 40°C, the mesophilic microorganisms are competed out by thermophilic class of microorganisms, which are able to withstand higher temperature. At temperature of 55°C and above, many pathogenic microorganisms are destroyed and this limits the rate of decomposition. To ensure aeration and lower temperature (<65°C), the compost pit is designed to allow passage of air, which mixes the compost intermittently. During thermophilic phase, high temperature accelerates the degradation of protein, fats and complex carbohydrates, such as cellulose and hemicelluloses, the major structural components of plants. Once the supply of these compounds gets exhausted, the compost temperature gradually decreases and mesophilic phase again takes over for the final phase of 'curing' or maturation of the remaining organic matter.

Organisms involved in composting process

Diverse communities of microorganisms (both unicellular and multicellular) play their role at different stages of composting. Constant change in the microenvironment around the community of organisms provide selective advantage to those organisms who can survive best at a given time. Bacteria make up 80–90% of the billions of microorganisms found in a gram of compost. At highest compost temperature, bacteria of the genus *Thermus* are isolated. Actinomycetes are filamentous type of bacteria and

play an important role in degrading lignin, cellulose, chitin and proteins. Special class of enzymes present in these bacteria are capable of degrading debris, such as woody stems, bark, or newspaper and cardboard boxes, etc.

Fungi represent a class of organisms that also consists of molds and yeast. It can decompose many complex polymer waste material present in the soil or compost. Most fungi live in the outer layer of the compost and are present during all phases of temperature change during the process of composting.

Protozoans are single-celled microscopic organisms found in water droplets trapped in compost matrix. Their role in composting is that of a secondary consumer and sustain on organic material, bacteria and fungi. In addition to above, compost also has the presence of a large variety of insects, mites, snow bugs, ants, earthworms and other types of worms. Vermiculture and methane emission are a part of the process of composting. Presence of worms in the compost or soil is an indication of the fertility of the soil in terms of the availability of nutrients essential for the growth of the plants. Composting, in essence, represents a natural way of regenerating soil fertility by utilising the waste produced in the environment by humans.

11.3 Management and Disposal of Biomedical Waste

'Biomedical waste can be defined as any waste generated during the diagnosis, treatment or immunisation of human beings or animals or research activities pertaining thereto or in the production or testing of biologicals or in health camps'. Government has made guidelines for handling of biomedical waste, which are provided under Biomedical Waste Management Rules (BMWM), 2016, The Biomedical Waste Management Rules, 2016, categorises the biomedical waste into four major categories based on the segregation pathway and standard colour code that has been assigned for the containers in which these are to be segregated, these are yellow, red, white and blue (Table 11.3).

Table 11.3: Details of colour code and types of waste

S. No.	Category	Type of Waste
1.	Yellow	(a) Anatomical Waste • Human tissues, body parts and fetus below the viability period • Animal carcasses, tissues, organs, body parts, animal houses (b) Soiled waste, items contaminated with body fluids, blood, cotton swabs, discarded old and blood components. (c) Discarded or expired medicines like antibiotics, cytotoxic drugs along with glass or plastic ampoules, vials, etc. (d) Chemical waste like disinfectants, chemical used in the production of biologicals, insecticides, discarded solvents and reagents used for laboratory preparations, heavy metals (broken thermometer), batteries, etc. (e) Beddings contaminated with blood or body fluids, discarded linen, mattresses, mask and gown. (f) Microbiology, biotechnology and other clinical laboratory waste, like blood bags, specimens, human and animal cultures used in research, residual toxins, dishes and devices used for culture.
2.	Red	Contaminated plastic waste: waste generated from disposable items like intravenous tubes, bottles, catheters, syringes without needles, fixed needle syringes with their needles cut, urine bags, vaccutainers and gloves.
3.	White	Sharps including metals; used, discarded and contaminated metal sharps, needles, syringes with fixed needles, needles from needle tip cutter, blades, scalpels or any other contaminated sharp object that may cause puncture and cuts.
4.	Blue	Metallic body implants, broken or discarded and contaminated glass; medicine vials ampoules

Box 6

Biomedical wastes are also classified on the basis of risk factors involved as per WHO guidelines. These are:

- **Risk Group 1** is comprised of no or low individual and community risk. For example, a microorganism that is unlikely to cause human or animal disease.
- **Risk Group 2** categorises the condition of moderate individual risk and low community risk. Pathogen that causes human or animal disease and is likely to be a serious hazard to laboratory workers, the community, livestock or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available and the risk of spread of infection is limited.

- **Risk Group 3** classifies high individual risk and low community risk condition. A pathogen that usually causes serious human or animal disease but does not ordinarily spread from one infected individual to another. Effective treatment and preventive measures are available.
- **Risk Group 4** is a group with high individual as well as community risk. In this group, the risk is due to pathogens that usually cause serious human or animal disease and that can be readily transmitted from one individual to another, directly or indirectly. Effective treatment and preventive measures are not usually available.

As we know, the biomedical wastes generated in—healthcare facilities, diagnostic laboratories, biotechnology laboratories and institutions, medical research centres, veterinary institutes, mortuary and autopsy centres, and blood banks — are in large amount and highly infectious to human health, animals, living organisms and environment. It is the responsibility of the health care provider to segregate these biomedical wastes appropriately, and it is also important to segregate waste at the point of its generation from handling point of view. The containers, bins, bags, sharp boxes should be available in each health care facility, laboratories, medicals, etc. Trained staff is needed to segregate the waste. General information, posters, proper instructions regarding proper disposal of waste must be placed near dustbins, bags, containers, etc.

Box 7

WHO recommended segregation and collection scheme

Waste Categories	Colour of Container and Markings	Type of Container	Collection Frequency
Infectious waste	Yellow with biohazard symbol, highly infectious waste should be additionally marked 'HIGHLY INFECTIOUS'	Leak-proof strong non-chlorinated plastic bag placed in a container (bags for highly infectious waste should be capable of being autoclaved)	When three-quarters filled or at least once a day.
Sharp waste	Yellow, marked SHARPS with biohazard symbol.	Puncture-proof container	When filled to the line or three-quarters filled.
Pathological waste	Yellow with biohazard symbol.	Leak-proof strong plastic bag placed in a container.	When three-quarters filled or at least once a day.

Chemical and pharmaceutical waste	Brown, labeled with an appropriate hazard symbol	Plastic bag or rigid container	On demand
Radioactive waste	Labeled with radiation symbol	Lead box	On demand
General health care waste	Black	Plastic bag inside a container or container which is disinfected after use.	When three-quarters filled or at least once a day.

Box 8

Mayapuri Radiological Accident

In April 2010, scrap market of Mayapuri area in Delhi was affected by a serious radiological accident in which public radiation exposure was reported. In this accident, the source of radioactive unit containing Gamma unit having Cobalt-60 pencils were sold at auction to a scrap metal dealer in Mayapuri on February 2010. It was dismantled by workers without knowing its consequences. The cobalt-60 source was cut into eleven pieces. Eight people were hospitalised in AIIMS as a result of radiation exposure, where one later died.

MANAGEMENT AND DISPOSAL OF RADIOACTIVE WASTE

The Atomic Energy Regulatory Board (AERB) has been mandated by the Government of India to be the competent authority as per Atomic Energy (safe Disposal of Radioactive Wastes) Rules, 1987. The radioactive waste should be disposed according to guidelines issued as per the provisions of the said rules.

1. Atomic Energy (safe Disposal of Radioactive Wastes) Rules, 1987: https://www.aerb.gov.in/images/PDF/AE(SDRW)Rules%201987.pdf
2. Radiation protection rule: https://dae.nic.in/writereaddata/RPR2004.pdf

11.4 Bioremediation of Pesticides

Pesticides are the categories of diverse chemicals that are used either for protecting the standing crop or stored food grains from different types of pests like insects, rodents, weeds, bacteria, larvae of worms and even fungi. Broadly, chemical pesticides can be categorised into organochlorine, organophosphorus, organometallic, synthetic pyrethroids, carbamates, etc., which vary in their properties.

Box 9: Various types of pesticides along with their properties

Type of pesticide	Chemical Nature	Effects on animals	Example	Persistency
Organochlorine	Organic compound with chlorine atom, Lipid soluble	Nervous system disrupter, Toxic	DDT, Lindane, Endosulfan, Aldrin, Dialdrin, Atrazine (herbicide)	Very long
Organophosphate	Organic compound with phosphate group as basic structure	Nervous system disrupter (inhibits cholinesterase activity), highly toxic	Parathion, Malathion, Dimecron, Diazinon	Less persistent
Carbamates	Derivate of Carbamic acid	Nervous system disrupter (inhibits cholinesterase activity), highly toxic	Carbaryl, Carbofuran, IPC	Less persistent
Pyrethroids	Synthetic analogues of pyrethrum plant	Nervous system disrupter	Cypermethrin, Deltamethrin	Usually degraded by sunlight in a few days. Less persistent in soil (few days)
Organometallic	Synthetic organic compound with heavy metal	Can be absorbed through skin, GI tract and through respiration, toxic	Methylmercury	Very long
Inorganic Pesticides	Metal or non-metal and its compound	Coagulation of protein, affect the central nervous system and functioning of enzyme	Arsenic, Mercury, Cadmium, Sulphur	Very long

Use of these agrochemicals was also one of the key contributors in the success of green revolution during late sixties, which made our country self-sufficient for food.

Let us briefly understand the nature of different types of pesticides. DDT (an organochlorinous pesticide) was initially found to be a miraculous solution to the spread of malaria as it proved to be highly effective and useful in the control of *Anopheles* mosquito. Later, resistance

Box 10: The first pesticide

Dichlorodiphenyltrichloroethane (DDT) was the first ever pesticide synthesized in the laboratory, which is an organochlorine compound. Considering the efficacy in killing insects, including mosquitos, it was considered initially as a solution to the problem of insects and other categories of pests. Subsequently, a large number of other chemicals having the potential to kill insects and other pests quite efficiently were synthesized and its usage in agricultural practices became quite prevalent.

to DDT pesticides was noted among target organism, rendering them ineffective on the one side, and their use was found to be harmful to non-target species. Owing to their hydrophobic nature and lipid solubility, these chlorinated organic compounds have also been found to be non-biodegradable.

Organophosphate pesticides have phosphate group and they are acutely toxic due to their ability to inhibit cholinesterase resulting into accumulation of neurotransmitter acetylcholine across synapse during neuronal transmission. Excess of such organophosphates in their residual forms may turn out to be more harmful than the original one and therefore their excessive usage may be harmful in the long run.

Carbamate and synthetic pyrethroids pesticides get easily hydrolysed and degraded. Organochlorine and organometallic pesticides may persist long in the environment and owing to their solubility in lipid, they may also remain present even in the body of non-target organisms, i.e., organisms other than insects and pests including human beings.

These excessive agrochemicals, either in their original or metabolised forms, may also enter into plants and ultimately in different organisms of the trophic levels including human beings by the process called **biomagnification**. Quite significant levels of such agrochemicals have been found to be present in the tissues of many organisms besides being accumulated in soil and water.

Therefore, the removal of such a long-lasting toxic compounds from soil and water bodies is extremely crucial for ecosystem and the health of non-target organisms including human beings even if it is present in residual amount.

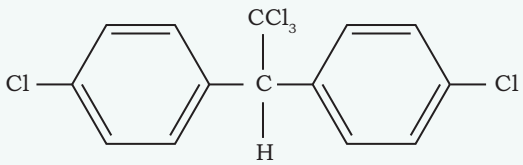
Bioremediation of pesticides using microorganisms

Considering the persistency of pesticides or its residues, either the removal or altering the toxic nature is extremely crucial and essential in minimising its adverse effects in humans, other non-target organisms and the ecosystem. Bioremediation is thus a process through which such toxic pesticides can be removed completely or its chemical nature can be altered, so as to have no or less adverse impact on the environment. In the process of bioremediation, the ability of microorganisms to degrade or alter the toxic nature of chemical pesticides into non-toxic substances is exploited. There are quite a good number of microorganisms capable of wide spectrum metabolism. These may be bacteria, fungi, algae and other microbial strains. Many microorganisms are known to utilise chemicals to derive energy on one hand and possess enzymes capable of degrading such pesticides on the other hand. Enzymatic reaction that is important from the perspective of degrading pesticides or other toxic chemicals are mainly catalysed by cytochrome P450, esterases, oxidases, peroxidases, transferases, etc. Role of these enzymes in the process of degradation reaction has been given briefly in Table 11.5. The process of transformation or degradation of pesticides is complex and may involve enzymatic reactions like oxidation, reduction, hydrolysis, transfer of groups and formation of conjugate with sugar or amino acid making it less toxic and water soluble.

Box 11: Enzymes and their role in Pesticide Bioremediation

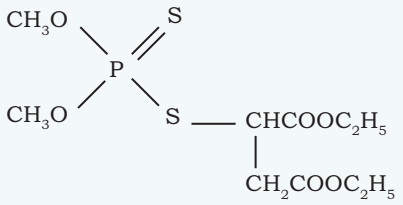
Enzyme	Function	Role in Bioremediation
Cytochrome P450	Oxidation and reduction reaction	Oxidative breakdown of the pesticide or any xenobiotic (synthetic) compound
Esterase	Hydrolysis reaction	Hydrolytic breakdown of organophosphate or carbamate pesticide
Oxidase	Redox reactions	Oxidation reduction reaction
Peroxidase	Redox reaction	Oxidative degradation of organometallic compounds
Transferase	Transfer of glutathione group, hydrolytic and redox reaction	Transfer of glutathione to some electrophilic pesticides and also its oxidative degradation

Box 12



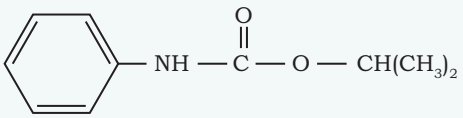
p,p'- DDT

(a) Organochlorine Pesticides



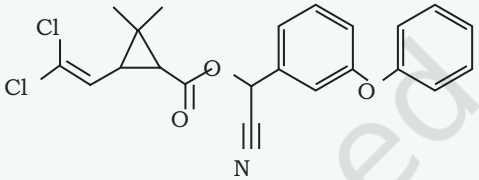
Malathion

(b) Organophosphate Pesticides



Isopropyl phenyl carbamate

(c) Carbamate Pesticides



Cypermethrin

(d) Synthetic pyrethroids Pesticides

Examples of (a) Organochlorine, (b) Organophosphate, (c) Carbamate and (d) Synthetic pyrethroids

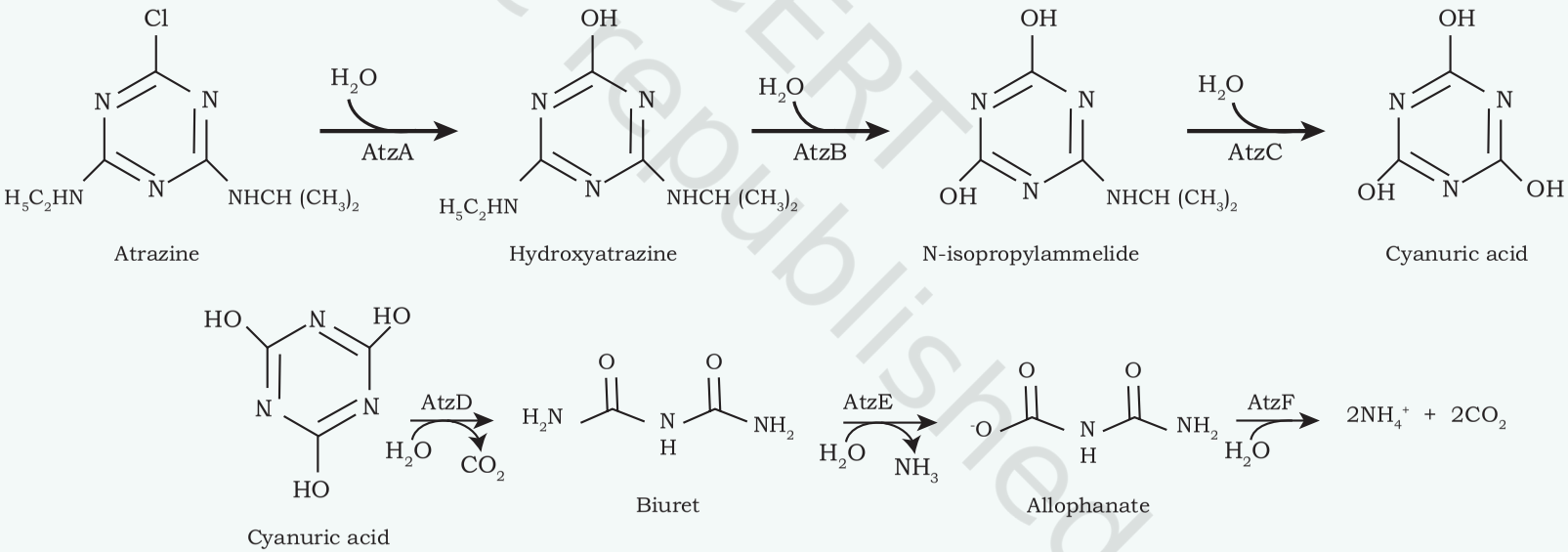
A number of microorganisms have been identified with the potential to bioremediate a number of toxic substances, e.g., *Pseudomonas*, *Aerobactor*, *Acinetobacter*, *Plesiomonas*, *Neisseria*, *Sphingomonas*, *Flavobacterium,* etc. The whole process of bioremediation of any persistent chemical present in the system also depends on physicochemical conditions like pH, temperature, water, availability of oxygen and substrate, etc., of the environment on one hand and varieties of soil and water microflora present on the other hand. Simultaneous use of a conglomerate of such microorganisms for biodegradation or bioremediation of toxic chemicals has also been observed to yield a better result.

Strategies for bioremediation

Bioremediation of toxic substances may take place naturally or it may be maneuvered using specific type of microorganisms and other strategies. We have already understood earlier that microorganisms have either already present in the soil/water or introduced and has the potential to either degrade or remediate the toxic substances into non-toxic substances or elements. Such an onsite remediation strategy is referred to as *in situ*

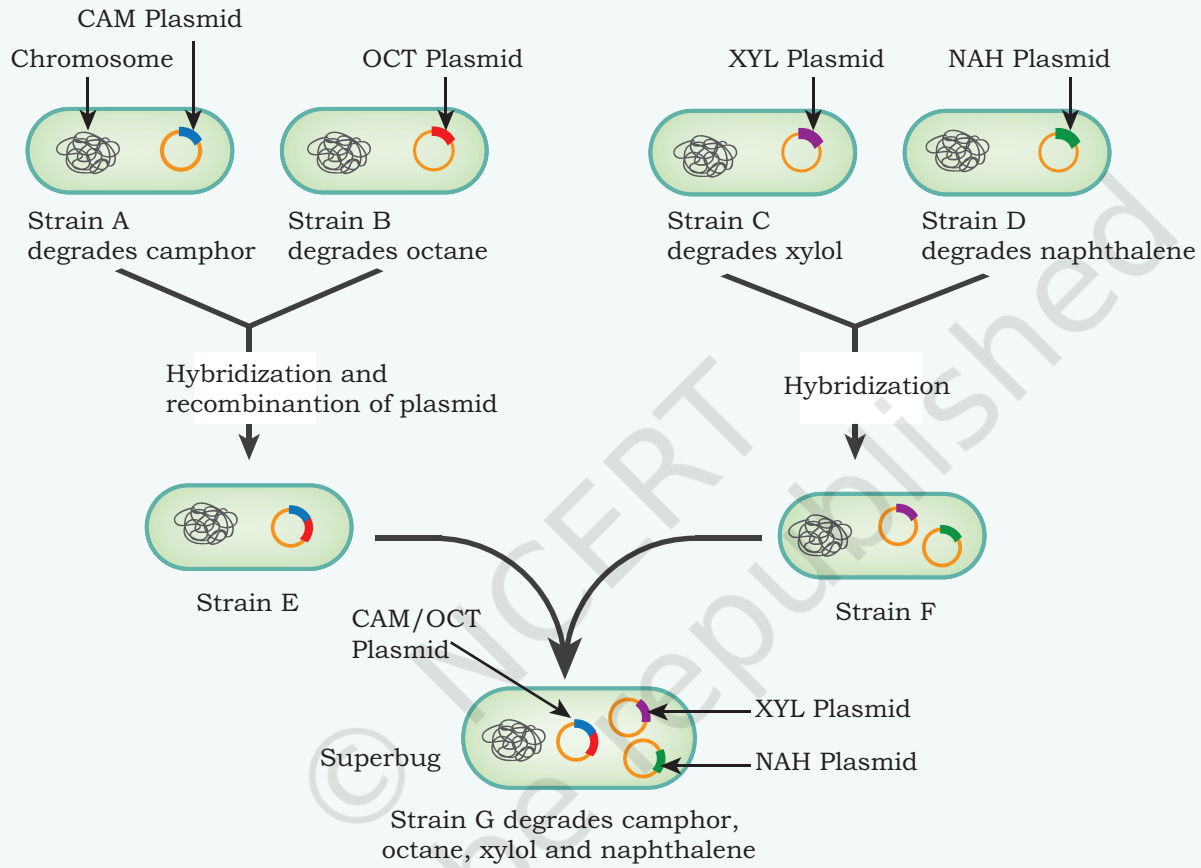
Box 13: Mechanism of Pesticide Bioremediation

After its release each pesticide undergoes a series of reactions for exhibiting its property in the target organism, i.e., pests. In case the same is not completely used up, then depending on the type of processes and reactions in which it enters, it is quite likely that the same will remain present in its original form or in other form, which may lead to various harmful consequences. Bioremediation of these agrochemicals present in soil or water is one of the best solutions. However, involving microorganisms in such a process of bioremediation of pesticides is dependent on a number of factors. Such factors include environmental conditions like temperature, humidity, pH, salinity, nutrition, presence of CO_2 and O_2, etc. Also, the microbial species present and its adaptability with the pesticide is crucial for its bioremediation. The structure of pesticide is yet another factor that affects the process of bioremediation. For example, a polymeric xenobiotic compound is comparatively less biodegradable than a lower molecular weight compound. The xenobiotic substances are subjected to enzymatic action present in the microorganisms during the bioremediation process. The most immediate enzyme action on the pesticides present in soil or water are oxidation–reduction reaction, hydrolysis and transferase activities of cytochrome, peroxidase esterase and transferase enzymes. Needless to mention, the conditions for the sustenance and emzymatic activity of microorganisms must be conducive. Also, a few microorganisms have been found to possess genes and the product of such genes have been found to be capable of degrading pesticides. A series of *Atz* genes (namely *Atz* A to *Atz* F) have been identified in ***Pseudomonas***, which helps in utilising Atrazine pesticide as its carbon source for energy and completely degrade the pesticide through the following mechanism.



Box 14: Superbug and Bioremediation

The presence of hydrocarbon degrading genes on plasmids of four different strains *Pseudomonas* was successfully exploited by an India born US scientist Anand Mohan Chakrabarty (4 April 1938 –10 July 2020) and these genes were inserted using rDNA technology into one strain of *Pseudomonas putida* which is capable of degrading camphor, naphthalene, octane and xylene. Creation of this superbug by rDNA technology is shown in the Figure below. This breakthrough was recognised by the award of a US patent.



bioremediation. There are many instances where *in situ* bioremediation strategies have been adopted to clean-up petroleum spillage and for restoration of degraded ecosystem due to excessive mining or accumulation of toxic substances including pesticides. It is possible that some of the toxic substances are degraded or remediated into non-toxic ones by microorganisms naturally present in the system in the course of time. This situation is referred to as intrinsic *in situ* bioremediation. However, such an intrinsic mechanism of bioremediation is dependent on many factors, like type and population of microorganisms, soil type, availability of nutrients and

other substances that support growth. Undoubtedly, such an intrinsic pathway of *in situ* bioremediation mechanism involves very less disruption of the contaminated site with low public exposure and is cost effective. However, this process not only takes comparatively long time but it has also been found that complete bioremediation of the toxic substances is not achieved through this process.

In order to address these difficulties in the *in situ* bioremediation process, it is possible to adopt a strategy to encourage microbial growth in an area selected for bioremediation. For this, oxygen, nitrogen and other growth-promoting substances are circulated in the contaminated soil through injection and extraction wells created for the purpose (Fig. 11.6). Obviously, oxygen will serve the purpose of electron acceptor (which is produced during the process of being converted into water by the toxicant called electron donor). Such a process in which the microbial growth is promoted externally in the contaminated soil or water for *in situ* bioremediation of toxicants is referred to as engineered *in situ* bioremediation.

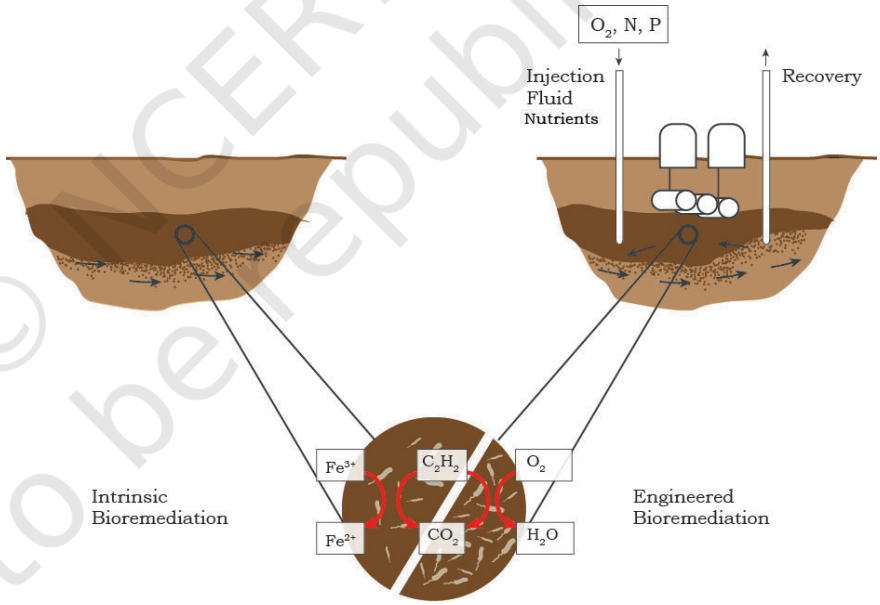


Fig. 11.6: In Situ bioremediation

For the purpose of bioremediation, another technique can also be adopted in which the contaminated soil is physically taken out and treated separately for the removal or biodegradation of toxicants present in it. This is normally done for a very heavily contaminated soil. There are various strategies adopted for the purpose as described below:

Slurry bioreactors: In such bioreactors, the contaminated solid or soil is agitated vigorously in the presence of water and nutrients capable of enhancing the ability of microbes present in soil to degrade the toxicant under the optimum condition. Mobile slurry bioreactors are

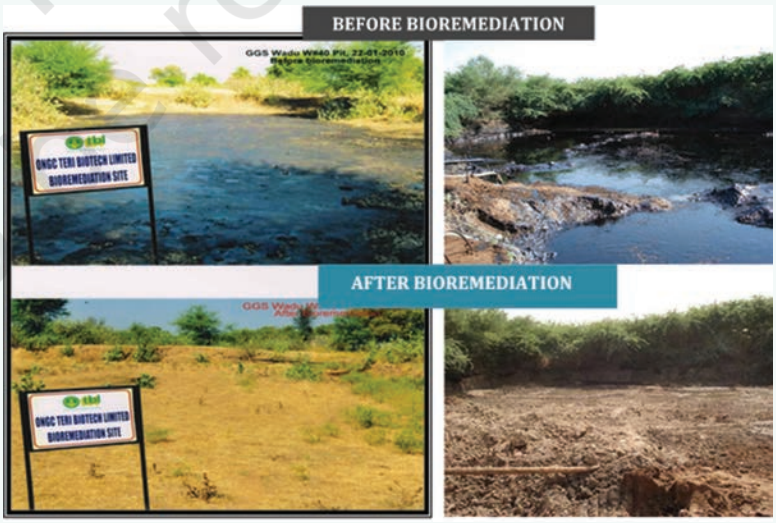
Box 15

Oilzapper: A solution for oil spills and treatment of oily sludge

Oil spill incidents are global phenomena posing a serious threat to the ecosystem and its flora and fauna. Exploration, production, transportation, storage and refining processes of petroleum may accidently lead to oil spill in soil or water bodies causing hydrocarbon pollution. Physical (storing of oil sludge and drill cutting in secured pits) and chemical methods of remediation of these hydrocarbons are expensive as well as non-environment friendly. The Energy and Resource Institute (TERI) with the support of Department of Biotechnology, Government of India, came out with its invention called 'Oilzapper', which can zap the spilled petroleum oil using a consortium of five non-pathogenic bacterial strains. These bacteria can biodegrade different fractions of oil like aliphatic, aromatic and asphalting compound of oil sludge or crude oil. In this cost effective technology, Oilzapper is immobilised onto a carrier material which is powdered corncob, for its application in oil contaminated environment. TERI has developed fermentation facility of different capacities ranging from 15000 litres, 1500 litres, 300 litres to 20 and 10 litres for large scale production of Oilzapper. 'Oilzapper' have been successfully used by various oil companies across India as well as abroad. Considering the wide scale implication of 'Oilzapper', TERI and ONGC (Oil and Natural Gas Company) developed a joint venture namely Ongc Teri Biotech Limited (OTBL), which takes care of its implementation. Till date, 'Oilzapper' has cleaned more than 750000 metric tones of oil sludge and oil contaminated soil in India and in a few Middle-east countries.

Oilzapper packed in sterile polybags

Bioreactors for production of Oilzapper (13kl,1.5kl,300L,30L,10L)



Courtesy: The Energy and Resources Institute (TERI)

also available so that the bioremediation of contaminated soil can be done very close to the contaminated site.

Land Farming: In land farming nutrient, moisture and other conditions for an optimal microbial growth are applied to bed of the land farming site to facilitate bioremediation process.

Composting: In this process, the contaminated soil is mixed with agricultural wastes of organic substances like stray or corncobs to enable optimal supply of air and water for bioremediation process. Excavation and transport of contaminated soil for composting is a challenge from the point of view of exposure of contaminants to labourers.

Biopiles: This is a process in which the contaminated soil is excavated and mixed with leachate materials capable of degrading and draining out the toxic substances present in it.

Phytoremediation

There are many examples in which whole plants do play an important role in either uptaking or remediating chemical nature of toxic substances present in soil or water. Students have studied about *Brassica napus*, *Helianthus annus* plants in Class XI (Chapter 1) which can accumulate mercury and lead from contaminated soil. Besides this, microorganisms found in the rhizosphere play an important role in the remediation process of toxic chemicals present in the environment (soil and/or water). Since most of the toxic substances present in soil are due to agricultural activity and therefore, microorganisms present in the rhizosphere (region of soil under the influence of secretions of root or microorganisms present in it) facilitates the phytoremediation process through a variety of biochemical processes, like chelation, methylation, changing pH of soil, altering redox reaction and many other actions. The process also includes the ability of a number of plants to accumulate toxicants (especially compounds with heavy metals), altering bioavailability of such toxicants.

The role of nitrogen fixing bacteria, also known as **diazotrophs,** in increasing the soil fertility is well known, which can convert atmospheric nitrogen into the compound (ammonia and urea) that plants can easily uptake. Few of the common examples of microbes involved in the process of phytoremediation are given in Table 11.4. Also, these diazotrophs facilitate the production of a few plant

hormones like indole acetic acid (IAA), and gibberellins that facilitates crop plant growth besides remediation of toxicants containing lead, zinc, nickel, etc. Also, there are a few aquatic plants like *Hydrila verticilliata* and *Nymphaea alba* which help in the reduction of chromium in polluted water and therefore used in phytoremediation.

Table 11.4: Microbes in Phytoremediation

Types of microorganism	Plant	Effect
Azotobacter chroococcum	Maize, Mustard	Reducing soil pH, production of plant hormones, remediation of heavy metals like lead, zinc
Rhizobium leguminosarum	Maize	Reducing soil pH, production of plant hormones, remediation of heavy metals like lead, zinc
Rhizobium sp.	Pea, Lentil	Production of siderophore, release of hormones, remediation of nickel and zinc

SUMMARY

- Large population and various human activities lead to the drastic shortage of fresh water in India.
- A large part of the population is unaware of the treatment of sewage waste before discharge.
- Sewage water consists inorganic and organic matter suspended in soluble form, which can be detected by Biological Oxygen Demand (BOD).
- Sewage water contains the microflora of human intestinal tract as well as many soil and water species, some fungus and viruses.
- Sewage disposal plants are operated for treatment of waste water before its release into streams. The waste water treatment plants are designed based on their capability to provide primary, secondary (biological), and tertiary (advanced) treatment.
- Primary treatment removes large floating objects, secondary treatment removes suspended organic material through microbiological degradation, whereas tertiary treatment removes phosphorus and nitrogenous nutrients and all suspended organic matter from waste water.

- All non-liquid waste materials generated from households, streets, industrial, commercial and agricultural activities are called solid wastes.
- Solid wastes can be categorised into two groups: (i) biodegradable waste and (ii) non-biodegradable waste.
- Decomposition of solid wastes are associated with various diseases, like malaria, dengue, SARS, plague; attract animals, flies, vermins and can play a major role in the transmission of diseases.
- Solid waste management refers to the process of collecting, treating, and disposing of solid material that is discarded or is no longer useful.
- The handling and managing of solid waste involves two approaches—dumping and recycling the waste.
- Organic waste can be recycled through the process of composting. Fruits and vegetables waste, animal dung and fallen leaves from plants form an excellent soil conditioner and fertiliser (compost).
- Health care solid waste contains many infectious pathogens, which needs incineration or safe burial immediately. The handler of the waste needs to take all precautions (wearing mask and gloves) while handling waste.
- Industrial solid waste can be of different kinds and the main concern associated with industrial waste is the proper disposal of chemical, biological and toxic metals, which is handled by the respective industries.
- Composting is the most powerful biological, chemical and physical activity, available in natural environment, where organisms under suitable conditions carry out biological degradation of material.
- Biomedical Waste Management Rules 2016 (BMWM) categorises the biomedical waste into four major categories based on the segregation pathway and standard colour code assigned for containers.
- Yellow colour code is for anatomical waste, soiled waste, discarded or expired medicines, chemical waste, bedding, microbiology and biotechnology and other clinical laboratory waste.
- Red colour code is for contaminated plastic waste: waste generated from disposable items like intravenous tubes, bottles, catheters, syringes without needles, fixed needle syringes with their needles cut, urine bags, vaccutainers and gloves.

- White colour code for sharps including metals; used, discarded and contaminated metal sharps, needles, syringes with fixed needles, needles from needle tip cutter, blades, scalpel or any other contaminated sharp object that may cause puncture and cuts.
- Blue colour code is for metallic body implants, broken or discarded and contaminated vials, ampoules.
- As per WHO guidelines, the biomedical waste is also classified based on risk factors involved into four major groups.
- It is important to segregate waste at the point of its generation from handling point of view.
- Common Biomedical waste Treatment Facility (CBWTF) is available within 75 km of travelling distance of a health care facility.
- A symbol indicating 'Biohazard' should be placed in all colour coding and labels.
- The radioactive waste should be disposed of according to the guidelines issued and as per the provisions of the said rule.

EXERCISES

1. Which microorganisms are used in sewage water treatment and what is their role?
2. Explain as to how biological oxygen demand represents the condition of sewage water?
3. How does any toxic substance get biomagnified among organisms? Explain in brief.
4. What are xenobiotic compounds? How do these compounds affect the productivity of soil?
5. Discuss the process of aerobic and anaerobic decomposition of sewage waste water treatment.
6. What are the different types of solid wastes produced?
7. Discuss the role of different microorganisms in the process of composting of solid waste.
8. How are pesticides harmful for non-target organisms?
9. Briefly explain as to how microorganisms can bioremediate toxic pesticides into harmless and non-toxic compounds.
10. Which of the following compounds can be removed from waste water during treatment using lime?
    (a) Organic compound

(b) Phosphorous salts
(c) Ammonia
(d) Urea

11. Maximum decomposition of water takes place during:
(a) Primary treatment only
(b) Primary and Secondary treatment both
(c) Secondary treatment only
(d) Secondary and tertiary treatment both

12. Which enzyme is mainly responsible for hydrolytic breakdown of pesticides?
(a) Peroxidase
(b) Oxidare
(c) Cytochrome P/450
(d) Esterase

13. Which of the following is not a strategy of bioremediation?
(a) Sludge digestion
(b) Composting
(c) Slury bioreactor
(d) Biopiles

14. **Assertion:** Bioremediation is a process through which toxic pesticides can be completely removed.
Reason: Microorganisms can be used for bioremediation of toxic chemicals.
(a) Both assertion and reason are true and the reason is correct explanation of assertion.
(b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.
(c) Assertion is true but reason is false.
(d) Both assertion and reason are false.

15. On the basis of the colour coding and type of container for disposal of health care waste, choose the correct match:
(a) Yellow — recyclable plastic waste
(b) Red — metallic body
(c) White — sharp metals, needle, syringes
(d) Blue — general health care waste

16. What is the basis of classifying organisms into four risk groups?

UNIT V

Recent Innovations in Biotechnology and Entrepreneurship

Chapter 12: Recent Innovations in Biotechnology

Chapter 13: Entrepreneurship

John Bertrand Gurdon
(2nd October 1933–Present)

Sir John Bertrand Gurdon (born on 2 October 1933) is an English Developmental Biologist. He is best known for his pioneering research in nuclear transplantation and cloning. In 1956, he joined a Ph.D. programme with Embryologist Michael Fischberg and started to work on nucleus transfer from differentiated cells to eggs cells. Later, he successfully replaced a nucleus of fertilised egg cell of frog with a nucleus of mature cell from the intestine of tadpole. This egg cell with the nucleus from intestinal cell grew successfully into a new frog. This experiment proved that mature cell also carries the required genetic information required to differentiate into every cell type. Gurdon's work laid down the foundation for cloning, which later resulted in the first successful cloning of mammal "Dolly". In 2012, Gurdon was awarded the Nobel Prize for Physiology or Medicine, jointly with Shinya Yamanaka, who discovered the induced pluripotent stem cells. His Nobel Lecture was called "The Egg and the Nucleus: A Battle for Supremacy".

Chapter 12 Recent Innovations in Biotechnology

Biotechnological innovations have grown steadily over the past 10 years or so, and have benefited the human lives in agriculture, medical science, environment and energy. These technological innovations include GM crops, diagnostics, bacteria that can eat oil, growing human organs in labs for transplants, and advances in biofuel, that can help reduce our nation's carbon footprint. Innovations could help improve cost effectiveness and resource efficiency of biogas energy technology in a sustainable manner. We now even have plants and meat grown in labs that can be genetically regulated to taste and look in a particular way reflecting on the importance of synthetic biology.

12.1 Environmental Biotechnology

Ever since the industrial revolution started in the 1750s, the number of industries in different sectors increased exponentially. With this, came the unprecedented degradation of the environment, polluting the air, water and soil with various pollutants such as heavy metals, pesticides, dyes, carbon dioxide and other greenhouse and poisonous gases, micro-pollutants, etc.

In addition to the issue of environmental degradation, it has also become inevitable to find alternatives for non-renewable fossil fuels or finding more efficient ways of extracting and using fossil fuels. Biotechnology offers viable options, primarily because it is environment-friendly and uses natural systems. In environmental biotechnology, different forms of biological systems, whether naturally occurring or genetically engineered, are used.

The basic concepts and approaches in the field of environmental biotechnology are bioremediation (that includes a wide array of applications such as waste treatment, degradation, vermi-technology, etc.), prevention of environmental problems, detection and monitoring of contaminants and genetic engineering. You have learned about bioremediation in Chapter 11 of Unit IV. In this section, the focus will be on the second aspect, i.e., prevention, and will primarily deal with the production of biofuels and avenues in the field of biodegradation and manufacture of biodegradable products.

12.1.1 Biofuel

Biofuels are the fuels produced from biological products, which could be living organisms or from waste generated from biological products, such as from landfills, recycled vegetable oil, etc. In many places specific crops, such as soybeans, jatropha, pongamia, palm oil, algae, etc., are grown for the production of fuel. Fuels produced from such crops are known as biofuel or **agrofuels**. On the basis of their characteristics, biofuels can be broadly divided into biodiesel, bioalcohol, biogas and biomass in different forms.

Biodiesel

Biodiesel is made from raw materials such as animal fats, vegetable oils, waste cooking oil, soybean, rapeseed, jatropha, mustard, flax, sunflower, palm oil, canola, hemp, field pennycress, *Pongamia pinnata*, algae, etc., by the process called trans-esterification. Although in many European countries, a 5 per cent biodiesel blend is widely used, it can be used in its pure form without blending it with fossil diesel. The advantage of biodiesel is that it

contains higher hydrogen and oxygen and less carbon when compared with fossil fuels. This improves the combustion of biodiesel and reduces the particulate emissions from unburnt carbon. Another advantage of biodiesel is that it can be directly used in regular diesel engines without making any changes or modifications to the engines.

Bioalcohol

Bioalcohols, such as bioethanol, are produced from wheat, corn, sugarcane, molasses, sugar beets, potato, fruit waste, etc., by the process of fermentation. The basic steps in the production of bioalcohol include treatments which release sugars from the stored starch or cellulose followed by fermentation of the sugars by microorganisms, distillation and drying.

Box 1: Ethanol Blending in Petrol

Ethanol is widely used across the continents. Although it can be blended in any percentage with petrol, the blending percentage varies and ranges from about 2% to almost 30%, depending upon the norms adopted by the country. In India, the government has permitted oil marketing companies to sell ethanol blended petrol with percentage of ethanol up to 10%. Bureau of Indian Standards (BIS) specification recommends to achieve 5% ethanol blending across the country as a whole. As more ethanol is blended, the lesser will be the consumption of fossil fuel. In addition, ethanol is renewable and also reduces carbon dioxide and other greenhouse gas emissions. However, the use of ethanol has practical problems, like reduced efficiency of the engine. It makes engines harder to start and causes sputtering and oxidation of aluminium in the carburettors. Further, it causes steel components of the engine to rust. In order to overcome this issue, automobile manufacturers have to make necessary changes in the engine.

As far as the source of biofuel is concerned, micro algae and macro algae are being explored for the purpose. Micro algae includes microscopic photosynthetic forms, and the macro algae are the large multicellular plant-like organisms, commonly known as seaweeds. Different varieties of seaweeds have varied content of oil and sugar. The mass production of biofuels from algae is being explored because of several reasons, such as the ease with which it can be grown and harvested. It is renewable as it can be grown and harvested year after year, and it can produce both bioalcohol and biodiesel, it is non-edible and

can serve as an alternative to corn, sugarcane, soybeans and other sources which are also consumed as food. This is also important from the perspective that increased use of agriculture fields to produce biofuels or biodiesel which will adversely affect food production for human consumption. However, in spite of the advantages of using algae for the production of biofuel, at present it is still not cost-effective and research is underway to overcome such challenges.

Biogas

Biogas is a useful energy source or biofuel produced from organic substances such as dung, night soil wastes, manure, crops, organic industrial waste, waste water, etc., and mainly consists of methane and carbon dioxide. It is produced by breaking down of organic material with the help of bacteria in anaerobic conditions. This process takes place in an anaerobic digester and is accelerated by creating the optimal conditions for microorganisms to multiply, which leads to a highly efficient breakdown of materials.

Biogas could play a key role in the developing market for renewable energy. Utilisation of biogas is expected to be doubled in next five years. Biogas generation serves three important functions: waste removal, environmental management and energy production.

In India, under the National Biogas and Manure Management Programme, the Ministry of New and Renewable Energy installed about 49.3 lakh biogas plants in the country, introducing novel technologies, such as cryogenic separation, *in situ* upgrading, hydrate separation and biological methods. Biogas plant is considered to be the most sustainable option for households having feed material, so as to become self-dependent for cooking gas and highly organic enriched bio-manure. It provides the solution to protect the households from the problems of indoor air pollution at the same time saving on the cost of refilling of LPG cylinders. Biogas is considered to be the future of renewable and sustainable energy.

Inspite of its many benefits, the method of its production is yet to be perfected so as to simplify and improve the process of biogas generation to make it more efficient and

user-friendly. Biogas has been found to damage vehicles by corroding the metal parts of the engine when used in automobiles as a biofuel. This greatly increases the cost of maintenance.

Biomass

Biomass, or solid biofuels, are an important source of energy, especially for the economically weaker section of the society. Some examples of solid biofuels are wood, sawdust, coal, grass cuttings, domestic refuse, charcoal, agricultural waste, non-food energy crops, dried manure, etc. Most of the available biomass can be conveniently used directly on stove, furnace, hearth, etc. Certain forms of biomass such as sawdust, wood chips, and other agricultural wastes are preferably converted into pellets. Cow dung cakes are another popular form of solid biofuel. However, the problem that comes with the combustion of biomass or solid biofuel is that it emits considerable amounts of pollutants, such as particulate matters and polycyclic aromatic hydrocarbons (PAHs), which are harmful to health.

12.1.2 Biodegradation

The natural waste management and recycling system breaks down everything from yard waste to crude oil keeping the planet clean and healthy. Increase in population has resulted in the generation of heaps of wastes, many of which are non-biodegradable, such as plastics and fluorinated carbons, which takes hundreds of years to decompose. Microbes have been identified that can break down such non-biodegradable materials. For example, scientists have discovered a strain of bacteria that can literally eat plastics. They have also found ways to improve such activity of the bacteria so that the task can be done faster. However, this technology is still developing, but it is promising for environment-friendly recycling.

Bioplastics

An alternative to reduce non-biodegradable products is the production of materials that are made using biological materials. For example, instead of using styrofoams or

plastics in the manufacture of disposable or single use or use-and-throw products, plant materials such as leaves, bamboo, wood chips, corn starch, seaweeds and natural biopolymers, such as polysaccharides, proteins or lignin, natural rubber, etc., can be used. Since plastics are made from fossil fuels, such approaches to do away with plastic products will help reduce the use of fossil fuels and hence, thereby reducing the plastic based pollutants. Though less than 10% of oil is used to make plastic products, this is important keeping in view its impact on the environment. Technological innovations have also been made in the field of bioplastic production. In 1999, the production of biopolymers from seeds and leaves of genetically modified plants had been established which is economically competitive with polymers produced from petrochemicals. Biodegradable plastic, e.g., Polyhydroxybutyrate (PHB) is being produced commercially by fermentation with the bacterium *Alcaligenes eutropus,* and can be produced in transgenic plants. The genetically engineered *Arabidopsis* plant may be used to upscale PHB production as the three genes involved in PHB synthesis from *A. eutropus* produced PHB globules in their chloroplasts without affecting plant growth and development. The large scale production of PHB may be done using plants such as leaves of populus, the only limitation being its high cost as compared to synthetic polymers.

Biotechnology in paper industry

Pulp making is a crucial step in the manufacture of paper during which wood chips are cooked by heating under pressure using caustic soda and sulfur. During this process, lignin is separated from cellulose fibers. Biopulping (treatment of wood chips with lignin-degrading fungi) improves the effectiveness of pulp making, thereby reducing the demand for energy and chemicals, it improves paper quality and ultimately decreases the environmental impact of pulp production. By increasing such efficiency, it helps in conserving raw material, energy, chemicals, etc. On the contrary, GM trees that produce less of lignin are being considered to be a promising solution to ease the process of pulp making. As in the case of biopulping, biobleaching uses enzymes such as xylanase to reduce the use of chlorine in

bleaching. However, considering the impacts of deforestation to obtain wood for paper industries, alternatives are also being explored to completely replace the use of wood for the manufacture of paper. Non-wood materials such as bagasse, bamboo, jute, hemp, esparto, flax, grass, kenaf or reed, etc., are now used in paper making, whereby biotechnology contributes immensely in paper industry.

Biotechnology in oil recovery

Microbial technologies are also being used in Enhanced Oil Recovery (EOR). EOR is a stage in the production of oil, which involves the extraction of residual oil from the initial first two phases (primary and secondary phases) of production. The use of suitable microbes and their metabolic products can enhance the efficiency of EOR. The ability of bacteria to feed on hydrocarbons has also been exploited to clean-up after an oil spill. These oil-eating microbes play an important role in maintaining the ecosystem. A genetically engineered *Pseudomonas* bacterium has also been developed, which can be used to 'eat' oil spills. Such 'superbugs' have immense environmental benefits. The details of superbug construction is given in Box 10 of Chapter 11.

12.1.3 Bioremediation

Bioremediation has gained importance with advances in biotechnology, especially environmental restoration, using microorganisms to reduce the toxicities caused by chemical pollutants including heavy metals in soil or water such as As, Cr, Hg, Cd, Zn, etc. Three key principles must be considered for selecting the most appropriate strategy to treat, contaminated wastes:

(1) Possibility of pollutant to allow biological transformation to less toxic products.

(2) Bio availability of the contaminant to micro-organisms.

(3) The efficiency of bio-activity.

Recent advances in molecular genetics of biodegradation and studies on enzyme-tailoring through recombinant DNA–RNA technologies are being utilised for the removal of heavy metals and toxic chemicals from contaminated sites. Bioremediation has been discussed in detail in Chapter 11.

12.2 Plant Biotechnology

Plant biotechnology such as GM technology and molecular-assisted breeding has generated products that have helped to achieve enhanced yields in a more sustainable manner. Development of pharmaceuticals, recombinant therapeutic proteins, plant-made pharmaceuticals, transgenic plants, artificial seeds, and plant-made vaccines or antibodies (plantibodies) are currently in advanced research in plant science. Recently, attempts are being made to use genome editing technique namely CRISPR-Cas9 for crop improvements.

12.2.1. Innovations in plant biotechnology through GM crops

You have already studied about GM crops and its application in Chapter 4 of the book. Almost 29 countries adopted GM crops in which soybeans, maize, cotton and canola form the major crops. The transgenic technology initially developed was limited to the control of weeds and pests, whereas, the second generation of GM crops has assisted farmers in the production of plants with tolerance to abiotic stress, improved nutritional features, can overcome sexual incompatibilities between plants and species, and overcome barriers that allow the introduction of genes from unrelated organisms such as bacteria, fungi or other plants and also from viruses. Innovations made in plant biotechnology through GM crops are described under various heads in the following sections.

Herbicide tolerant GM Crops: Weeds (plants growing where they are not wanted, e.g., Striga) decrease crop yields and quality primarily by competing with crop plants for light, water and nutrients. Farmers apply herbicides or weedicides (e.g. glyphosate) for the eradication of weeds in the fields, but the main problem with this is the loss of crop plants also as these herbicides are non-selective. Newer techniques, based on biotechnology tools, have been developed which are quite effective for weed management as well as in increasing the yields and income. There are several biotechnological strategies for weed control, but the most commonly employed approach is the over-production of herbicide target enzyme (usually in the chloroplast) in

the target plant (crop), so that it becomes insensitive to the herbicide. The popular example for such an approach is the introduction of a modified gene from an *Agrobacterium* that encodes for a resistant form of an enzyme into crop plants for tolerance against the most extensively used herbicide glyphosate (sold as Roundup) which is effective against many weeds. Roundup Ready GM crop plants, such as canola, soybean, corn and cotton, tolerant to glyphosate have already been commercialised.

Disease resistant GM crops

A wide range of diseases caused by viruses, fungi, bacteria, insects, mites and plant nematodes lead to significant crop losses each year. Disease causing microbes and strategies to make resistant plants are discussed below.

(i) Viruses

Viruses are among the most ubiquitous pests in agriculture. The transgenic technology has been used successfully in many plants against several different viruses such as virus causing yellow crookneck disease in squash, potato mosaic virus, the potato leaf roll virus, barley yellow dwarf virus in wheat and papaya ringspot virus (PRSV) for developing resistance to virus by introducing gene for viral coat proteins. A viral coat protein acts like a vaccine, causing the plant to develop resistance to the particular virus. Transferring the gene for a viral coat protein, a part of the outer shell of a virus that does not cause disease, into the plant may confer protection to the plant and induces resistance without the introduction of the actual virus.

(ii) Fungi

Fungal diseases cause significant damage to crops including fruits and vegetables, and is characterised by wilting, mouldy coatings, rusts, blotches, scabs and rotted tissue. Finding adequate substitutes for fungicides like methyl bromide has intensified the need to develop genetic engineering approaches. Defensins are antimicrobial proteins that enhance the plant's tolerance to pathogens and are present in insects, mammals (including humans), crustaceans, fish and plants. Defensins from moths and butterflies, the fruit fly, pea seeds and alfalfa seeds all

show potent antifungal activity too. The first transgenic application of defensins was the incorporation of the antifungal defensin from alfalfa into potatoes. The transgenic potatoes were resistant to the fungal pathogen *Verticillium dahlia*.

Resveratrol from the white grapevine is a secondary metabolite that acts as a natural defensin which protects the plant from *Botrytis cinerea* infection. A gene of resveratrol when inserted into the host plant provides resistance against *Botrytis cinerea* in wheat and barley. Rpi-vnt1.1 gene isolated from *Solanum venturi* is transferred to potato to provide resistance for late blight caused by *Phytophthora infestans*. Similarly, wheat gene encoding oxalate oxidase is being transferred to *Castanea* sp. to provide resistance against chestnut blight fungus; and chitinase gene from insects is being transferred to apple to provide resistance to apple scab fungus.

(iii) Bacteria

Most food crops are susceptible to bacterial infections causing leaf and fruit (lesions), soft rots, yellowing, wilting, stunting, tumors, scabs or blossom blights. When tissue damage occurs on the blossoms, fruit or roots of food crops, yields may be reduced. GM crops have been developed against various bacterial pathogens in which lysozyme genes are being transferred into potatoes to provide resistance against blackleg and soft rot diseases caused by *Erwinia carotovora*. Gene from *E. carotovora* and *Pseudomonas syringae* (pv tabaci) is being inserted into rice to make it resistant to wild fire disease.

(iv) Insect

Widely used transgenic pest-protected crops are those that express insecticidal proteins derived from genes cloned from the soil bacterium *Bacillus thuringiensis* (Bt). This insecticidal protein called crystal (Cry) proteins or delta-endotoxins bind specifically to receptor protein in the insect gut destroying cells and killing the insect. A number of plants offering resistance to insects using this strategy have been developed worldwide. In India, Bt cotton is the only transgenic plant permitted by the Government for

cultivation at commerical level since 2002. Bangladesh became the first country in the world to approve the commercial planting of Bt brinjal, which started in 2014.

In addition, the widening spread of fall-army worm infestations prompted the researchers to strategise on its control by using pyramided, i.e., stacking of two or more genes in a single crop plant for insect resistant crop improvement. Stacking of two genes, one with increased water use efficiency and other for resistance to insect pests have resulted in the formation of hybrid maize seeds. Similarly, Bt gene was stacked with the drought-tolerance trait that expresses the *B. subtilis* cold-shock protein B (cspB).

(v) Plant nematodes

Transgenic strategies to combat nematodes that infect bananas, soybeans, rice and potatoes are emerging areas. Incorporation of the genes in potatoes produced upto 70 per cent nematode resistance in field trials. Using genetic markers, scientists bred plants containing the resistance gene with domesticated varieties, circumventing the poor performance characteristics of the wild variety. Producing a resistant variety of soybean from wild strain is one such example, in which by using specific genetic marker, scientists first identified a particular trait and its subsequently breeding with plants containing resistance gene with domesticated variety. The new varieties are not transgenic but have resulted from combining the use of modern genetic markers with conventional breeding techniques.

There are many other applications of such genetic modification of crops through which a tolerant and resistant variety of crops can be produced. One of the examples is a GM maize CIEA-9 that has been developed in Mexico, which has enhanced adaptation to severe drought and extreme temperature in which sugar trehalose is broken down by a glycoside hydrolase enzyme trehalase. By antisense RNA technology, trehalose expression is silenced in maize and such a biotech maize requires approximately 20 per cent less water and can withstand higher temperature.

Box 2: Few GM plants and their acquired charateristics

Crop	Name of trait/ gene transferred	Applications
Stress resistant GM crops		
Arabidopsis and Tobacco	Mannitol	Withstand high saline conditions and enhanced germination rates and increased biomass
Rice	Embryogenesis abundant protein gene from barley	More tolerant to drought and salinity than conventional varieties of rice
Soybeans	*HaHB4* transcription factor gene from sunflower plant	HB4® soybean named EcoSoy® tolerant to drought and herbicide
Wheat	*HaHB4* transcription factor gene from sunflower plant	HB4® wheat named EcoWheat® has increased the yield upto 20% and drought tolerant
GM crops for enhanced paper quality		
Arabidopsis	Coenzyme A-ligase	Resulted in transgenic plants with upto 45% less lignin
Eucalyptus	Gene encoding an *Arabidopsis thaliana* protein	20% more wood than the conventional variety and is ready for harvest in five and a half years instead of seven
GM crops for improved product		
Potato	Gene involved in starch synthesis	60% more starch than non-GM plants which make potatoes take up less fat during frying, resulting in a lower-fat product
Maize and Soybean	Two stacked genes	Confer tolerance to the herbicides glyphosate and 2,4-D-choline
Apple	Gene involved in non-browning	Apple named Arctic®Gala had non-browning trait

GM technology has also helped scientists in the fortification of many crop plants and a few of there have been depicted in Table 12.1.

Table 12.1: Biofortified GM crops

Crop	Nutrient incorporated	Name of the trait/ gene transferred	Benefit
Golden Rice	Vitamin A (Vit. A)	Genes encoding phytoene synthase, carotene desaturase and lycopene beta-cyclase which will lead to synthesis of beta-carotene	Golden rice is an efficient source of vitamin A in human

Soybeans	High oleic acid	Gene encoding fatty acid desaturase was down regulated	Seeds have increased ratio of oleic acid to linoleic acid content
Tomato	Increased shelf life	Polygalactouronase expressed in antisense orientation	Increased shelf life

12.2.2. Genome Engineering technology: Application of CRISPR-Cas9

In Chapter 5, Section 5.4.2, you learned about genome editing with CRISPR-Cas9 technology, in which guide RNA bound to target DNA and Cas9 is used to edit genes in diverse plants. Scientists at the University of California San Diego have developed the first CRISPR-Cas9-based gene edited plants, with the objective of producing drought and disease-resistant crops. Breaking from the traditional inheritance principles that stipulate that an offspring acquires genetic materials equally from each parent (Mendelian genetics), the new research employs CRISPR-Cas9 editing to transfer specific traits from a single parent to successive generations. It has been used to improve nutritional quality, increase yield, and develop resistance to various biotic and abiotic stress in several crops, including rice, tomato, and soybean, etc. As we know that the traditional methods take years to develop a different trait, CRISPR-Cas9 is comparatively rapid, than other genetic manipulation techniques. Furthermore, CRISPR-Cas9 technology is relatively simple, precise, and can be used to create many beneficial traits in plants as it targets multiple genes. However, the revolution brought by CRISPR-Cas9 in plant molecular biology has raised various concerns for its off-target effects, and at times, low efficiency. In addition, there are regulatory issues and several challenges for its safety and use in the future. Efforts are being undertaken to improve its on-target efficiency and several modifications of the Cas9 enzyme. However, the work carried out is in preliminary stage and there is still more to be done.

12.3 Regenerative Medicine

Regenerative medicine is an emerging multidisciplinary specialty in medicine with the goal of cell and organ replacement to restore the loss of function resulting from degeneration, trauma or other disease processes. It can also protect vulnerable diseased cells from death. The oldest strategy is replacement of the diseased or lost organ by a new one, which is termed as **transplantation**. The organ transplantation may be of three types:

1. **Autograft** – Transplantation of cells, tissues or organs between sites within the same individual, e.g., skin graft.
2. **Allograft** – Transplantation of organs or tissues from a donor to a non-genetically identical individual of the same species. This is the most common practice for organ transplant like renal, liver, heart, lungs and pancreas.
3. **Xenograft** – Transplantation of an organ or tissue between two different species. 'Pig valves', have been used to repair or replace a defective heart valve in humans.

Majority of organs like liver, kidney, pancreas consists of differentiated cells with limited capacity for regeneration. In case of major organ damage, these differentiated cells cannot proliferate and replace the damage cells. Stem cell technology can be helpful in this situation where the stem cells have the capability to proliferate and differentiate, replacing dead or damaged cells. There are several types of stem cells depending on their differentiation and origin as depicted in Chapter 9.

12.3.1 Stem Cell Technology

Stem cells exhibit an intrinsic ability to assemble into complex structures. When placed within a hydrogel (often Matrigel) and in the presence of suitable exogenous factors, the stem cells can be coaxed into forming structures that contain organised clusters of cells. The recent availability of stem cell derived organoid systems to provide 3D self-organised tissue models provide a compelling new class of biological model to serve as both tissue and organ substitutions (Fig. 12.1).

Organoids are ultra-small, self-assembled three-dimensional tissue cultures that are derived from stem

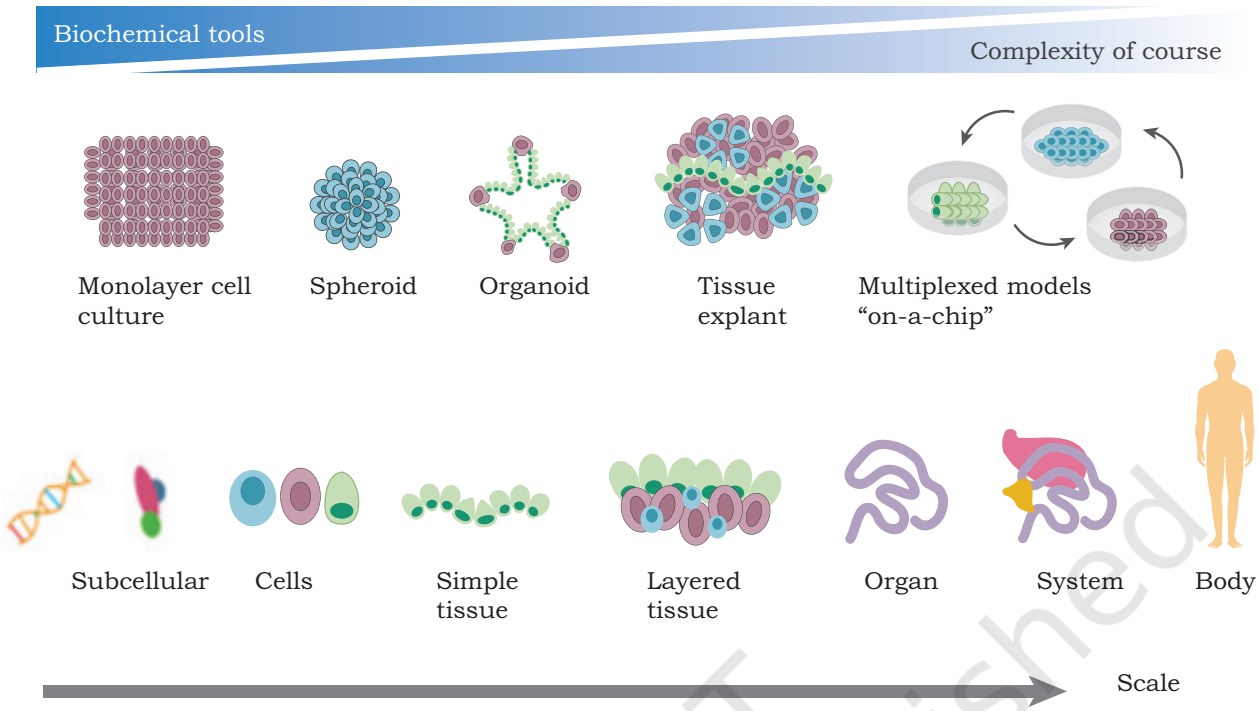


Fig. 12.1: All organisms follow a system from the subcellular level to the whole body. A number of models have been developed across organismal hierarchy, to address definite questions across biology and medicine. Each model system has specific characteristics; in general, with increasing scale comes increasing system complexity and challenges in cell culture and the reduced availability of biochemical and quantitative tools, which can limit study insights. Organoid models provide a unique opportunity to incorporate moderate system complexity while still affording many tools for probing structure and function. When compared to tissue explants, organoid systems can mimic similar cell-cell and cell-matrix interactions while maintaining the ability for long-term cultures, maintaining signaling cues that are important for survival.

cells. Organoids can be created from both pluripotent stem cells (PSCs) and adult stem cells (ASCs) by simulating the biochemical and physical characteristics of tissue development and homeostasis. Such cultures can be maneuvered to replicate most of the complexity of the organ, or may be differentiated to express certain characteristics, like producing particular subsets of cells only. For example, the shape and tissue architecture of mesenchymal stem cells from bone marrow directly impacts their differentiation into osteoblasts or adipocytes. A round shape promotes adipogenic differentiation, and cells with wide spreading favour an osteoblastic differentiation.

Organoids reiterate a number of biological parameters that include spatial organisation of tissue-specific cells that are heterogeneous, cell-matrix interactions, cell–cell

interactions, and certain physiological functions generated by tissue-specific cells within the organoid.

Organoids bridge a gap in the existing model systems by providing a stable system that can be extended to the manipulation and mimicking the *in vivo* physiology. The foremost aim of engineering organoid systems is to enhance the system utility in downstream applications. Therefore, it is necessary to create improved *in vivo* mimics for tissues and organs and to improve the organoid system to achieve high-throughput formats or multi-tissue organoid compatibility in larger multiplexed systems, e.g., human-on-a-chip. Bioengineers have long aspired to deconstruct biological systems and manipulate or reconstruct the system in a controlled manner. Bioengineering approaches have enabled us to understand cell behaviour and cell organisation, that are the fundamental processes in organoid formation. Several improved systems are on the horizon.

Cells in monolayers have the same unlimited access to nutrients and growth factors. But, in *in vivo*, the position of cell inside a tissue determines whether it can get enough nutrients. In response to the downsides of monolayer cultures, biomedical companies have developed various types of **3D cell culture systems** to exploit its potential for predicting more realistic representations of drug response *in vivo*.

Tumor spheres represent a model of cancer stem cell expansion; tissue-derived tumor spheres and organotypic multicellular spheroids are characteristically obtained from mechanical dissociation and cutting of the tumor tissue. However, there is a distinct ordered self-assembly in organoids as opposed to spheroid cultures, and the former is more dependent on a matrix for its generation.

Organoids have recently attracted attention as a model, primarily as they may serve as a better *in vitro* model when compared to 2D or even 3D co-culture systems. Applications for organoid research include organ development, disease modeling, drug penetration in the centre of the tumor mass, drug screening and toxicity testing. To induce spheroid formation, one can choose from methods such as suspension culture, non-adherent surface methods, hanging drop methods, and microfluidic methods (Fig. 12.2).

Though 3D cell cultures provide realistic models of cell assembly into tissues or organs, the new variables as to how the tissues function, or how the pathological processes disturb the cellular functions, need to be carefully addressed. Three-dimensional models are far more expensive and time-consuming than 2D cultures, as very few automation and reproducible applications are available. The 3D cultures further require careful planning and expert handling.

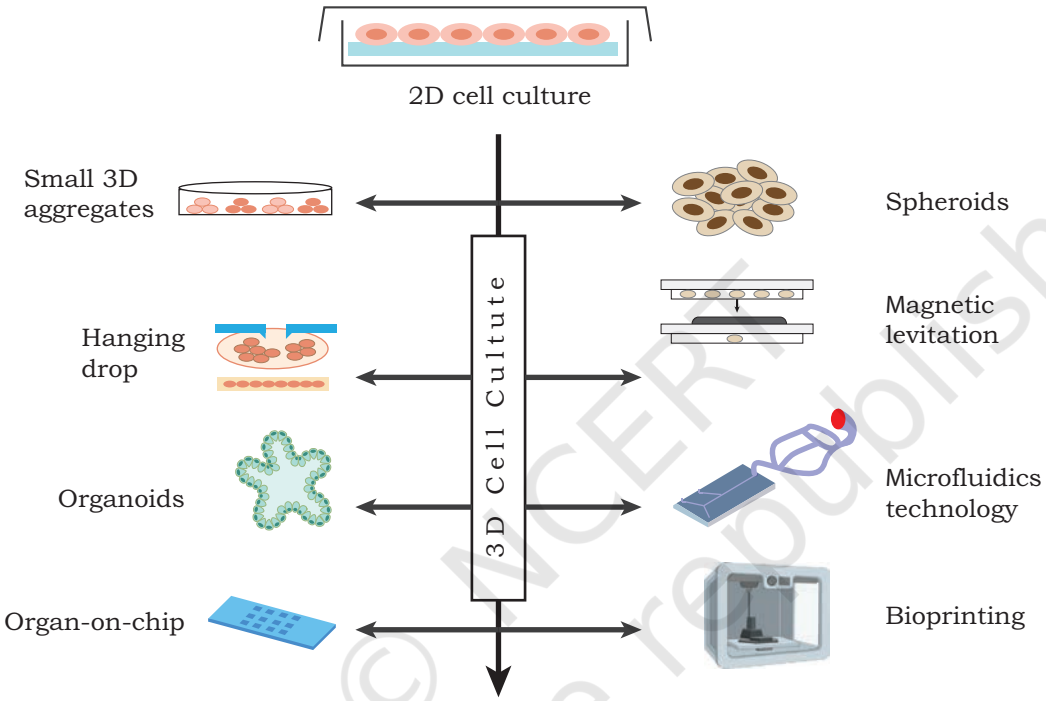


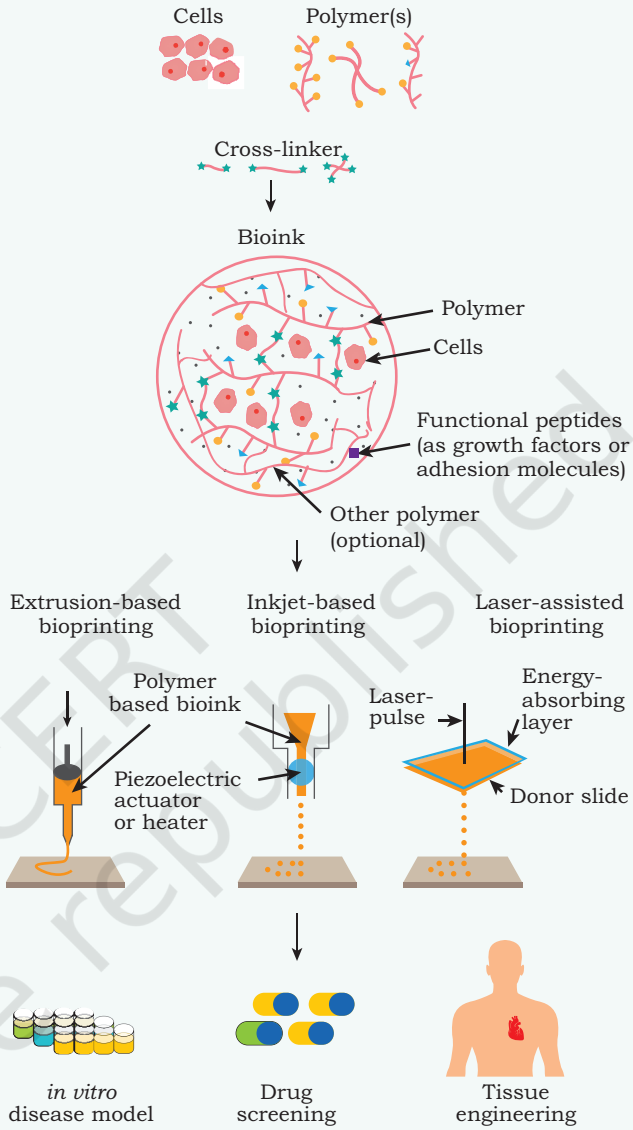
Fig. 12.2: 2D and 3D culture

Rapid scientific development in this field has led to several new strategies involving cell based therapy, polymer and material science, nanotechnology, bioengineering and 3D bioprinting making an artificial organ or a substitute of the organ. Currently, tissue engineered vascular graft, trachea, cardiac patches are in clinical trials. In near future, swift development in the field of regenerative medicine will empower the physicians to replace the majority of the diseased or dead cells in the human body.

Box 2: Bioprinting

Several therapies based on the principles of regenerative medicine are currently being practiced which are approved by regulatory bodies. The usual practice in regenerative medicine includes identification of the diseased organ dimension by imaging like Computerised Tomography (CT) scan or Magnetic Resonance Imaging (MRI). The tissue engineered scaffold with biopolymer embedded with cells and nanoparticles containing growth factors can be made by a computer assisted 3D Bioprinter with exact dimensions calculated from the MRI/ CT scan. The concept is depicted in the diagram.

Cartilage injury is common in sports injury as well as degenerative disease in old population which can lead to significant disabilities. 'Carticel' is an innovative therapy where autologous cartilage cells (chondrocytes) from articular cartilage fragment removed during surgery are expanded in *in vitro* and then implanted at the site of injury. 'laViv' is a technology where autologous fibroblasts are injected in the face for the removal of wrinkles or beautification of nose as a part of cosmetic surgery. 'Dermagraft', a patch of allogenic fibroblast dermal substitute, is used for diabetic ulcer. Autologous platelet, derived from the peripheral blood, is used for wound healing.



Overview of cell-based therapy, polymer and material science, nanotechnology, bioengineering and 3D bioprinting for tissue engineering, drug screening and creating in vitro disease model

12.4 Nanobiotechnology

Nanoscience is the study of materials which are in nanoscale range, that includes size in nanometer, that is less than one micron. Fig. 12.3 gives clarity on the concept of scales,

where an atom is 0.1 nm and a small molecule of sand is 1 mm. The diagram shows the size comparison among an atom, which is less than 1 nm, a fullerene molecule containing 60 atoms of carbon (C60), a molecule of DNA, protein, a virus, bacteria, red blood cell, pollen and a sand particle (Fig. 12.3). The technology of using material in nanoscale is called nanotechnology. Nanobiotechnology thus implies application in the field of biotechnology at nano scale. The use of nanotechnology in medicine for diagnosis and therapy is termed as nanomedicine.

The conversion of any bulk material to nanoscale results in the alteration of its physicochemical, biological, mechanical, optical and electronic properties. These newly acquired novel properties of the materials due to conversion into a nanoscale can be utilised for different useful purposes.

The typical example of change in property of the bulk material when it is converted to nanoparticles of different sizes is observed in gold. Gold is a golden coloured metal widely used for making ornaments and jewellery.

Fig. 12.4 (a) shows golden colour of the bulk metal. The nanoparticles smaller than 30 nm are ruby red, up to 100 nm are pink and larger appear darker in colour demonstrating change in one of the physical characteristics

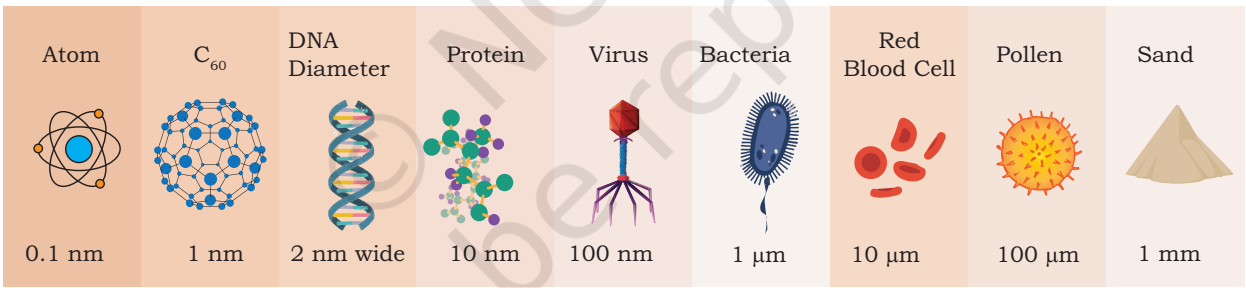


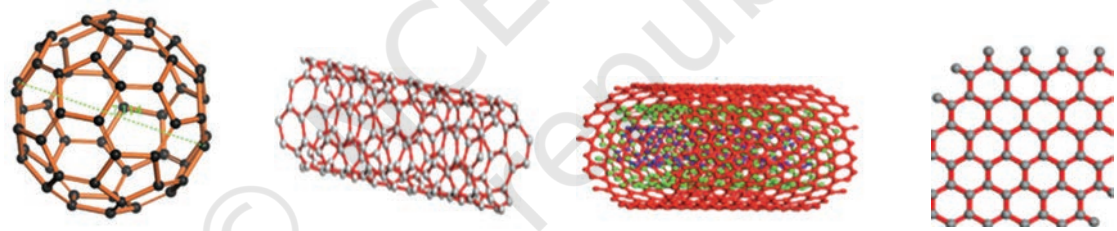
Fig. 12.3: Concept of scales (0.1 nm –1 mm)

with nanoform that is also size dependent. The bulk material, gold, of unit weight (1 mg) when converted to nanoparticles in a reducing size scale for example, with diameter of 500 nm, 100 nm and 10 nm, increases the number of particles exponentially accompanied by an enormous increase in the surface area [Fig. 12.4 (b)]. This phenomenon can tremendously increase the surface reactivity, which can then be used for different applications in the field of nanobiotechnology.

(a) (b)

Fig. 12.4: (a) Gold as a noble metal (b) Colour change in gold based on the size.

Nanoparticles can be made from a large number of inorganic and organic materials as mentioned in the following table (Table 12.2). Depending upon the composition, nanoparticles can be categorised as biodegradable and non-biodegradable. Properties of nanoparticles depend on its size, shape, surface charge and composition. Nanoparticles made from one material like carbon, depending upon its composition, may acquire unique size and shape like fullerene (C_{60}), single wall and multiwall carbon tube and graphene (Fig.12.5).



(a) (b) (c) (d)

Fig. 12.5: (a) Fullerene (C_{60}), (b) single wall, (c) multiwall carbon tube and (d) graphene

Table:12.2: Composition of some of the nanoparticles widely explored in nanobiotechnology

Inorganic	
Metal	Iron oxide, Gold, Silver, Copper, Zinc oxide, Titanium oxide, Cadmium, Selenium
Non-metal	Silicon oxide, Calcium phosphate, Ceramic
Organic	
Polymer	Naturally derived like chitosan from shell, alginate from algae, Cellulose, Lignin

Synthetic polymers	Polycaprolactone (PCL), polylactic acid or polylactide (PLA), Poly Lactic-co-Glycolic Acid (PLGA)
Protein	Albumin, Gelatin
Lipid	Cholesterol, Fatty acids, Phospholipids, Liposome

Similarly, cadmium–selenium (CdSe) nanocrystals of different sizes produce different colours of fluorescence, which does not quench with light (Fig. 12.6). The fluorescent CdSe nanocrystals are called Quantum Dots (QDs), which can be used for fluorescence based diagnostic tests.

12.4.1 Application of Nanotechnology

Nanotechnology is an enabling technology, which is useful in diverse sectors, such as chemicals, textile, consumer products, cosmetics, health (Nanomedicine), energy, agriculture, various industries and for environment as shown in the given diagram (Fig. 12.7).

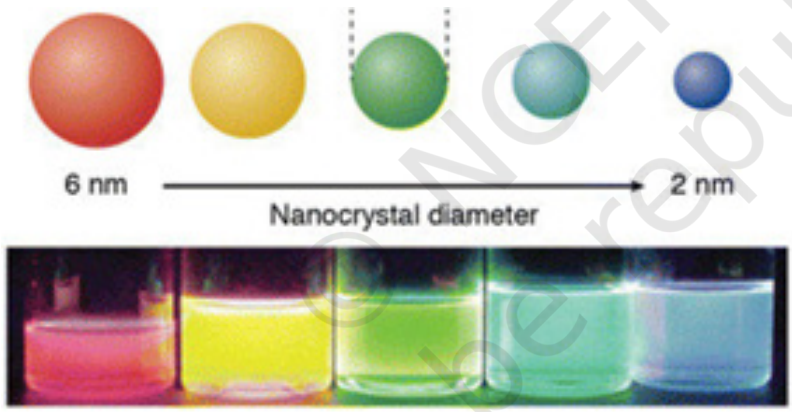


Fig. 12.6: The QDs of different sizes showing different colour of fluorescence

Some of the applications of nanotechnology are described in the following sections.

(a) Medicine (Nanomedicine): Drug or other biomolecules can be loaded in nanoparticles or nanocarriers, which can be targeted to the diseased site for delivery. This kind of nanotechnology based **Drug Delivery Systems** (DDS) can increase the bioavailability of the drugs at the diseased site with

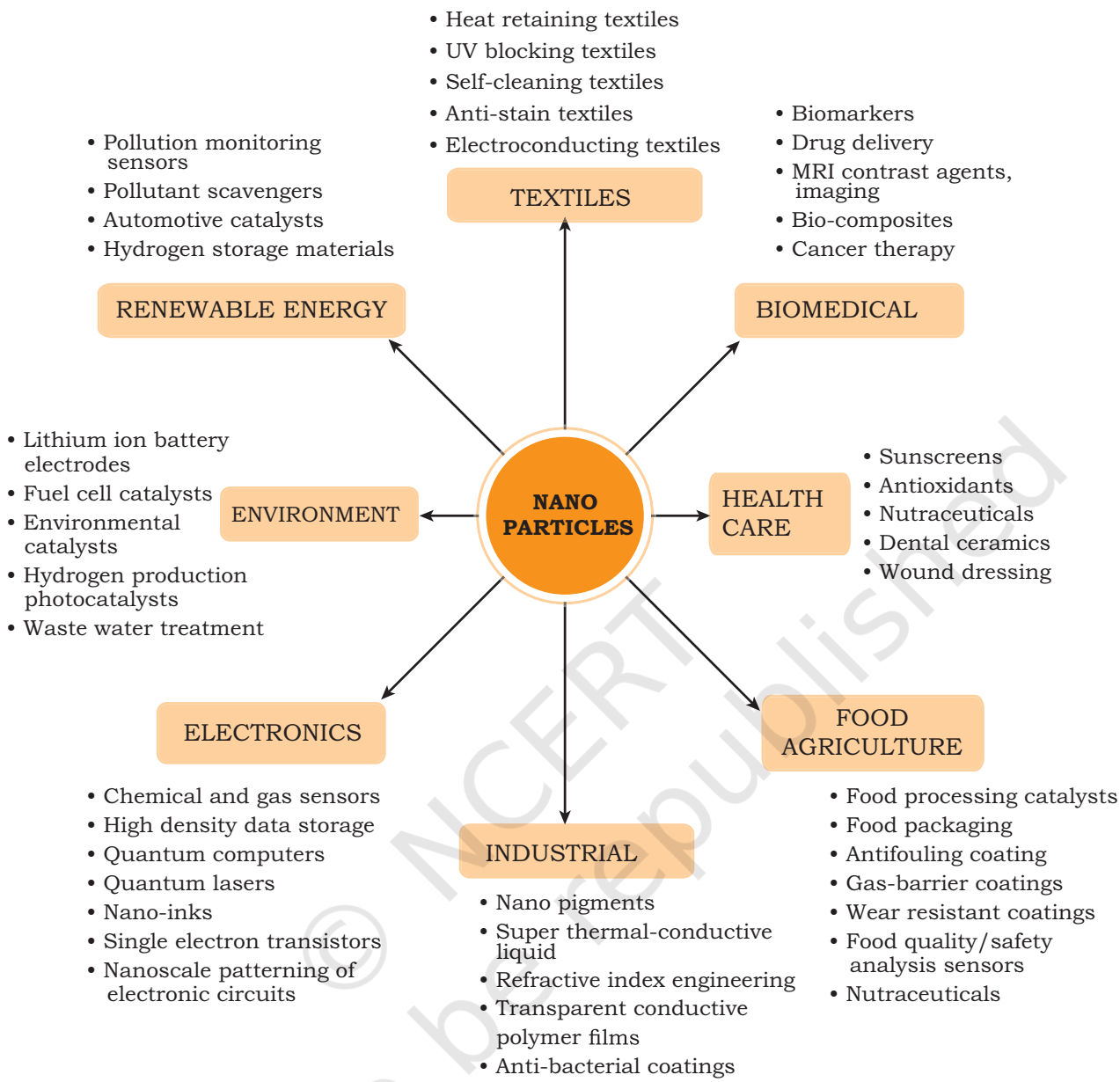


Fig. 12.7: Nanotechnology an enabling technology relevant to every aspect of life

low systemic toxicity and reduced amount of drug required for cure. Currently, systemic administration of drug by oral or parenteral routes need higher dosage to maintain high serum levels, so that effective concentration can be achieved at the diseased site, which is associated with toxicity involving other organs. Antifungal drug, amphotericin B has a high toxicity. When it is delivered in liposomes, which

is a spherical vesicle consisting of one or more phospholipid bilayers, it shows less toxicity and higher efficacy. Systemic toxicity with anticancer chemotherapeutic drugs is a major problem in cancer management. The anticancer drugs can be targeted to cancer micro-environment by enhanced permeability and retention (EPR) effect. The endothelial cells in the tumor has gaps or fenestration making them leaky in comparison to healthy tissue. If small size nanocarriers (20 to 30 nm) are loaded with drug and administered, it will be accumulated in the tumor area through the leaky vessels with retention of the drug loaded nanoparticles in the tumor area, this phenomenon is called EPR effect, which is shown in Fig. 12.8. An example of such kind of DDS is Paclitaxel loaded albumin nanoparticles or liposomal doxorubicin currently being used for cancer therapy. Silver nanoparticles have wide spectrum antimicrobial properties and are being used in different wound dressings.

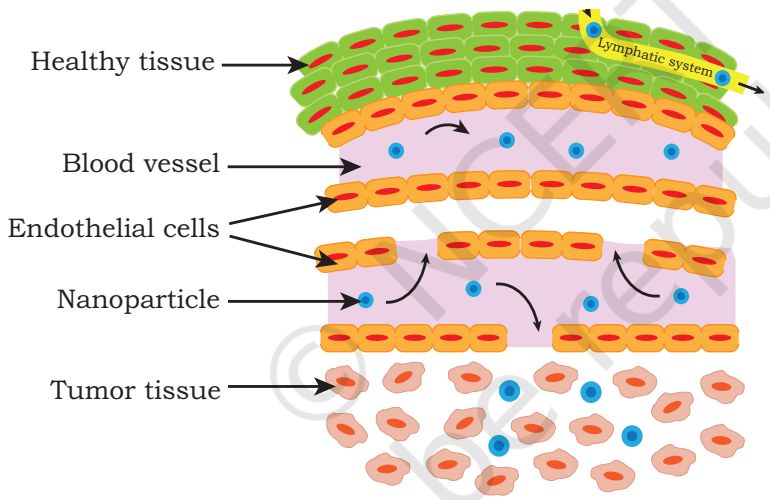


Fig. 12.8: Accumulation of drug loaded nanoparticles in tumor by enhanced permeability and retention (EPR)

(b) Cosmetics: Zinc oxide and titanium oxide nanoparticles are very useful for protection against ultraviolet light and widely used in sunscreen cosmetic preparations. Liposomal preparations and nanoemulsions are commonly used for better penetration of essential oils, vitamins and other biomolecules, which can prevent age related skin changes, reduce pigmentation and provide many other beneficial effects.

(c) **Agriculture and food packaging:** Nanotechnology is being used for precision farming techniques enhancing the absorption of nutrients. New generation of nano-pesticides is more effective in controlling plant diseases with less amount of pesticide thereby reducing human exposure. Nanomaterials are used in food packaging for longer preservation and storage. Silver and iron nanoparticles can be used for the treatment and disinfection of livestock and poultry.

(d) **Textile:** Nanomaterials can be used for several improvements of the textile like stain and water repellence, wrinkle-free features, fire retardancy, high tensile strength, durability, textured surface and so on. Nanotechnology can impart electrical conductivity to fibers without compromising their comfort and flexibility. The garments can sense and respond to external stimuli *via* electrical, colour or physiological signals and keep itself cool in summer and warm in winter. In near future, nanotechnology enabled garments can monitor the health conditions too.

(e) **Nanobiosensors:** Nanotechnology has revolutionised the area of sensor development for the detection of biological molecules, which may be biomarkers of diseases. Point of care devices can be made, which are low cost and more effective and the tests can be done at bedside, in villages with small volume of test materials. The scientists are working with the concept of 'Lab on a Chip', where many tests can be done simultaneously using nanotechnology and microfluidics.

The above-mentioned examples give a clear insight into the enormous scope of nanotechnology applications. In future, this technology renders immense hope and may lead to innovations in various biomedical applications ranging from molecular imaging, drug delivery, gene therapy to biosensors and biomarkers. However, the use of nanotechnology raises fundamental questions about the need for new regulations and we should understand the safety issues of using nanoparticles in the body and environment.

12.5 Synthetic Biology

Traditionally, scientists have used two major strategies to study organisms. First is a reductionist approach that involves going from higher level description to the lower level description, i.e., from the whole animal or plant to the tissue level, then to the cellular organelle level and finally at the molecular level (DNA, protein, etc.). We use several names for this general approach, e.g., anatomy, histology, molecular biology, biochemistry and so on. The reductionist approach has been highly successful leading to the generation of a huge data at various levels.

Now the challenge is to collect this data and weave it in the form of databases and computer models of tissues, metabolic pathways and so on. This integrative approach of data collection and doing biology through computer, is more popularly known as bioinformatics, systems biology and so on.

In June 2004, a third major approach was announced at MIT USA that focused on building organisms from scratch. People asked: Could one chemically synthesise a chromosome, assemble mitochondria, construct a nucleus, a cell or tissue and ultimately an organism, just like an engineer constructs buildings from brick and mortar? This new engineering approach of building organisms from the bottom up, is called **synthetic biology**. Therefore, synthetic biology may be defined as a rationale design approach of constructing biological components leading to certain functions. The logic is that if engineering principles have worked in constructing electronic gadgets, buildings, bridges and so on, why not use them in constructing organisms? However, to enable such an activity, one needs to build (a) standards and (b) rules of composition.

Synthetic biology bridges the gap between biologists and engineers to design and build innovative biomolecular networks, components and pathways, and to utilise these constructs to reprogramme and rewire organisms. Synthetic Biology offers innovative approaches for engineering new biological systems or re-designing existing ones for useful purpose.

Integrating interdisciplinary expertise has enabled synthetic biology to address the unpredictable challenges linked to the complex intricacy of cellular systems. Synthetic biology has enthused researchers to innovate and bioengineer biological systems to accomplish explicit tasks. Its applications are— biomanufacturing of high-value biomolecules, diagnostics, therapeutics leading to cheaper drugs, 'green' means to fuel our cars and targeted therapies for attacking 'superbugs' and diseases, such as cancer. These re-engineered organisms will change our lives over the coming years. While synthetic biology has demonstrated its potential to solve high-impact problems, a plethora of basic research studies are still going on to discover the engineering limits for biological systems.

While people were collectings the datasheets of gene, another thought emerged: how about chemically synthesising a series of genes in one step, instead of assembling them? This gave rise to the technology of long DNA synthesis leading to a major change in the way genetic engineering was practised (Fig. 12.9).

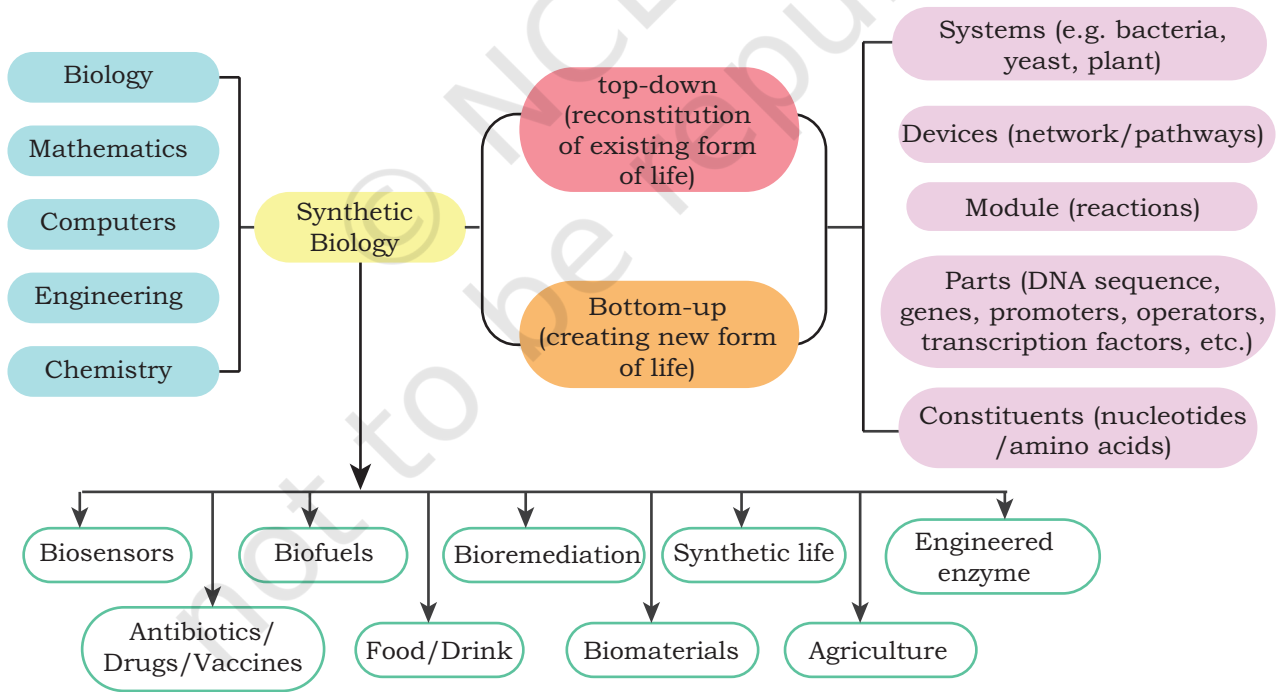


Fig. 12.9: Summary of synthetic biology indicating the amalgamation of associated disciplines and their varied applicability.

Instead of constructing plasmids and loading them with genes, the new strategy was to edit the plasmid in the computer, save the document as a text file of nitrogen bases (ATGC), email the sequence to the company and get the recombinant vector in a month or so. The process that would normally take six months to an year could be completed in less than a month. This strategy has been so successful that a chemically synthesised and a fully functional yeast chromosome was made by scientists at the John Hopkins University.

12.6 Future Prospects

Biotechnological innovations will undoubtedly be at the core of discoveries benefiting the society in future, depending primarily on how the future unfolds. For example, as microbes make their impact, complex ecological systems and exploitation of biological knowledge will offer innovations in a wide spectrum including medicine, health care, food systems, industries and creating smart materials.

Currently, scientists are working towards creating microbial factories, where bacteria can synthesise chemicals and help in the reduction of air, soil and water pollution caused by chemical factories. They are also trying to clear environmental pollution by designing microbes that can eat harmful chemicals and convert them into environment friendly end products. People have been successful in synthesising natural plant products in bacteria and save plants from destruction. Bioremediation treatment technologies for contaminated soils and groundwater can involve: (1) solid-phase biotreatment; (2) slurry-phase treatment; (3) *in situ* treatment; and (4) combination of biological, physical and chemical treatment.

Plant biotechnology with a focus on seed-varietal improvement, such as GM technology and molecular-assisted breeding, has generated products that help agriculture to achieve enhanced yields in a more sustainable manner. GM technology has brought significant improvements in income, life quality and per acre productivity. Plant-made pharmaceuticals have become a major focus point since 2010, when realistic opportunities for commercial

development emerged. Plant-manufacturing platforms for pharmaceuticals or molecular pharming open interesting prospects for low-income countries, where large quantities of medicines need to be provided on a regular basis. Cost-effective local focus and needle-free deployment can be of great help for the treatment of tropical diseases. In the industrial sector, plant biotechnology has the potential not only to generate more productive biomass feedstocks and minimise inputs, but also to develop more efficient biofuels, chemicals and bio-material conversion processes. A number of non-food crops, improved with sustainable management, have gone through the regulatory process.

Medical applications of biotechnology include regenerative medicine, tissue engineering, transplantation, stem cell research, nanobiotechnology and synthetic biology. The interdisciplinary field of nanobiotechnology is bringing the science of the incomprehensibly small devices closer to reality. Although the expectations from nanobiotechnology in medicine are high and the potential benefits are endlessly enlisted, the safety of nanomedicine is not yet fully defined. If synthetic biology becomes widespread in future then the rubber used in tyres, the bioacrylic used in glues and paints, the surfactant used in shampoos and soaps, additives and flavours used in food and so on would be made in side bacteria.

Nevertheless, there are also aspects of synthetic biology which may be harmful for humanity, e.g., creating new viruses that may give rise to new diseases and designing bioweapons, which can be misused against humanity. It is therefore more important to promote ethical and humane practices in synthetic biology and have strict regulations to prevent its misuse.

India has become a key contributor in biotechnology sector playing a key role in the global vaccine market including DPT, BCG and measles and other vaccines. Low cost diagnostics kits and medical interventions along with vaccines were promptly developed by the vibrant biotechnology sector of India. Recent unprecedented pandemic caused by Corona Virus (COVID-19) posed a severe threat to human health. Biotechnological innovations helped to develop vaccines that saved

mankind. In summary, the future of biotechnology research is strong and promising. Its development requires entrepreneurship as business skills are critical for managing research projects and a technical team. Technology experts can add real time analysis to the cutting-edge research. The Draft National Biotech Development Strategy 2020–24 aims to build and nurture a vibrant start-up, entrepreneurial, and industrial base, connecting academia and industry. It focuses strengthening and nurturing of a strong basic research and innovation driven ecosystem across research institutes and laboratories, public and private sector, with complete engagement of start-ups, small, and large industries.

SUMMARY

- Environmental biotechnology focuses on bioremediation that includes a wide array of applications, such as waste treatment, degradation, vermi-technology. It also includes the second aspect, i.e., prevention, that will primarily deal with the production of biofuels and avenues in the field of biodegradation and manufacture of biodegradable products.
- Biofuels are the fuels produced from biological products from living organisms or from waste generated from biological products such as from landfills, recycled vegetable oil, etc. Fuels produced from crops are known as biofuels or agrofuels. On the basis of their characteristics, biofuels can be broadly divided into biodiesel, bioalcohol, biogas and biomass in different forms.
- Biodegradation using biotechnology helps in environment-friendly waste recycling.
- Bioremediation of heavy metals in soil or water such as As, Cr, Hg, Cd, Zn, etc. is being done by plants and microorganisms such as bacteria and fungi using biotechnology.
- GM technology has evolved to generate a number of products and crops with improved traits, such as resistance crops, against herbicides, biotic and abiotic stresses, etc., as well as with improved nutritional quality and quantity.
- Regenerative medicine is an emerging multidisciplinary specialty in medicine with the goal of cell and organ

replacement to restore the loss of function resulting from degeneration, trauma and other disease processes.

- Stem cells exhibit an intrinsic ability to assemble into complex structures. When placed within a hydrogel (often Matrigel) and in the presence of suitable exogenous factors, the stem cells can be coaxed into forming structures that contain organised clusters of cells.
- The recent availability of stem cell derived organoid systems to provide 3D self-organised tissue models provides a compelling new class of biological model to serve as both tissue and organ substitutions.
- Organoids are ultra-small, self-assembled three-dimensional tissue cultures that are derived from stem cells. Organoids have been created from both pluripotent stem cells (PSCs) and adult stem cells (ASCs) by simulating the biochemical and physical characteristics of tissue development and homeostasis.
- Nanoscience is the study of materials in the range that includes size in nanometer, which is less than one micron, i.e., 10^{-9} to 10^{-12}. The application of material in nanoscale is called nanotechnology.
- Nanobiotechnology implies the use of nanotechnology in the field of biotechnology. Nanomedicine is the strongest arm of nanobiotechnology used for diagnosis, drug/gene delivery and therapy.
- Nanobiosensors: Nanotechnology has revolutionised the area of sensor development for the detection of biological molecules, which may be biomarkers of disease. Point of care devices can be developed, which are low cost, more sensitive and the tests can be done at bedside villages with a small volume of test materials. The scientists are working with the concept of "Lab on a Chip", where many tests can be done simultaneously using nanotechnology and microfluidics.
- Synthetic biology may be defined as a rational design approach of constructing biological components leading to certain functions.
- The application of automation and artificial intelligence (e.g., in designing and building plasmids) may help to reduce the time and cost to improve the return on investment.

EXERCISES

1. What are the advantages of biodiesel?
2. Enlist the differences between biodegradation and bioremediation.
3. Explain how biofuel is better than fossil fuels.
4. Enumerate the challenges in growing 3D cultures.
5. What are the applications of stem cells in generating organoids and spheroids.
6. What are nanomaterials?
7. Are there specific health risks from nano products?
8. Organoids can be created from:
   (a) Both Totipotent and Pluripotent Cells
   (b) Both Pluripotent and Multipotent Cells
   (c) Both Adult Stem Cells and Pluripotent Cells
   (d) Both Adult Stem Cells and Multipotent Cells
9. Incineration is:
   (a) Extracting metals from their ores using microbes
   (b) Treating waste, which involves the combustion of organic substances
   (c) Degrading harmful chemicals and materials using microbes
   (d) Remediation of harmful metals from the environment using microbes.
10. Ultra small, self-assembled, three dimensional tissue cultures derived from stem cells are called:
   (a) Spheroids
   (b) Organoids
   (c) Monolayer Cells Culture
   (d) Tissue Explants
11. What is the colour of the nano gold particles?
   (a) Yellow
   (b) Orange
   (c) Red
   (d) Variable
12. Quantum dots can be used in:
   (a) Crystallography
   (b) Optoelectronics
   (c) Mechanics
   (d) Quantum physics

13. Fabrics are extensively made out of nano materials like _____.
    (a) Carbon nano tubes
    (b) Fullerenes
    (c) Mega tubes
    (d) Polymers
14. **Assertion**: Bt cotton is a transgenic plant.
    Reason: Bt toxin provides resistance to plants against insects.
    (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.
    (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.
    (c) Assertion is true but reason is false.
    (d) Both assertion and reason are false.
15. **Assertion**: Biodiesel is made from raw materials such as vegetable oils, animal fats, etc.
    Reason: Biodiesel reduces the particulate emissions from unburnt carbon.
    (a) Both assertion and reason are true and the reason is the correct explanation of the assertion.
    (b) Both assertion and reason are true but the reason is not the correct explanation of the assertion.
    (c) Assertion is true but reason is false.
    (d) Both assertion and reason are false.

Chapter 13 Entrepreneurship

Entrepreneurs are the harbingers of prosperous society. An entrepreneur not only creates employment but also contributes to the economic development of the country. With the growth of scientific temperament in society people who have created enterprises based on scientific discoveries have grown in large numbers. One such field of entrepreneurship is based on biotechnology. This chapter highlights the role and relevance of entrepreneurs in general and biotech entrepreneurs in particular. The chapter also has exhibits of cases from the real world about entrepreneurs who have caused a niche for themselves by successfully launching biotechnology-based enterprises. Besides, the chapter also discusses the contemporary legislations governing biotechnology-based enterprises.

13.1 Concept of Entrepreneurship

The root word for the term 'entrepreneur' is the French word 'enterprendre' that means 'to undertake'. Entrepreneur thus, is an individual who undertakes an activity foreseeing business opportunity. An entrepreneur organises resources needed for starting the enterprise and also bears the risk involved in the process. Thus, there

are three prominent roles that an entrepreneur fulfils—an innovator, organiser and a risk bearer.

While performing the three roles mentioned above, an entrepreneur does significant tasks for self and the society. As an innovator, an entrepreneur is the person who brings out new products into the market. As an organiser, he/she organises the factors of production in order to take advantage of opportunity in the market. Finally, as a risk bearer he/she shoulders all uncertainties associated with the venture.

Box 1

1. Howard Stevenson (1983) defined that "Entrepreneurship is the process by which individuals pursue opportunities without regard to the resources they currently control."
2. "Entrepreneurship is the persistent progression towards an innovative solution to a key problem. It's the **constant hunger for making things better** and the idea that you are never satisfied with how things are." – *Debbie Roxarzade, founder and CEO of Rachel's Kitchen*
3. "At its core, [entrepreneurship] is a mindset – a way of thinking and acting. It is about **imagining new ways to solve problems and create value.** Fundamentally, entrepreneurship is about...the ability to recognise [and] methodically analyse [an] opportunity, and ultimately, to capture [its] value." – *Bruce Bachenheimer, Clinical Professor of Management and Executive Director of the Entrepreneurship Lab at Pace University*
4. "To be a successful entrepreneur you must have a **passion for learning** – from customers, employees and even competitors." – *James Bedal, CEO of Bare Metal Standard*

13.1.1 Importance of entrepreneurship

Entrepreneurship is widely regarded as the best way to augment the growth of an economy. Entrepreneurs have played a significant role in developing some of the best economies of the world like that of USA and Japan. In India too we have had several communities who have played a significant role of entrepreneurs for the economic development of our nation. Entrepreneurs are the prime movers of innovation and the introducers of new products into the market. Therefore, they play a key role in improving

the lives of our societies. Enumerating the importance of entrepreneurship, we see that they:

(a) help in capital creation by bringing together the resources of the people.

(b) are employment creators and their role is pivotal. By creating employment opportunities, the standard of living of people increases and also the purchasing power of the people augments.

(c) help in developing total community. If employment is diversified among small entrepreneurial units, it promotes overall standard of living leading to stability and higher quality of community life.

Entrepreneurship is regarded as the panacea for the unemployed in an economy. Self-employment empowers an individual and helps in bringing about the social change for community development.

13.1.2 Qualities of a successful entrepreneur

India has had some of the finest entrepreneurs in history that laid the foundation of Indian economy. We can see that they all possess certain distinct qualities, some of which are summarised below:

1. **Initiative:** Being an innovator is a significant characteristic of an entrepreneur. Entrepreneur takes initiative at the right time to launch a venture, enduring all the struggles of initiation of a new venture.
2. **Knowledge and skill:** An entrepreneur is a skilled individual who possesses the relevant knowledge relating to the industry, economy, consumer choices, technology, etc., and prospects of growth for the same in future.
3. **Risk taker:** An entrepreneur is ready to take the uncertainties associated with the enterprise. An entrepreneur has the foresight to accept the long-term prospect of success for the enterprise. In doing so, he/she is ready to withstand the short-term risks associated with the venture.
4. **Adaptability:** An entrepreneur is adaptable to the changing business environment. As an entrepreneur starts and grows the business, they constantly monitor the business environment and adapt themselves to the changes taking place in it.

Box 2: Story of Dr Krishna Ella, founder of Bharat Biotech International Ltd.

As students we sometimes ask our teachers or ourselves "Why am I learning this? Will I even use it in real life?". Well, if your life goals are similar to those of Dr. Krishna M.Ella,that is, to create massive positive changes through scientific innovation,then you just might.

Dr. Ella is the Chairman and Managing Director of Bharat Biotech International Limited (BBIL), which he co-founded with his wife in 1996 in Hyderabad, Telangana. A person hailing from a rural background and born in a village Nemili in Tamil Nadu, started his early education from the same village, had his share of ups and downs to achieve the feat what he is today. He completed his higher schooling and intermediate course in Life Sciences from Madras followed by completing his higher degrees in Agriculture from Maharashtra and later from Karnataka. In order to gain marketing experience, he worked with the Agricultural Division of a Pharmaceutical company in India as well.

He also pursued Masters in Science (specialisation in Plant Pathology) at the University of Hawaii and doctoral programme at the University of Wisconsin, Madison, after getting a scholarship. Followed by his Ph. D. in 1993, Dr. Ella joined the Medical University of South Carolina, Charleston, where he made a shift from Plant Pathology to human and yeast molecular biology. While conducting regular research, he started thinking of innovative ideas for the development and production of Recombinant Hepatitis B Vaccine.

In 1996, Dr. Ella returned to India and with the encouragement of his wife and his mother, set up a small laboratory in the city of Hyderabad. Their prime objective was to innovate in the development of affordable vaccines and biotherapeutics. As they wanted India to be known as the country that innovated and the name 'Bharat Biotech International Ltd., was chosen. BBIL was established in 1997 in Hyderabad with the funds and assistance from angel investors. Since its inception, BBIL has produced over 4 billion doses of various human vaccines for diseases such as Hepatitis B, Rabies, Diphtheria, Rotavirus, and corona virus, etc., most of them for the Universal Immunisation Programme of India and for more than 120 developing countries to safeguard global public health.

COVAXIN® is Bharat Biotech's 17th Vaccine and the first COVID-19 Vaccine which made in India. Dr. Ella and his team developed COVAXIN®, the inactivated whole virus and adjuvanted vaccine, in a record time of just eight months. COVAXIN® has been in use as a life saving vaccine against the COVID-19 pandemic since December 2020 across India and many other developing countries. WHO has also approved COVAXIN for emergency use against COVID-19.

Dr. Ella once said, "Solving today's problem is not innovation, it is the ability to predict the future problem and finding solutions to solve an important problem, something that matters to public health." He strongly believes in the potential of upcoming generation of students to shape the future of India. "For students of science, their focus on academic performance today can yield a better life for them and also for everyone around them. Many years from now, there will be a new generation of science students reading about a different entrepreneur in their textbook. You can be that entrepreneur too!"

(***Courtesy: Bharat Biotech International Limited (BBIL)***)

5. **Self-confidence:** An entrepreneur is confident of the decisions they take. This self-belief is a key factor that makes entrepreneurs successful and acts as a catalyst in making them take up risks.
6. **Wealth creators:** Entrepreneurs are wealth creators that translate great ideas into commercial success. Those who succeed after taking these risks turn out to be wealth creators for themselves and for the nation they belong to, by providing valuable employment opportunities to the people. Thus, it is seen that every successful and economically developed nation has nurtured and promoted the entrepreneurial spirit among its people.

13.1.3 Difference between Entrepreneur and Intrapreneur

Entrepreneur and intrapreneur have similar values—that of innovation and creativity, the two are often used interchangeably. However, an entrepreneur and intrapreneur have distinctive differences. An entrepreneur is a person who takes risks to start a business venture in order to earn profit. They foresee the opportunities for products and services and coordinates with the factors of production to establish an enterprise. On the contrary, an intrapreneur is an employee of an organisation who promotes innovation among the employees of the organisation. They are hired to bring about success in a business venture.

13.1.4 Planning and Resourcing an Enterprise

Planning and resourcing an enterprise are critical steps in transforming a business idea into a sustainable venture. Planning involves setting clear objectives, defining the business model, analyzing the market, and outlining strategies for operations, marketing, and growth. It includes identifying potential risks and devising contingency plans to address them. Resourcing focuses on securing the necessary inputs to support the business, such as financial capital, skilled personnel, technology, raw materials, and infrastructure. This process requires evaluating resource availability, sourcing strategies, and cost-effectiveness

while ensuring alignment with the enterprise's goals. Effective planning and resourcing ensure that the enterprise is well-prepared to navigate challenges, leverage opportunities, and achieve long-term success

13.2 Sources of Funds

Define Start-up

In entrepreneurial terminology, the term 'Start-up' has become a popular word. Under the Startup India Action Plan, startups that meet the definition as prescribed under G.S.R. notification 127 (E) dated 16 January, 2019 are eligible to apply for recognition under the programme. The startups have to provide support documents at the time of application.

Eligibility criteria for startup recognition:

(a) The startup should be incorporated as a private limited company or registered as a partnership firm or a limited liability partnership.

(b) Turnover should be less than INR 100 crores in any of the previous financial years.

(c) An entity shall be considered as a startup up to 10 years from the date of its incorporation.

(d) The startup should be working towards innovation or improvement of the existing products, services and processes and should have the potential to generate employment and create wealth. An entity formed by splitting up or reconstruction of an existing business shall not be considered a 'Startup'.

Box 3: Six sources of financing the new venture:

1. **Personal investment:** The first investor in a business is the 'entrepreneur' himself/herself. By putting in his/her own money, the entrepreneur proves to bankers and other investors, his/her long-term commitment to the venture.
2. **Venture capital:** Venture capitalists are investors that look for technology driven enterprises and companies that have a high growth potential. Some of the high growth potential sectors are Information Technology, Biotechnology, etc. The Venture Capitalists take an equity position in the company in order to help in developing a high-risk venture. They look for high return on investment when the business is developed, after which they may exit.

3. **Angel investors:** Angel investors are generally, wealthy individuals who invest in promising startups. These investors are very often leaders in their own fields, who invest money, ideas and experience in the promising ventures. They tend to finance the early stages of business.
4. **Business incubators:** Business incubators, also called accelerators, are investors who support high-tech sector by giving support to them in various stages of development. There are incubation centers in academic institutions and industrial cooperatives as well. Incubation phase lasts generally for two years only.
5. **Government grant and subsidy:** Government agencies provide financing such as grants and subsidies in order to promote business.
6. **Bank loans:** These are the most commonly used source of funding business ventures. For being eligible for a bank loan, a good idea itself is not enough, it should be complimented by an appealing business plan. Loans for start-ups typically require a personal guarantee from the entrepreneur.

13.3 Entrepreneurship in Biotechnology

13.3.1 Significance of Biotechnology Entrepreneur

Biotechnology entrepreneurship consists of all the activities that an entrepreneur does to build and sustain an enterprise based on biotechnological innovation. It is an enterprise built by the amalgamation of science and business.

Some definitions of Biotechnology Entrepreneurship given by experts are as under:

1. 'Biotechnology' by accepted definition, involves the use of living organisms or parts of living organisms through biological processing to develop new products or provide new methods of production—'Damian Hine and John Kapeleris (2006)
2. The concept of bio-entrepreneurship was elaborated by A.D. Meyers (2008) as "Bio-entrepreneurship is the process of creating value from life science innovation. It is referred to by several names including—bioscience entrepreneurship, life science entrepreneurship or bioscience enterprise. Whatever the descriptor, the fundamental notion is about moving a life science discovery or invention from the research phase through development to a commercial market."

Biotech entrepreneurs are the backbone of biotech industry. They are the innovators with a vision that their idea will impact the life of masses for better. Biotech

entrepreneurs start companies for the reasons given as under:

1. Biotech entrepreneurs believe that their ideas can solve a real-life problem of people around the world.
2. Biotech entrepreneurs are found to be altruistic in nature while starting their enterprise.
3. Financial rewards are also motivators to prove to the world that their discoveries can be commercial successes.

13.3.2 Assimilation of two distinctly different disciplines

Biotechnology is pure science, which when takes the form of an enterprise becomes commerce (a distinctly different discipline altogether). This intertwining of two disciplines can be a challenge for first time entrepreneurs. Scientific research requires academic skill in the subject while setting up biotechnology enterprise demands knowledge of economic conditions, decision making ability and risk-taking ability between a General entrepreneur and Biotech entrepreneur is as under:

Table 13.1: Comparison between General and Biotech Entrepreneurs

Basis	General Entrepreneur	Biotech Entrepreneur
Idea	Must be competitive	Must be competitive
Team work	Necessary and must be experienced	Necessary and must be experienced
Risk	Takes all risks with perseverance	Takes all risks with perseverance
Gestation period	Low to high depending on the type of enterprise	High
Degree of uncertainty	Present, but not scientific in nature	Uncertainty in business apart from other types carries inherent scientific uncertainty.
Capital requirements	Low to high depending on the type of enterprise	High
Relevance of academic qualification	Moderate to negligible	Very high
Regulatory framework	Moderate	High

13.3.3 Process of starting a Biotech enterprise

Starting an enterprise requires intense planning and does not happen without a proper roadmap in place. The element of 'chance' should be avoided and rationale should be present for every action taken while starting an enterprise. As the words of Louis Pasteur go, "chance only favors the prepared mind", an entrepreneur must be prepared for the outcome of his actions. This is true for all enterprises and also for biotech entrepreneurs. Following are the steps that are critical to starting a biotech enterprise:

Step 1: Need assessment

The entrepreneur should conduct a thorough assessment of the market demand for the product. The entrepreneur must be absolutely sure that there is a real market need for the product to be offered. Also, one has to be sure that the technology of interest is protected by intellectual property (IP). Finally, the entrepreneur needs to secure the assets, IP rights and assurances from the inventors and key personnel (in case the promoter is not the only one involved in the business).

Step 2: Identification of founders and key personnel

A new entrepreneur must look for like-minded individuals, who can join the team and bring the idea into reality. Generally, we see that a new entrepreneur works collectively with a team of people with expertise in diverse disciplines. Selecting this key group of people is a challenge, yet this group is indispensable to the success of an entrepreneurial idea.

Step 3: Getting a legal expert

This individual is a key partner. A legal advisor will be the individual the promoter will go to for advice and guidance. He will help the promoter move through the corporate and business issues during all the stages of establishing and growing his company.

Step 4: Incorporation as a Limited Company

It is advisable for the entrepreneurs of Biotechnology based start ups to register their companies as Private Limited Companies under the provision of the Companies Act 2013. Due to the nature of the business that demands

high capital investment and an above average gestation period, a Limited enterprise would be the best fit for a Biotechnology Startup.

Step 5: Design a marketing and business strategy

A well-designed business and marketing strategy are the next requirements. It will not be possible to raise money unless it clearly describes the market problem and need for the product, how the new product will solve the need, how much money the new product will generate, how the fund generated will be utilised and the expected return on investment.

Step 6: After the seed capital is raised the technology development should be the focus

It is important to highlight the key product development milestones in biotechnology ventures. The product is the pivot in a biotech enterprise and hence innovation in the same must be consistent to keep the market sentiments positive to establish and grow.

13.4 Concept of IPR

The proprietary aspect is the key feature of biotechnology of modern times. In the past, innovations in biotechnology came out only of publicly funded laboratories. In present times, biotech innovations are well protected within legal framework of Intellectual Property Rights (IPR).

With the Trade Related Aspects of Intellectual Property Rights (TRIPS) Agreement of World Trade Organisation (WTO), the Intellectual Property Rights attained the authority to enforce the law internationally. According to TRIPS, the intellectual property rights are:

Box 4: Copyright and Related Rights

1. Rights of artists, painters, musicians, sculptors, photographers and authors for copyright in their works.
2. Rights of computer programmes, whether in source or object code for a copyright in their programmes and compilation of data.
3. Rights of performers, producers of phonograms and broadcasting organisations in respect of fixation on their programmes for copyright in their work.

4. Right of traders in their trademarks.
5. Right of manufacturers and producers on geographical indication in relation to such products and produce.
6. Right of designers for their distinctive design striking to the eye.
7. Right of the inventor for patent of his/her invention.
8. Rights of plant breeders and farmers.
9. Rights of biological diversity.
10. Right of computer technologist for their layout design of integrated circuits.
11. Right of businessmen for protection of their undisclosed information on technology and management.

13.4.1 The aspects of IPR involved in Biotechnology

(a) Patent

The Patents Act 1970, along with the Patents Rules 1972, came into force on 20th April 1972, replacing the Indian Patents and Designs Act 1911. The Patents Act was largely based on the recommendations of the Ayyangar Committee Report headed by Justice N. Rajagopala Ayyangar. One of the recommendations was the allowance of only process patents with regard to the inventions relating to drugs, medicines, food and chemicals.

Later, India became signatory to many international arrangements with an objective of strengthening its patent law and coming in league with the modern world. One of the significant steps towards achieving this objective was becoming the member of the TRIPS system. Being a signatory to TRIPS, India was under a contractual obligation to amend its Patents Act to comply with its provisions. India had to meet the first set of requirements on 1 January 1995 to give a pipeline protection till the country starts granting product patent.

Section 5, an important section of the Indian Patent Act,1970 was deleted by the 2005 Amendment Act to allow product patents in the area of biotechnology, chemicals and pharmaceuticals. The basic criteria for a patent to be

granted are novelty, non-obviousness (inventive step) and utility. For a patent to be granted in India, it should not be covered in the negative list in Section 3, which provides an extensive list of what are not considered as inventions under the Indian Patents Act. The inventions related to DNA molecules or sequences must not be contrary to public order and morality.

The biotechnology industry is devoted to the development of commercially valuable therapeutic, biochemical and pharmaceutical products and processes among others. Many of these products and processes revolve around the manipulation of DNA molecules and their encoded proteins. In the last thirty years, great strides have been made in the field of biotechnology and particularly in recombinant DNA research. However, with this progress has come a degree of uncertainty regarding the obviousness of certain biotechnological inventions.

For example, procedures of cloning genes and transporting them between organisms have become common place. The accessibility of these methods along with the central doctrine of molecular biology, i.e., DNA is transcripted into RNA, which in turn is translated into functional or structural protein molecules, has created a somewhat disarrayed legal structure.

In India, only inventions are patentable while discoveries are not. There is a clear distinction between inventions and discoveries as the law specifies that only inventions create patentable subject matter. Indian patent law provides for a demonstrative list where it has mentioned the subjects that are not patentable. Any subject matter, which does not fall within the domains of the demonstrated list, does establish a patentable subject matter. The list has been updated and altered to comply with the provisions of the TRIPS.

(b) Plant Breeder's Rights and Farmer's Variety Act

Plant breeder's rights (PBRs) are used to protect new varieties of plants by giving exclusive commercial rights for about 20–25 years to market a new variety or its reproductive material. The variety must be novel, distinct, uniform and stable. This protection stops anyone from growing or selling the variety without the permission of

owner. Exceptions may be made, however, for both research and the use of seed saved by a farmer for replanting.

(c) Trademark

The Trademark Act, 1999 under Section 2 (zb) defines 'trade mark' as "a mark capable of being represented graphically and which is capable of distinguishing the goods or services of one person from those of others and may include shape of goods, their packaging and combination of colours…" Besides, the Act also provided for the definition of 'mark' under Section 2(m), which enumerates a mark to include a device, brand, heading, label, ticket, name, signature, word, letter, numeral, shape of goods, packaging or combination of colours or any combination thereof.

Two essential ingredients for Trademark registration

1. The mark is capable of being represented graphically.
2. Capable of distinguishing goods and services of one person from those of others.

(d) Copyright

Copyright is a right given by the law to the creator of literary, dramatic, musical and artistic work and the producers of cinematograph films and sound recordings. In fact, it is a bundle of rights including, *inter alia*, rights of reproduction, communication to the public, adaptation and translation of the work. There could be slight distinctions in the alignment of the rights dependent on the work.

Some of the exclusions are the uses of the work—

(a) for the purpose of research or private study
(b) for criticism or review
(c) for reporting current events
(d) for judicial proceeding
(e) for performance by an amateur club or society if the performance is given to a non-paying audience
(f) for the making of sound recordings of literary, dramatic or musical works under certain conditions

(e) Trade Secrets

In India, **trade secrets** refer to confidential business information, such as formulas, processes, designs, or methods, that provide a competitive advantage and are kept

secret by a company. Unlike patents or trademarks, trade secrets are not registered, and their protection depends on the company's ability to maintain confidentiality through internal measures, like non-disclosure agreements (NDAs) and secure information practices. Although India does not have a specific law dedicated to the protection of trade secrets, it is safeguarded under **Indian contract law** (such as through NDAs and confidentiality clauses) and **tort law**, which allows for remedies in cases of unauthorized use or disclosure. The **TRIPS Agreement** (Trade-Related Aspects of Intellectual Property Rights), to which India is a signatory, mandates member countries to protect **undisclosed information** that has commercial value, as long as reasonable steps are taken to maintain its secrecy. In India, the legal protection for trade secrets is primarily enforced through civil suits for breach of contract or misappropriation, with remedies such as damages or injunctions. India's legal framework is also aligned with international standards under TRIPS, although trade secret protection in India is more reliant on contract law than dedicated trade secret legislation.

13.5 Biopiracy

When there is commercial exploitation of biochemicals or genetic materials which occur naturally, it is known as biopiracy. Generally, indigenous people have traditional understanding of biological features and genetic diversity of the natural environment passed on from one generation to another. Few of the traditional knowledge relevant to global survival has the elements listed below:

1. Farming or agriculture
2. Medicinal plants
3. Varieties of food crops

There have been cases of infringement of rights towards traditional materials in recent times. Here are some examples:

1. The biopiracy case of the Neem tree (*Azadirachta indica*) highlights how multinational corporations exploited traditional knowledge. Native to India, neem has been used for centuries in traditional medicine and agriculture for its antimicrobial and pesticidal properties. In the 1990s, W.R. Grace and Co. and the

United States Department of Agriculture obtained a patent for neem oil extraction and use as a pesticide, claiming it as a novel invention despite its well-documented traditional use. In 2000, the EPO revoked the patent, marking a victory for indigenous knowledge and setting a global precedent against biopiracy while emphasizing the need to protect biodiversity and the rights of local communities.

2. The biopiracy case of turmeric involved the patenting of its traditional use in wound healing, a practice well-known in Indian culture for centuries. In 1995, two researchers from the University of Mississippi Medical Center were granted a U.S. patent for using turmeric powder to heal wounds, claiming novelty in their application. This sparked outrage as turmeric's medicinal properties were part of India's ancient Ayurvedic knowledge. The Indian Council of Scientific and Industrial Research (CSIR) challenged the patent, presenting documented evidence from ancient texts and publications that turmeric's wound-healing properties were already well-established. In 1997, the U.S. Patent and Trademark Office (USPTO) revoked the patent, recognizing the lack of novelty. This case became a landmark victory in the fight against biopiracy, emphasizing the need to safeguard traditional knowledge and prevent its unjust appropriation by corporations.
3. The biopiracy case of Basmati rice involved the U.S.-based company **RiceTec Inc**., which, in 1997, was granted a patent by the United States Patent and Trademark Office (USPTO) for certain strains of Basmati rice and methods of breeding them. Basmati, a variety of aromatic long-grain rice, is traditionally grown in the Indian subcontinent and is deeply tied to the cultural heritage and agricultural practices of India and Pakistan. The patent allowed RiceTec to label their rice as "Basmati" in international markets, threatening the livelihood of Indian farmers and undermining the unique geographic identity of traditional Basmati. The Indian government and advocacy groups strongly opposed the patent, arguing that Basmati rice and its qualities were a product of traditional agricultural knowledge and not an invention. After a widespread international campaign, several claims in the patent were successfully challenged, leading to a partial

revocation of the patent. This case underscored the need for international mechanisms like **Geographical Indications (GIs)** to protect traditional products and highlighted the importance of combating biopiracy to preserve the rights of local farmers and communities.

In order to curb the biopiracy of traditional medicines, Traditional Knowledge Digital Library (TKDL) has been set up which is a pioneering initiative of India to prevent the misappropriation of country's traditional medicinal knowledge at International Patent Offices. Its beginning dates back to the Indian effort on revocation of the patent on wound healing properties of turmeric at the USPTO.

SUMMARY

- Entrepreneur is an individual who undertakes an activity foreseeing business opportunity. They organise resources needed for starting the enterprise and also bear the risk involved in the process.
- Entrepreneurship is widely regarded as the best way to augment the growth of an economy. Entrepreneurs have played a significant role in developing some of the best economies of the world like that of USA and Japan. In India also we have had several communities who have played the significant role of entrepreneurs for the economic development of our nation.
- Entrepreneurs are seen to display certain inherent qualities like that of initiative, knowledge and skill, risk-taker, adaptability, self-confidence and they are also the wealth creators.
- The two terms—entrepreneur and intrapreneur, are often used interchangeably. But each has a distinctive definition. An entrepreneur is a person who takes risks to start a business venture in order to earn profit. On the contrary, an intrapreneur is an employee of an organisation who promotes innovation among the employees of the organisation.
- In entrepreneurial terminology, the term 'Startup' has become a popular word. A startup should be working towards innovation or improvement of the existing products, services and processes and should have the potential to generate employment and to create wealth. An entity formed by splitting up or reconstruction of an existing business shall not be considered a 'Startup'.

- There are seven sources of starting a new venture viz. Personal Investment, Venture Capital, Angel Investors, Business Incubators, Government Grant Subsidy and Bank Loans.
- Biotechnology entrepreneurship consists of all the activities that an entrepreneur does to build and sustain an enterprise based on biotechnological innovation. It is an enterprise built by the amalgamation of science and business.
- There are six steps that are critical to starting a biotech enterprise, these are—Need assessment, Identification of founders and key personnel, Getting a legal expert, Incorporate the company as a Limited Company, Design a marketing and business strategy, and Focus on technology development.
- The proprietary aspect is the key feature of biotechnology in modern times. In the past, innovations in biotechnology came out only of publicly funded laboratories. In present times, biotech innovations are well protected within legal framework of Intellectual Property Rights (IPR). Aspects of IPR involved in biotechnology are Patent, Plant Breeder's rights and Farmer's Variety Act, Trademark, Copyright and Trade-secrets.
- Biopiracy is a major issue in efforts to commercialise biotechnological knowledge. When there is commercial exploitation of biochemicals or genetic materials, which occur naturally, it is known as biopiracy. Generally, indigenous people have traditional understanding of biological features and genetic diversity of the natural environment passed on from one generation to another. There have been cases of infringement of rights towards traditional materials in recent times. A case of biopiracy by multinational corporations is that of the Neem tree of India. Another case was when patent was granted to researchers for the use of turmeric in wound healing, which was revoked later. Yet another case was when rice similar to Basmati was granted patent in USA, which was also revoked later.
- In order to curb the biopiracy of traditional medicines, Traditional Knowledge Digital Library (TKDL) has been set up which is a pioneering initiative of India to prevent misappropriation of country's traditional medicinal knowledge at International Patent Offices.

EXERCISES

1. Define the term 'entrepreneurship'. Describe its importance.
2. What are the qualities of an entrepreneur?
3. Differentiate between entrepreneur and intrapreneur.
4. What are the steps of preparing a feasibility report?
5. Define a 'start-up'. What are the sources of funds for a new venture?
6. Elaborate the significance of a Biotechnology Entrepreneur.
7. Identify the similarities and differences between General Entrepreneur and Biotechnology Entrepreneur.
8. Explain the process of starting a Biotech Enterprise.
9. Explain the concept of IPR and aspect of IPR in Biotechnology.
10. Explain the role of IPR in Biotechnology Enterprise.
11. What are the three central criteria for grant of Patents of any scientific inventions?
12. 'Angel' usually provide what type of financing?
    (a) Debt
    (b) Equity
    (c) Stock Sales
    (d) None of the above
13. A patent is granted for a specified amount of time because of the assumption:
    (a) That during this time, the firm will cover its development costs
    (b) That firm will earn a sufficient profit during this period
    (c) To limit the monopoly of the firm
    (d) That it will stimulate the idea and development of a better product
14. A short-term, internal source of funds can be obtained by reducing all of the following EXCEPT _____.
    (a) short-term assets
    (b) cash
    (c) fixed assets
    (d) Inventory

15. A typical researcher entrepreneur usually _____.
    (a) is highly creative and enjoys the process of research
    (b) does not encourage change
    (c) is not willing to take risk
    (d) dislikes change
16. Which of the following elements is NOT an important element of the financial data and projections section of a business plan?
    (a) SWOT analysis
    (b) Projected income statements
    (c) Break-even analysis
    (d) Cost controls
17. Which of the following cannot be covered under the copyright protection?
    (a) Computer software
    (b) Computer hardware
    (c) Poems and songs
    (d) Models and sculpture
18. Which of the following is false?
    (a) A business plan is often prepared by an existing company to ensure that growth is properly managed.
    (b) A business plan is usually not required when obtaining finance for a startup.
    (c) If a business plan is completed for a start-up, it may help the entrepreneur avoid costly mistakes.
    (d) All of the above.
19. Which of the statements is/are true with respect to entrepreneurship?
    (i) Entrepreneur is an individual who undertakes an activity foreseeing business opportunity.
    (ii) He/She organises resources needed for starting the enterprise and also bears the risk involved in the process.
    (iii) There are three prominent roles that an entrepreneur fulfils that of an innovator, organiser and a risk bearer.

    Options:
    (a) Only (i)
    (b) Only (i) and (ii)
    (c) Only (i) and (iii)
    (d) (i), (ii) and (iii) are true

20. Seed capital assistance is _____.
    (a) a long-term assistance.
    (b) initial assistance
    (c) a help for the purchase of seeds.
    (d) a short-term assistance.
21. Which one of the following is a pioneering initiative of India to prevent misappropriation of country's traditional medicinal knowledge at International Patent Offices?
    (a) Traditional Knowledge Digital Library (TKDL)
    (b) National Digital Library of India (NDLI)
    (c) Digital Library of Open Access Books (DOAB)
    (d) Universal Digital Library