

DAIRY BIOTECHNOLOGY



Sunita Grover

V. K. Batish

V. Padmanabha Reddy



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Dairy Biotechnology

Sunita Grover & V. K. Batish

Dairy Microbiology Division
NDRI, Karnal

V. Padmanabha Reddy

Dairy Microbiology Division
SVU, Tirupati



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Lesson 1 DEFINITION AND SCOPE OF BIOTECHNOLOGY

1.1 Introduction

Biotechnology has emerged as a wonder technology with immense potential with innumerable applications in almost all walks of our life by providing a tangible solution to the problems confronting human life. This technology holds a great promise to improve the quality of life on the earth by poverty alleviation and raising the standard of living at the global level particularly in third world countries including India. Biotechnology can help us in meeting our basic needs such as food, clothing, shelter, health and safety.

1.2 What is Biotechnology?

The literal meaning of Biotechnology as implied from this word is the study of tools from living things based on the split words contained therein i.e.

Bios - life

Teuchos - tool

Logos - Study of or essence

1.3 Definition of Biotechnology

There is no universal and complete definition of Biotechnology due to its wide range of usage. Although, Biotechnology has been defined differently in different countries, the most widely used and comprehensive definition of 'Biotechnology is any technique that uses living organisms or substances from these organisms to make or modify a product, to improve plants or animals or to develop micro-organisms for specific uses' (Office of technology Assessment of the United States Congress).

Biotechnology is defined as “any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use”.

Biotechnology is a multi-disciplinary concept involving many disciplines or branches of learning including all areas of life sciences such as Microbiology, Genetics, Molecular Biology, Biochemistry, Fermentation technology or Bio-process engineering and Bioinformatics etc.

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Biotechnology is not a new technology as it had been traditionally used by man since time immemorial without knowing the scientific principles involved in the process. The basics and advanced principles behind this powerful technology were unraveled and recognized only during the last few decades due to the tremendous progress and advancements made in the area of genetics, molecular biology that included the discovery of restriction endonucleases, polymerase chain reaction and completion of human genome project etc.

1.4 Scope of Biotechnology

Biotechnology as explained above has the newest roots in the science of molecular biology, genetic engineering and microbiology. Advances in these areas have been exploited in a variety of ways both for production of industrially important bio-chemicals including enzymes and pharmaceutically important proteins, hormones etc. and for basic studies in molecular biology. As a result of its endless potentials, the scope and prospects of biotechnology have widened dramatically for commercial exploitation. By integrating biotechnology in the process and product development at commercial scale, biotech based industries have grown enormously all over the world including India and in the process, have created new job opportunities, human resource development and poverty alleviation. Biotechnology has now become a key issue to boost the economy of different nationalities including both the developed and developing countries.



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Lesson 2

HISTORICAL DEVELOPMENT (TIME LINE) OF BIOTECHNOLOGY

2.1 Introduction

The term 'Biotechnology' was coined in 1917 by Karl Ereky, an Hungarian engineer. The term meant all the lines of work by which products were produced from raw materials with the aid of living organisms such as bacteria. There is a common misconception that Biotechnology includes recombinant DNA technology and Genetic Engineering only. Biotechnology is NOT new. Man has been manipulating living things to solve problems and improve his way of life for millennia. Roots of Biotechnology can be traced back to 6000 B.C. when Sumerians fermented beer. In 4000 BC Egyptians used yeast to prepare bread and wine.

Dahi / curd and other milk fermentations had been in use in India since times immemorial during the Vedic period. Early agriculture was concentrated on producing food. Plants and animals were selectively bred and microorganisms were used to make food items such as beverages, cheese and bread. 'New Biotechnology' has emerged as a Biological revolution and led to creation of a world of —Engineered products.

From historical perspective, biotechnology can be classified as ancient/old, classical and new/modern biotechnology.

2.2 Ancient Biotechnology

It began with early civilization. Our ancestors were producing wine, beer, and bread by using fermentation, a natural process in which the biological activity of one-celled organisms play a critical role. They also found that by manipulating the conditions under which the fermentation took place, they could improve both the quality and the yield of the ingredients themselves.

2.3 Classical Biotechnology

It followed ancient biotechnology which makes wide spread use of methods from ancient biotechnology especially fermentation methods adapted to industrial production. It produces large quantities of food products and other materials in short time to meet demands of increasing population.

2.4 Modern Biotechnology

It deals with manipulating genetic information. Microscopy and advanced computer technologies are used for in-depth knowledge of science. It is based on advancements in genetics research from the mid 1800's. In 1859, Darwin published his theory of evolution on the 'Origin of Species'. Use of biotechnology to produce new life forms emerged in mid 1900's and it was made possible by rDNA technology.

2.5 Milestones that led to the Development and Advancement of Modern Biotechnology

The major milestones in science that laid the foundation of the modern biotechnology and their time lines are listed below in chronological order (Table 2.1).

Table 2.1 Milestones in Biotechnology



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Milestones in Biotechnology	Year
• Darwin - published his theory of evolution on the 'Origin of Species'	1859
• Gregor Mendel, an Austrian Botanist and monk formulated basic laws of heredity with classical green peas experiment. He is considered as Father of Genetics	1865
• Johan Friedrich Miescher, a Swiss biologist isolated nuclei of white blood cells and identified nucleic acids	1869
• Walter Sutton determined that chromosomes carried units of heredity identified by Mendel.	1903
• Wilhelm Johannsen, Danish Botanist named the units of heredity as -Genes.	1909
• Frederick Griffith identified the genetic material as the transforming principle	1928
• Thomas Hunt Morgan was awarded Nobel Peace Prize in Physiology / Medicine for research in -Gene Theory.	1933
• George W. Beadle and Edward L. Tatum proposed One Gene - One Enzyme Hypothesis	1941
• Avery, MacLeod & McCarty experimentally proved that the transforming principle was DNA	1944
• Erwin Chargoff proposed that DNA bases follow Chargoff's -rules (%A = %T and %G = %C)	1947
• Barbara McClintock first reported on "transposable elements" - known today as "jumping genes."	1947
• Alfred Hershey and Martha Chase experimentally proved that DNA was the hereditary/genetic material that programs cells to make copies	1952
• Rosalind Elsie Franklin and Maurice Wilkins elucidated the helical structure of DNA	1953
• James Watson and Francis Crick discovered the double helical structure of DNA for which they got the noble prize	1953
• Francis Crick proposed Central Dogma of Life	1957
• Matthew Meselson & Franklin Stahl proposed that DNA replication was semi-conservative	1958
• Marshall Nirenberg and H. Gobind Khorana Deciphered Genetic Code	1961
• Hamilton Smith and Daniel Nathans discovered restriction endonucleases and received Nobel Prize Hamilton Smith discovered <i>HindII</i> in <i>Haemophilus influenzae</i> Daniel Nathans used <i>HindII</i> to make first restriction map of SV40	1970
• Norman E. Borlaug developed wheat varieties producing high yields and received Noble Peace Prize	1971
• Paul Berg produced first recombinant DNA molecule using EcoRI	1972
• Herbert W. Boyer, Stanley N. Cohen & Chang were the first to transform <i>E. coli</i> with recombinant plasmid	1973
• Mary Clare King determined that 99% of human DNA is identical to chimpanzee	1975
• Hybridoma technology was discovered by Georges J. F. Kohler (W. Germany) and Cesar Milstein of Argentina (now working in U.K.). Received Nobel Prize in Medicine	1975
• The NIH released the first guidelines for recombinant DNA experimentation	1976

• **1977 onwards - Biotechnology era opened up (Dawn of Biotech era)**

• **1977**

- o Genentech, Inc. (Company founded by Herbert Boyer and Robert Swanson in 1976) produced first human protein somatostatin from a transgenic bacterium which was considered as the advent of the Age of Biotechnology.
- o Walter Gilbert and Allan Maxam devised a method for sequencing DNA

• **1978**

- o Recombinant Human Insulin – produced at California by Herbert W. Boyer
- o David Botstein discovered Restriction Fragment Length Polymorphism (RFLP) technique for studying polymorphism

• **1980**

- o U.S. Supreme Court ruled that genetically altered life forms could be patented, thereby, allowing Exxon oil company to patent oil eating micro-organism.
- o Kary Mullis and others at Cetus Corporation in Berkeley, California developed Polymerase Chain Reaction (PCR). Sold patent for \$300 Million in 1991

• **1981**

- o First transgenic mice produced with rabbit beta-globin gene

• **1982**

- o USFDA approved sale of genetically engineered human insulin (first recombinant product to be marketed in US)
- o Michael Smith at the University of British Columbia, Vancouver, developed Site directed mutagenesis for making precise amino acid changes anywhere in a protein.

• **1983**

- o An automated DNA sequencer was developed
- o A screening test for Huntingtons disease was developed using restriction fragment length markers.
- o Eli Lilly received a license to make insulin commercially
- o First transgenic plant (a tobacco plant resistant to an antibiotic) was developed.

• **1984**

- o Alec Jeffreys' introduced technique for DNA fingerprinting to identify individuals

• **1985**

- o Genetically engineered plants (transgenic plants) resistant to insects, viruses, and bacteria were field tested for the first time
- o NIH approved guidelines for performing experiments in gene therapy (developed by Friedmann and Roblin in 1972) on humans

• **1986**

- o The FDA granted a license for the production of first recombinant vaccine (for hepatitis) to Chiron Corp.
- o The EPA approved the release of the first genetically engineered crop, gene-altered tobacco plant.

• **1987**

- o Invention of YACs (yeast artificial chromosomes) to clone DNA fragments larger than 100 kb and up to 3000 kb
- Calgene, Inc., Davis, California received a patent for the tomato polygalacturonase DNA sequence, used to produce an antisense RNA sequence that could extend the shelf-life of fruits (FLAVR SAVR tomatoes).

• **1988**

- o Harvard molecular geneticists Philip Leder and Timothy Stewart awarded the first patent for a genetically altered animal, a mouse that was highly susceptible to breast cancer.

• **1989**

- o National Center for Human Genome Research was created to map and sequence all human DNA by 2005.

• **1990**

- o UCSF and Stanford issued their 100th recombinant DNA patent and earning \$40 million from the licenses by 1991.
- o The first human gene therapy test involved a girl suffering from severe immune problems due to a defective gene for the enzyme adenosine deaminase (ADA) Doctors removed some of her bone marrow cells, inserted functional ADA genes into these cells, and then inserting the "corrected" cells back in place.
- o First transgenic dairy cow was created by GenPharm Int. Inc.
- o Creation of genetically engineered cotton plants developed by Calgene Inc., Davis, California.

• **1992**

- o U.S. Army began "genetic dog tag" program which involved collection of blood and tissue samples from all new recruits for identification of soldiers killed in combat.

• **1993**

- o Kary Mullis - Nobel Prize – Chemistry : PCR

• **1994**

- o Flavr Savr tomato was given approval by FDA for human consumption
- o First linkage map of the human genome was prepared
- o Recombinant GM-CSF approved for chemotherapy-induced neutropenia

• 1995

- o First full gene sequence of a living organism was completed for *Hemophilus influenzae*
- o Duke University Medical Center transplanted hearts from genetically altered pigs into baboons, proving cross-species operations.
- o Leptin gene identified to cause weight loss in experimental animals

• 1996

- o Genome of *Saccharomyces cerevisiae* was sequenced
- o A new inexpensive diagnostic biosensor test for the first time allowed instantaneous detection of the toxic strain of *E. coli* 0157:H7
- o The discovery of a gene associated with Parkinson's disease provided an important new avenue of research into the cause and potential treatment of the debilitating neurological ailment

The Department of Biochemistry at Stanford and Affymetrix introduced a technological breakthrough in gene expression and DNA sequencing technology with the introduction of DNA chips, small glass or silica microchips that contained thousands of individual genes that can be analyzed simultaneously and are extensively used these days

• 1997

- o Dolly was cloned from the cell of an adult ewe
- o DNA microarray technology was developed
- o Scientists at Athersys in Cleveland, Ohio used a combination of natural and synthetic DNA to create a "genetic cassette" that could be customized and used in gene therapy. Genes on the artificial chromosome were expressed and replicated in cells for over 6 months.

• 1998

- o First genome *Caenorhabditis elegans* sequenced
- o Human embryonic stem cells cultured *in vitro*
- o Scientists at Japan's Kinki University cloned eight identical calves using cells
- o RemicadeTM (infliximab) – marketing of a novel monoclonal antibody for treatment of Crohn's disease
- o Japan's Kinki University cloned eight identical calves wherein cells were taken from a single adult cow.
- o Antibody therapy against breast cancer, HER2neu (Herceptin)- based on molecular targeting of tumor cells

• 1999

- o 1,274 biotechnology companies were set up in the United States
- o At least 300 biotechnology drug products and vaccines were under human clinical trials
- o Development of Golden rice as a fortified food to meet shortage of dietary vitamin A
- o The insect resistant corn hybrid seed developed by US based Mycogen seeds

• **2000**

- o Human Genome Project and Celera Genomics Inc. – announced a major mile stone in mapping the genome
- o The world's first cloned pigs produced from adult pig cells by Scotland based PPL Therapeutics Inc. – to produce organs for human transplant
- o Genetically engineered golden rice modified to make pro-vitamin A by German scientists Prof. Ingo Potrykus and Peter Beyer
- o First crop field tested in Kenya – virus resistant sweet potato

• **2001**

- o Genome sequence of rice decoded- first food crop to be sequenced
- o Chinese National hybrid Researchers developed super rice plant varieties which can produce double the yield of normal rice

• **2002**

- o The first draft of a functional map of yeast proteome, an entire network of protein complexes and their interactions – completed by Germany based Cellzome AG, Heidelberg, Germany
- o Human Cloning – on progressive path
- o An International Consortium sequenced the genomes of malarial parasite Plasmodium and Anopheles mosquito
- o Draft version of human genome published – ahead of schedule
- o Vaccine for cervical cancer developed
- o Stem cells discovered - Cancer Research UK scientists developed a new technique for transplanting stem cells into patients for advanced leukaemia and lymphoma

• **2003**

- o Complete knowledge of Human Genome (National Human Genome Research Institute) coordinated by the U.S. Department of Energy and the National Institutes of Health and Celera Genomics
- o Coronavirus responsible for SARS (Severe Acute Respiratory Syndrome) –genome sequenced

• **2004**

- o Beginning of Genomics and Proteomics era
- o Stem cell era
- o International Rice Genome Sequencing Project (IRGSP) completed the Japanese rice variety Nipponbare genome sequence
- o The first omega-3 rich mammals (mice) produced. The transgenic pigs were created using technology for mice capable of making omega-3 fatty acids.
- o Several vaccines for diseases like Brain cancer, Anthrax, Plague and HIV/AIDS developed
- o The FDA approved the first anti-angiogenic drug for cancer, AVASTIN® (bevacizumab)

• **2005**

- o Golden Rice 2 which produced up to 23 times more beta-carotene than the original variety of golden rice produced.

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- o Roche, the Swiss pharmaceutical maker made a drug Tamiflu for staving off bird flu.
- o A cow cloned from the cells of a carcass was successfully produced at University of Georgia
- o National Institute of Health in December launched a pilot project to determine the feasibility of The Cancer Genome Atlas to know the genomic changes involved in all types of human cancer.
- o Scientists at Harvard University could successfully convert skin cells into embryonic stem cells through fusion with existing embryonic stem cells.
- o A soybean genome project was launched jointly by USDA, Monsanto and Genaisance Pharmaceuticals.
- o A consortium of scientists led by the National Human Genome Research Institute published the dog genome, which belonged to a 12-year-old boxer.

• 2006

- o The genetic test, Oncotype DX™ for breast cancer was developed by the biotech company Genomic Health and was made commercially available.
- o A joint venture of Monsanto and Cargill i.e Renessen LLC received approval from USDA to begin selling the first crop improved through biotechnology with added benefits for use in animal feed. The product, Maveria™ High Value Corn with Lysine was improved to grow with increased levels of lysine, an amino acid that is essential for animal diets, especially those of swine and poultry.
- o Researchers developed pigs that produced high levels of omega-3 fatty acids. The biotech pigs were developed by inserting the "fat-1" gene from *Caenorhabditis elegans*. The biotech pigs were cloned, and six of the 10 clones produced increased levels of omega-3 fatty acids, which are believed to ward off heart disease.

• 2007

- o James Watson became the second person to publish his fully sequenced genome online after it was presented to him on May 31, 2007 by 454 Life Sciences Corporation in collaboration with scientists at the Human Genome Sequencing Center, Baylor College of Medicine which opened up an era of personalized medicine.
- o At Oregon Health and Science University, Shoukhrat Mitalipov and his team cloned a Rhesus Monkey and used the resulting embryo to create stem cells.

• 2008

- o Growing a new organ from a patient's own stem cells –opened the era of regenerative medicine
- o Stem cell technique named scientific breakthrough of the year. The process that involves turning back the clock on adult tissue and "reprogramming" it with the properties of stem cells, could lead to new treatments for diseases including Parkinson's and diabetes.

• 2009

- o The genome of a zebrafish was decoded by Indian Scientists at Institute of Genomics and Integrative Biology (IGIB), New Delhi
- o Samrupa was the first cloned buffalo born on February 6, 2009, at National Dairy Research Institute, India.

• 2010

- o King Tut and 10 other royal mummies recently became the first ancient Egyptians to get their DNA analyzed. The results, published in Feb., 2010 Journal of the American Medical Association, turned up a treasure trove of new information about the famous boy king, his family and Egyptian royalty in general.
- o Indian scientists Drs. Vinod Scaria and Sridhar Sivasubbu — the two young scientists from the Council of Scientific and Industrial Research (CSIR) have successfully sequenced the human genome of Indian origin.
- o J. Craig Venter created the synthetic life form by building a genome from scratch and used it to control a cell.
- o Cloned buffalo calf - Garima-II weighing 32 kg, born through caesarian operation through the new and advanced 'Hand-guided Cloning Technique', at NDRI, Karnal, on June 6, 2010
- o A male calf Shresth weighing 41 kg was born at NDRI on August 26, 2010 using hand-guided cloning technique.

• 2011

- o Researchers at **Nanyang Technological University** have genetically engineered suicide bombers i.e. *Escherichia coli* to attack and kill *Pseudomonas aeruginosa*, which is responsible for many infections in hospital patients whose immune systems are weakened.



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Lesson 3

FUTURE APPLICATIONS OF BIOTECHNOLOGY

3.1 Introduction

The present generation biotechnology has found immense applications as genetic engineering, recombinant DNA technology, protein engineering, animal cloning, stem cell technology as well as cell and tissue culture. The main target of biotechnology is to modify any available organism for the benefit of human beings by exploring genetic manipulation and engineering processes. Biotechnology is a vast subject, which has potential applications in almost all areas related to our basic interactions in daily life, including food, agriculture, medical field, environmental science, personal care and much more. Realizing the extensive scope and future prospects of the subject, developed nations started large-scale investment in biotech sector, encouraging clusters of small and large biotech companies, financing extended research activities and inculcating both the basic ideas and advanced levels of knowledge to youngsters in high schools and universities. The aim of these well-established economies was to lead the world in the latest technology, claim the research findings and rule the world market by being the first to supply biotech products. Whereas, the developing countries followed the trend, dreaming to uplift their national economy using biotechnology to increase job opportunities and attract global investors, thereby, yearning to gradually transform themselves into a developed nation. Biotechnology finds a large scope of application in medicine, agriculture, food, industry, environment and a host of other areas including development of species bioinformatics. Although, modern biotechnology has been extensively explored for providing solution to almost all the problems confronting human life, the major fields where biotechnology is actively used include completion of human whole genome sequence of different origin across the world, medicine (drugs, vaccines and diagnostics), Pharmacogenomics, Pharmaceutical products, gene testing, gene therapy, agriculture and cloning. Biotechnology is one of the fastest growing sectors across the world including India and elsewhere. This emerging field is going to take a leap forward in the next few decades ahead. Some of the most recent and advanced areas where biotechnology can find future applications with direct impact on human life are elaborated below.

3.1.1 Human genome project

The Human Genome Project has been an initiative of the U.S. Department of Energy (DOE) that aims to generate a high-quality reference sequence for the entire human genome and identify all the human genes. The DOE and its predecessor agencies were assigned by the U.S. Congress to develop new energy resources and technologies and to pursue a deeper understanding of potential health and environmental risks posed by their production and use. In 1986, the DOE announced its Human Genome Initiative. Shortly thereafter, the DOE and National Institutes of Health developed a plan for a joint Human Genome Project (“HGP”), which officially began in 1990. The HGP was originally planned to last 15 years. However, rapid technological advances and worldwide participation accelerated the completion date to 2003 (making it a 13 year project). There are approximately 30,000 genes in human beings. The whole human genome sequence available on the net as public domain will be the main focus of more intensive future studies for identification of specific genes and their linkage with common human diseases which

will eventually be explored in their diagnostics, treatment and cure.

3.1.2 Drugs, vaccines and diagnostics

Drugs, vaccines and the diagnostics are the key elements in human health care where, biotechnology has played a vital role in the disease control and management. In this context, synthetic human insulin turned out to be the first biotechnology product approved for human health care at commercial level in 1982. Since then, more than as many as 170 biotechnology related drugs and vaccines have been approved by FDA, of which 113 are currently on the market. In addition, another 350 biotechnology medicines, together targeting over 200 diseases, are in the final stages of development. Some examples of the biotech products already approved during 2000s include medicines to treat pneumococcal diseases in children, diabetes, cancer and haemophilia. Further advancements in rDNA technology is likely to revolutionize new initiatives on vaccine development in the future. In this context, DNA vaccines have recently started the testing process and very soon are expected to eventually replace other methods of vaccine production. Conventional vaccines are made from either live, weakened pathogens (disease causing agents) or killed pathogens. Although, vaccines produced using live pathogens confer greater and longer lasting immunity than those using killed pathogens, they may carry some risk of causing the full-blown disease to develop. However, the current focus is on exploring individual proteins as antigens in sub-unit vaccines by recombinant DNA technology. DNA vaccines contain only those genes of the pathogen which produce the antigen, and not those used by the pathogen to reproduce itself in host cells. Therefore, DNA vaccines are expected to combine the effectiveness of live vaccines with the comparative safety of those based on killed pathogens. Several preventive and therapeutic vaccines for HIV are currently in early trials. DNA vaccines are likely to be more extensively available to developing countries than conventionally produced vaccines. Application of DNA vaccines could be more relevant to the third world countries since, such vaccines can be cheaper in comparison to attenuated and live vaccines. Moreover, DNA vaccines are more stable at normal temperatures. Refrigeration costs can significantly increase overall budget by 80 per cent in the vaccination program run in the tropical countries using conventional vaccines. However, there are still some uncertainties about the potential for vaccine DNA to “invade” the host’s genome and possibly trigger genes relating to tumor development. Therefore, a great deal of caution needs to be exercised while developing new DNA vaccines ahead.

Disease diagnostics is another area where modern biotechnology can play a significant role by rapidly detecting the presence of potential pathogens-implicated in various diseases not only in processed foods but also in clinical and environmental samples from the hospitals. The two key areas of biotechnology currently being explored in disease diagnosis include immunological assays and molecular techniques based on PCR and RT-qPCR. Monoclonal antibody diagnostic tests have been extensively used for several years and are now one of the most profitable areas of commercial biotechnology. These diagnostic tests are actually quite inexpensive to produce, and present enormous opportunities for some developing countries to enter the international biotechnology market. The second area of biotechnology used for diagnostics is DNA technology. DNA probes, which use isolated segments of DNA to “attract” complementary gene sequences from pathogens, are already on the market. However, the major focus is now on exploring PCR techniques including RT-qPCR that have completely revolutionized the concept of diagnostics as these are extremely fast methods apart from being highly sensitive and specific. Moreover, they are relatively cheaper to produce, and are usually more stable in transit and in tropical climates than conventional diagnostics. Further advancements in new generation of biotechnology have led to the emergence of more sophisticated high throughput techniques like DNA chips and microarrays which have

opened new avenues in DNA diagnostics and bright prospects in disease diagnosis and detection of high risk food-pathogens in foods. Microarrays allow the detection and analysis of thousands of genes in a single small sample, giving the power of many DNA probes in one small array. Microarray technology is also expected to greatly increase the efficiency of drug discovery, although no drugs have as yet been developed using the technology.

3.1.3 Drug discovery and development

After the completion of the human genome project, pharma industry is now exploring the vast amount of knowledge generated from therein in the discovery and development of novel drugs. By judiciously using genomics approach, it is now possible to make drug therapy more precise and effective. Advancements in genomic technology have provided the necessary impetus to pharma industry to use this strategy in which engineered proteins specifically target diseases for better compliance and safety in the affected population, enhanced delivery and maximum efficacy. It is going to provide the platform for exponential growth and development of drugs and vaccines for the benefit of mass human population. With the unraveling of the human genome and the use of modern biotechnological tools to screen and produce active molecules have grown enormously resulting into the discovery of thousands of new molecules to be developed and tested in disease management.

3.1.4 Stem cells

Generally speaking, stem cells are self-renewing primitive cells that can develop into functional, differentiated cells. They have the unique ability to be manipulated by genetic engineering to give rise to specific cell types. With regard to human longevity, the focus is currently on exploring human pluripotent stem cells (hPSCs), which are unique because they can develop into all cells and tissues in the body. The pluripotency of human stem cells creates the vast potential for humans to grow cells, tissues, and even organs in a controlled laboratory setting, for use in applications from acute emergency care to treatment of chronic, debilitating diseases. Stem cells and their applications will be elaborated in a separate chapter.

3.1.5 Animal cloning

This technology has the potential to produce genetically matched cells for use in repairing organs damaged by degenerative disease. The process of making genetically identical copies became science-fact when in early 1997, Dr. Ian Wilmut and his colleagues at the Roslin Institute unveiled Dolly- the first cloned sheep. Dolly demonstrated that the nucleus of an adult cell could be successfully transferred to an enucleated egg to create cloned offspring. The birth of Dolly was a significant achievement because it demonstrated the ability of egg cytoplasm to “reprogram” an adult nucleus. Reprogramming enables the differentiated cell nucleus to express all the genes required for full embryonic development of the adult animal. Following Dolly’s creation, cloning has been used to replicate mice, goats, and cattle from donor cells obtained from adult mice, goats, and cattle, respectively. These examples of cloning normal animals from fully differentiated adult cells demonstrate the universality of nuclear reprogramming. Using nuclear transfer, multiple identical copies of animals can be produced that express only the genetic traits of the animal whose cells were used as the nuclear donors. While the frequency of success is currently low, it is expected to improve, as the fundamental mechanisms of nuclear reprogramming by egg cell cytoplasm become better understood. The scope and potential applications of animal cloning have been listed below

- Rapid multiplication of desired live stocks and their germ plasm
- Conservation of the rare endangered animal species
- Use of cloned animals as research models to study genetic(cystic fibrosis) and other diseases related to aging and cancer, drug discovery and evaluation of gene and cell therapy (human medicine)
- Transgenic applications- production of transgenic live stocks for expression of value added human pharmaceutical proteins
- Production of animal organs for xeno-transplantation in affected human population
- Improvement in the quality and quantity of foods and fiber products reducing environmental pollution and improving animal disease resistance.

3.1.6 Nanobiotechnology

Employing nanodevices - high-tech, miniaturized devices on the scale of billionths of a meter, nanomedicine manipulates human biology at its most basic levels. These tiny tools enable scientists to play on the size scale of biomolecules itself, just as a mechanic works on a car's engine using tools that are on the same scale as the engine. Nanotechniques may be our best armament in treating and even curing-stubborn diseases such as cancer and diabetes. Researchers have now designed clever ways to power nanomachines with biologically based components. Nanomotors, as well as nanotweezers - the mechanical and energy aspects of which are completely built from DNA are revolutionary innovations that will enable scientists to unleash microscopic robots within the human body on missions to correct the ravages of age.

3.1.7 Artificial organs

The medical makeover is just a few years away. We are talking about checking in to a clinic near you and checking out with new body parts. Advanced prototypes of nearly every single body part already exists in research laboratories. Not farfetched, tomorrow's body part shop is an extension of work that began in the mid-twentieth century. Dr. Willen Kolff, inventor of the kidney dialysis machine, emigrated to the United States from the Netherlands in the mid-1950's and became known as the "father of artificial organs" after he developed the artificial heart at the Cleveland Clinic, and created the nation's first artificial organ research program at the University of Utah in the 1960's. Research teams from around the world are working on projects to produce mechanical body parts that would vanquish many diseases and disabilities with which thousands of people struggle. Replacement parts for worn out or damaged human organs, along with applications of genetic engineering and stem cell research, hold great promise both today and in the not-so-distant future for extending the healthy human life span.

3.1.8 Pharmacogenomics

Pharmacogenomics is the study of how the genetic inheritance of an individual affects his/her body's response to drugs. It is a coined word derived from the words "pharmacology" and "genomics". It is hence the study of the relationship between pharmaceuticals and genetics. The vision of pharmacogenomics is to be able to design and produce drugs that are adapted to each person's genetic makeup.

Pharmacogenomics results in the following benefits.

1. Development of tailor - made medicines.

Using pharmacogenomics, pharmaceutical companies can create drugs based on the proteins, enzymes and RNA molecules that are associated with specific genes and diseases. These tailor-made drugs promise not only to maximize therapeutic effects but also to decrease damage to nearby healthy cells.

2. More accurate methods of determining appropriate drug dosages.

Knowing a patient's genetics will enable doctors to determine how well his/ her body can process and metabolize a medicine. This will maximize the value of the medicine and decrease the likelihood of overdose.

3. Improvements in the drug discovery and approval process.

The discovery of potential therapies will be made easier using genome targets. Genes have been associated with numerous diseases and disorders. With modern biotechnology, these genes can be used as targets for the development of effective new therapies, which could significantly shorten the drug discovery process.

3.1.9 Gene therapy

Gene therapy may be used for treating, or even curing, genetic and acquired diseases like cancer and AIDS by using normal genes to supplement or replace defective genes or to bolster a normal function such as immunity. It can be used to target somatic (i.e., body) or gametes (i.e., egg and sperm) cells. In somatic gene therapy, the genome of the recipient is changed, but this change is not passed along to the next generation. In contrast, in germline gene therapy, the egg and sperm cells of the parents are changed for the purpose of passing on the changes to their offspring.

There are basically two ways of implementing a gene therapy treatment

1. *Ex vivo*, which means “outside the body” – Cells from the patient's blood or bone marrow are removed and grown in the laboratory. They are then exposed to a virus (e.g. adeno virus) carrying the desired gene. The virus enters the cells, and the desired gene becomes part of the DNA of the cells. The cells are allowed to grow in the laboratory before being returned to the patient by injection into a vein.

2. *In vivo*, which means “inside the body” – No cells are removed from the patient's body. Instead, vectors are used to deliver the desired gene to cells in the patient's body.

3.1.10 Nutrigenomics- a new era in personalized functional/health foods

Nutrigenomics is the new emerging era in the development of third generation of health/functional foods and is expected to revolutionize wellness and disease management across the world. Nutrigenomics and nutrigenetics encompass the understanding of how nutrients affect health at molecular level within the body and how these effects vary between individuals. The key technologies underlying nutrigenomics address these two overlapping areas. Firstly, **genomics** including approaches such as DNA micro-arrays and RT-PCR which examine the interventions between nutrients and gene expression and **proteomics** which determine the outcome of the altered protein synthesis, activation and regulation. Both genomics

and proteomic approaches examine the molecular mechanisms of nutrients, identify potential targets for nutritional interventions and establish suitable health biomarkers and their up and down regulation for monitoring the responses. Secondly, the characterization of Single Nucleotide Polymorphisms (SNPs) promises an understanding of differences in response of individual nutrients at genetic level. As the field of nutrigenomics grows with the availability of complete human genome project as public domain, it will eventually be possible for an individual to be genetically profiled, and identifying a food he should be eating or avoiding and which dietary supplement, he should be taking, that in fact is the concept of personalized foods. Very soon need based customized health foods with specific bioactive functions intended for the target population will appear at the counters in the super markets and food outlets. This effort, however, will require a strong proactive synergy between Food and Pharmaceutical industry as well as Nutritionists, Biotechnologists on one side and Dietetic and Medical professionals on the other.

Due to rapid advancements in molecular biology and powerful biotechnological tools such as second and third generation sequencing, stem cell technology, organ cloning, transgenics, pharmaco and nutrigenomics, gene therapy etc, biotechnology is going to be the most sought after technology in the next millennium to address all the problems confronting human life effectively and hence could play a significant role in poverty alleviation and improving the living standard of our vast population.



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Lesson 4

NUCLEIC ACIDS – STRUCTURE AND FUNCTION OF DNA AND RNA

4.1 Introduction

Nucleic acids, particularly DNA, are the macromolecules considered to be the hereditary material which store the genetic information used in the development and functioning of all known living organisms. These universal molecules were first discovered by Friedrich Miescher in 1871. Nucleic acid structure is surprisingly simple, despite of its importance in cellular functions. There are two types of nucleic acids, Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA). The structure and functions of these molecules are described below:

4.2 Components

Nucleic acids are basically the polymer molecules of nucleotides which are essentially made up of three basic components, a heterocyclic nitrogenous base, a pentose sugar and a phosphate group.

4.2.1 Nitrogenous bases

Nitrogenous bases occurring in nucleic acids fall into two categories, viz., monocyclic bases (comprising of a hexagonal aromatic ring) called pyrimidines and bicyclic bases (comprising of one hexagonal and one pentagonal aromatic ring) called purines. They are polyfunctional bases having at least one N-H site for attaching with one organic substitute.

Purines (Fig. 4.1) are Adenine (A) and Guanine (G) and **Pyrimidines** (Fig. 4.2) are Cytosine (C), Thymine (T) and Uracil (U). The members of purines and pyrimidines share a similar structure, but differ in their side groups. Both of the nucleic acids i.e. DNA and RNA contain adenine, guanine and cytosine. However, thymine is found only in DNA and uracil only in RNA.

Purines

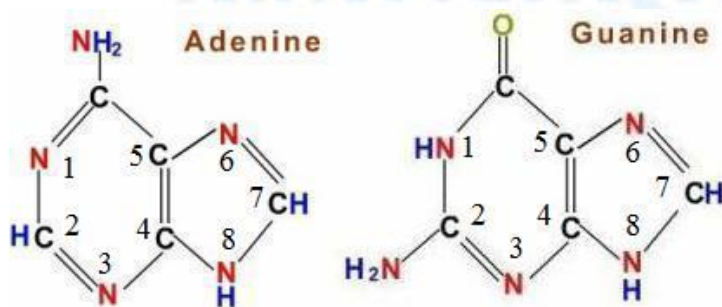


Fig. 4.1 Structure of purines

Pyrimidines

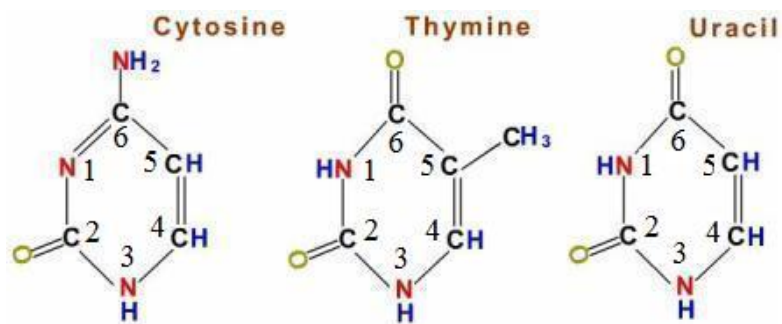


Fig. 4.2 Structure of pyrimidines

4.2.2 Cyclic five carbon sugar

The Cyclic five carbon sugar present in Ribonucleic acid is ribose where as in Deoxyribonucleic acid it is 2' deoxyribose sugar (Fig. 4.3).

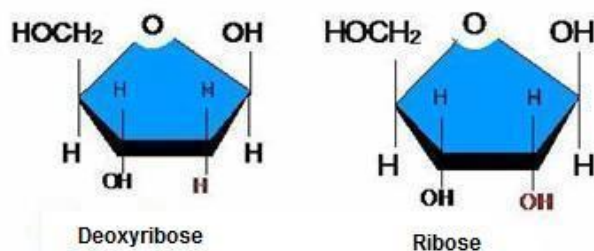


Fig. 4.3 Structure of pentose sugar (Ribose/Deoxyribose)

4.2.3 Phosphoric acid

The phosphate group is attached to 5' carbon of pentose sugar molecule (Ribose/deoxyribose) by phosphodiester linkage. This group is responsible for the strong negative charge on both the nucleotides and nucleic acids.

4.3 Nucleoside

Nitrogenous base with a pentose sugar molecule (Ribose/deoxyribose) is known as Nucleoside. Nitrogen bases are attached to 1' carbon atom of the sugar by N-glycosidic bond (Fig. 4.4).

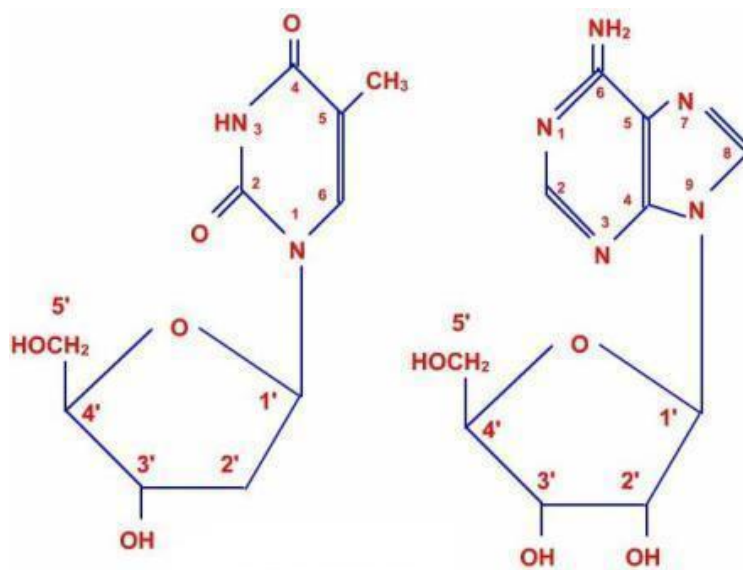


Fig. 4.4 Structure of Nucleosides

4.4 Nucleotide

Nitrogenous base with ribose or deoxyribose sugar molecule and phosphate group is known as Nucleotide i.e. ribonucleotide (RNA) or deoxyribonucleotide (DNA) (Fig. 4.5).

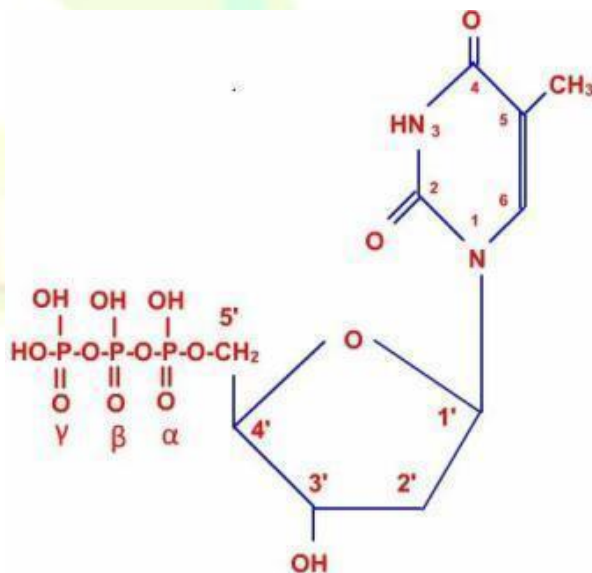


Fig. 4.5 Structure of Nucleotides

4.5 Polynucleotide or Nucleic Acid Strand

The polynucleotide strand is made of several repeating units called nucleotides consisting of nitrogenous bases which are capable of being covalently linked together to form a long chain (Fig. 4.6). The 3'-hydroxyl group on the ribose unit of the first nucleotide/deoxyribo nucleotide, reacts with the 5'-

phosphate group (phosphodiester bond) on its neighbor to form a chain. Further, purine or pyrimidine is linked to the sugar by a glycosidic bond between a nitrogen and the 1' carbon of the deoxyribose sugar.

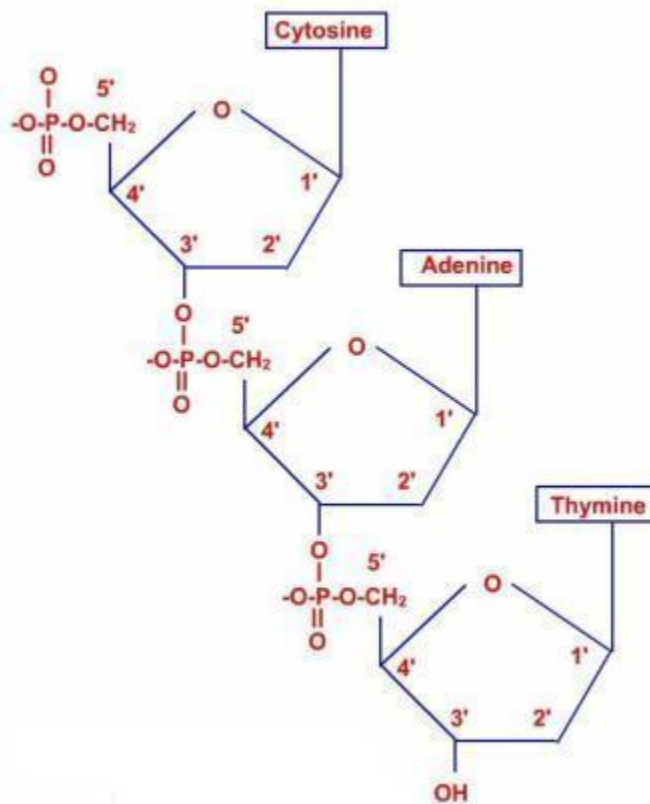


Fig. 4.6 Formation of polynucleotide

4.6 Deoxyribonucleic Acid (DNA)

The double helical structure of DNA was proposed by James Watson and Francis Crick in 1953 and nine years later, they along with Maurice Wilkins in 1962 received the Nobel Prize for this discovery.

4.6.1 DNA structure

The major role of DNA in a cell is to store the genetic information or instructions that are essential for carrying out various cellular functions like synthesis of biomolecules including RNA for the development of living cell. In prokaryotes, DNA is loosely packed in the cytoplasm and lacks distinct nuclear membrane. However, the cells of eukaryotic organisms contain DNA in their nucleus and in other organelles such as mitochondria or chloroplasts. DNA, in the form of plasmids, can also be located extrachromosomally both in prokaryotes and few eukaryotes such as yeast.

DNA consists of two polymer chains made up of nucleotides. These two long strands are intertwined (coiled) in the shape of a double helix which have the unique ability to wind and unwind to facilitate the duplication process. Each strand of polynucleotide consists of sugar-phosphate backbone made up of alternating 2' deoxyribose and phosphate groups. The third carbon of 2' deoxyribose sugar molecule is attached to the phosphate group by phosphodiester bond to the fifth carbon atom of adjacent 2' deoxyribose molecule. Because of these asymmetric bonds, each DNA strand of the helix has a unique direction i.e. the direction of one strand is opposite to the other and thus two strands are antiparallel to each other. As a result, one DNA strand has 3' end with terminal hydroxyl group and the second strand

has 5' end having a terminal phosphate group. Both chains are arranged in such a way that the nitrogenous bases, purine and pyrimidines, are inside the helix (variable) and the sugar-phosphate backbones are on the outside of the helix (constant) (Fig. 4.7).

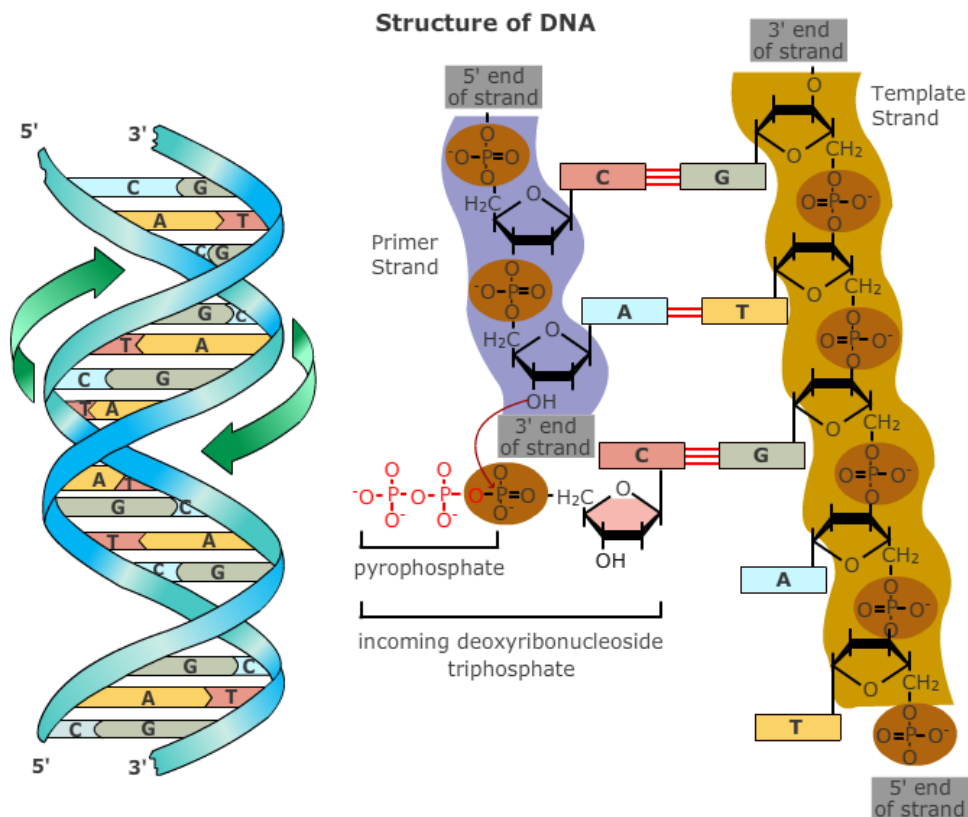


Fig. 4.7 The structure of DNA

In a double stranded DNA molecule, the nitrogen bases on one strand binds specifically with complementary bases on the opposite strand. Adenine always pairs with thymine with two hydrogen bonds and cytosine pairs with guanine by three hydrogen bonds. This kind of arrangement of two nucleotides binding together with the help of hydrogen bonds across the double helix is called a base pairing. As a result of this unique complementary base pairing, there are always the same number of A and T residues and G and C residues (this is known as Chargaff's rule and was one of the prime pieces of evidence that was needed to solve the structure of DNA) in a DNA molecule. Unlike covalent bonds, these hydrogen bonds can be broken and rejoined relatively easily. The GC pairing is 33% stronger than the AT pairing due to the extra hydrogen bond.

4.6.2 Structural features of the DNA double helix

The salient features of the double helical structure of DNA are given below (Fig. 4.8):

- DNA consists of two strands of polynucleotides that wind around each other like two strands of a rope.
- The sugar-phosphate backbone is on the outside which is hydrophilic in nature.
- The Nitrogenous bases are directed towards the inside of the duplex and account for the hydrophobicity of the DNA. Two bases in each base pair lie in the same plane.
- The bases are perpendicular to the axis of symmetry.

- It is a right-handed helix i.e. each strand appears to follow a clockwise path moving away from a viewer looking down the helix axis.
- The formation of the DNA double helix leads to generation of wide (major) and narrow (minor) grooves.
- One helical turn of the DNA duplex consists of 10 base pairs.
- Distance between two bases on each of the two strands is 3.4 Å. Therefore the total distance of helical turn is 34 Å.
- The two strands are antiparallel in DNA i.e. 3' OH terminus of one strand is adjacent to 5' – phosphate terminus of the other.
- The two adjacent nucleotides on each strand join with each other by strong chemical bonds called covalent bonds between sugar of one nucleotide and phosphate group of next nucleotide.

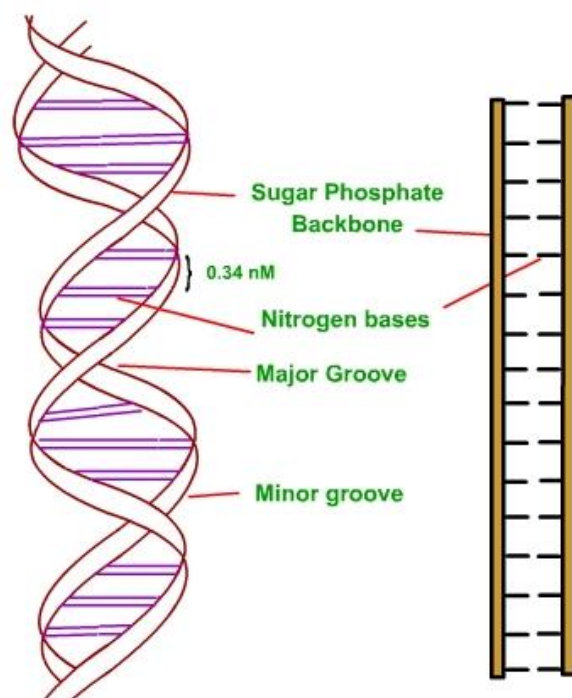


Fig. 4.8 Deoxyribonucleic Acid

4.7 Ribonucleic Acid

RNA is primarily a single stranded molecule containing purine and pyrimidine nitrogenous bases such as A, G, C and U and a ribose sugar. The major functions of RNA center around translating the genetic information contained in DNA into protein on ribosomal units. There are 3 types of RNA i.e. messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA) as briefly described below (Fig. 4.9):

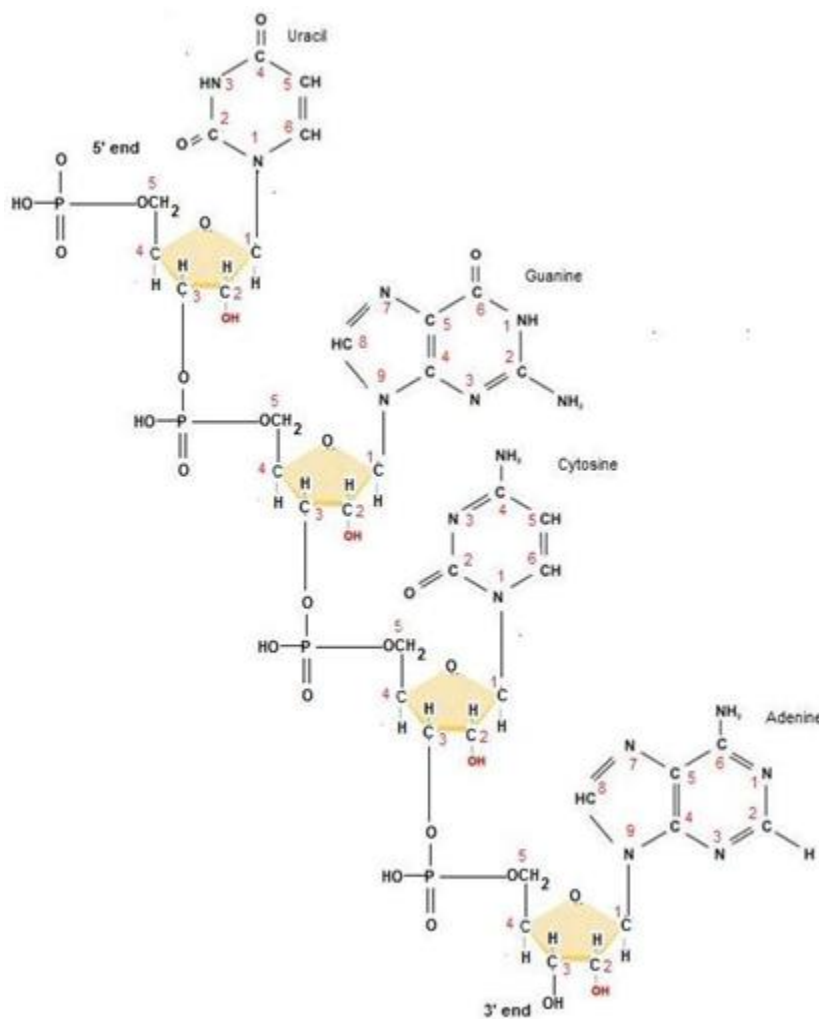


Fig. 4.9 Polyribonucleotide chain

4.7.1 Messenger RNA (mRNA)

mRNA constitutes the functional part of DNA and thus plays an important role in protein synthesis.

4.7.2 Transfer RNA (tRNA)

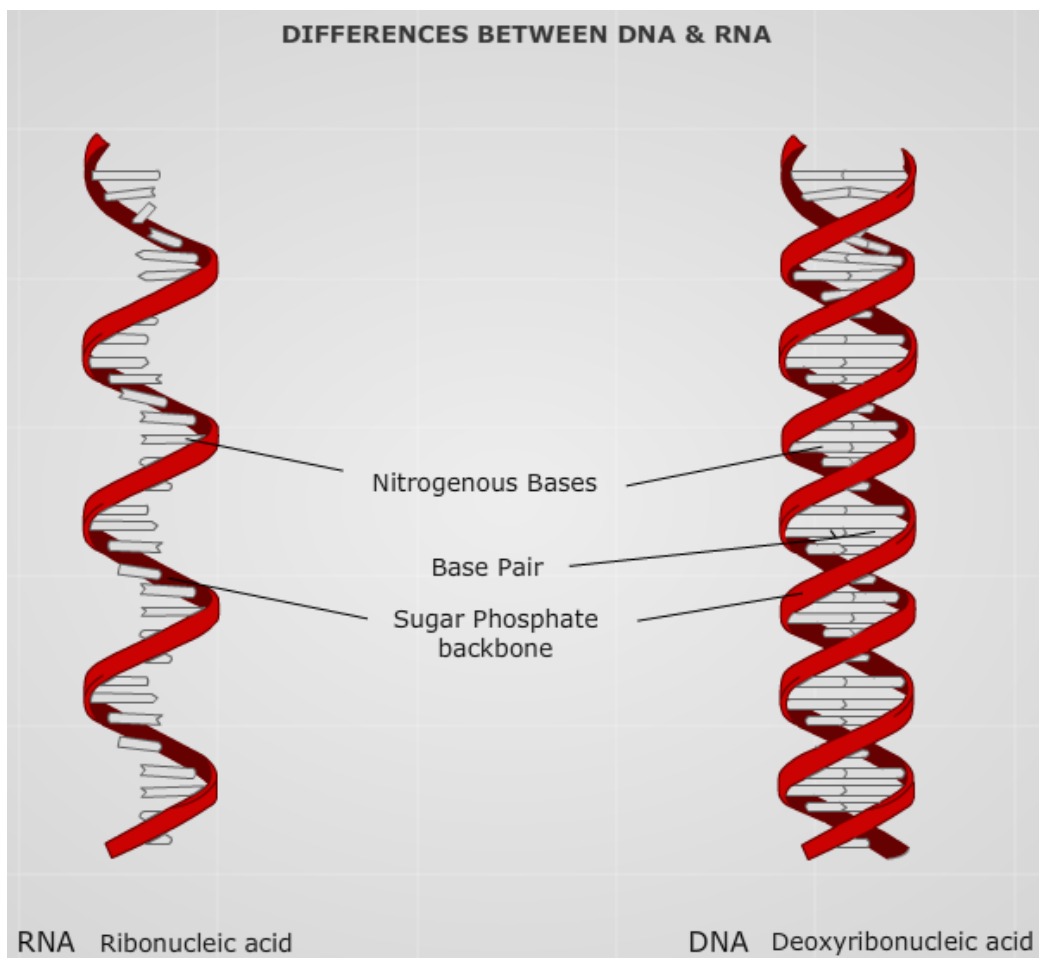
tRNA molecules act as adapters which carry specific amino acids from the cytoplasm on to the ribosomes during synthesis of proteins.

4.7.3 Ribosomal RNA (rRNA)

Ribosomal ribonucleic acid (rRNA) is the RNA component of the ribosome, the site of protein synthesis in all living cells.

4.8 Differences between DNA and RNA Molecules

The major differences between DNA and RNA in respect of their location, structure and function are delineated in Table 4.1



[RNA Vs DNA](#)

Table 4.1 Difference between DNA and RNA

Particulars	DNA	RNA
Location	Chromosome	Cytoplasm
Function:	Genetic material carrying hereditary information	Biosynthesis of protein molecules.
Structure	Double stranded molecule	Single stranded molecule
Nitrogen bases	Adenine, Guanine Cytosine, Thymine	Adenine, Guanine Cytosine, Uracil
Pentose sugar	2-deoxy ribose	D-Ribose
Monomers	Deoxyribonucleotides	Ribonucleotides
Types	chromosome or plasmid	Messenger RNA (mRNA), Ribosomal RNA (rRNA) and Transfer RNA (tRNA)



Lesson 5

DNA REPLICATION AND GENETIC CODE

5.1 Introduction

During the cell division the hereditary information is transferred from parent cell to the daughter cell by the faithful duplication of parental DNA molecule. This process of duplication of DNA is called replication. Three possible modes of DNA replication have been proposed which include conservative, semiconservative and dispersive replication (Fig. 5.1). However, semi conservative mode of replication was experimentally proved by Meselson and Stahl in 1958 which perfectly fits into the double helical model of DNA as proposed by Watson and Crick. In this mode of replication two strands of DNA are separated and each one acts as a template for the synthesis of daughter strand. The first generation progeny DNA produced after replication will constitute one parental and one newly synthesized DNA strand.

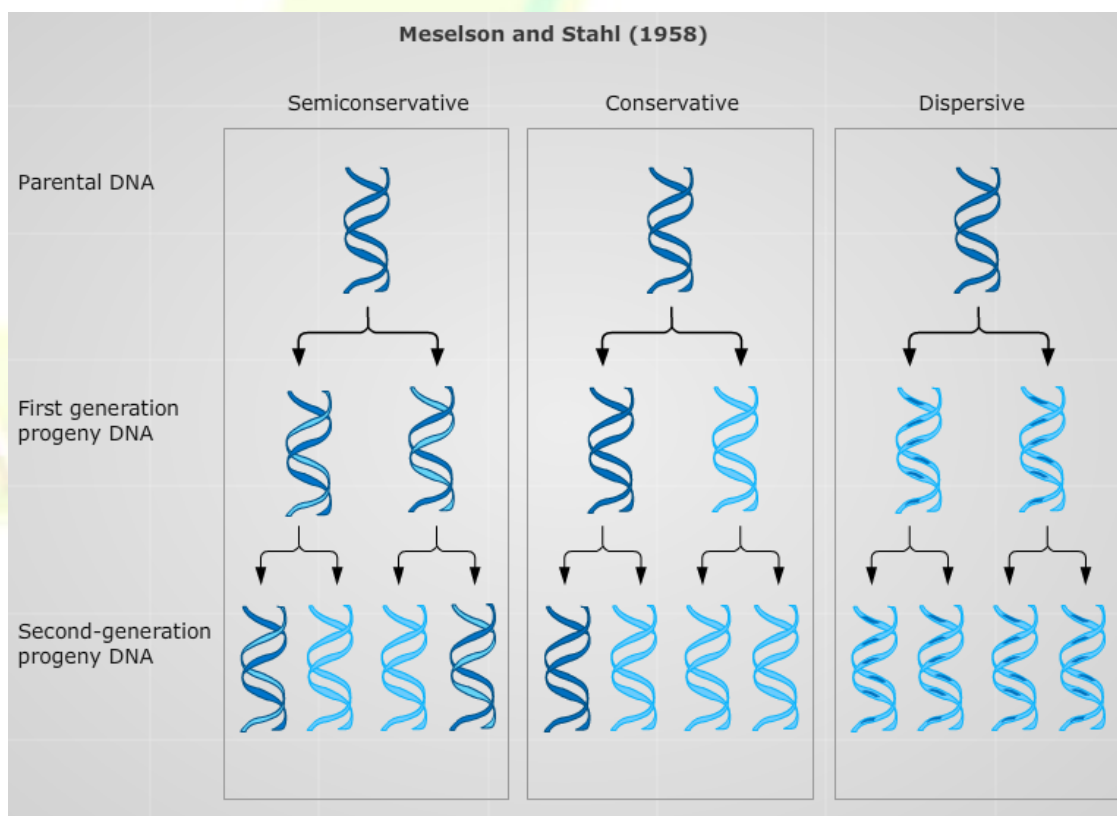


Fig. 5.1 Modes of DNA replication

The process of replication of DNA is described as below:

5.2 Components of DNA Replication Machinery

Several enzymes, protein factors and ATP molecules are required during the replication process of DNA.

5.2.1 Template

Double stranded DNA is used as template in DNA replication. It is generally circular in prokaryotes and linear in eukaryotes.

5.2.2 Enzymes

The key enzymes that take part in DNA replication are as follows:

DNA helicases bind to the double stranded DNA and catalyze the separation of the two strands.

RNA Primase synthesizes short RNA primers that provide free 3' hydroxyl group for the action of DNA polymerases.

DNA polymerase polymerizes the newly synthesized DNA strand. There are two main DNA polymerases i.e. DNA polymerase-I and DNA polymerase-III that participate in the replication process. DNA polymerase-III a multi subunit complex is the main replicative enzyme that polymerizes DNA in 5' to 3' direction by its 5' - 3' polymerization activity and proof reads the newly synthesized DNA by its 3' - 5' exonuclease activity. DNA polymerase-I catalyses the filling of gaps created during the RNA primer removal.

The **DNA gyrase** enzyme catalyzes the formation of negative supercoils that helps in the unwinding process to separate the two strands of the duplex DNA.

DNA Ligase catalyses the formation of phosphodiester bonds between the DNA fragments and fills the gaps.

5.2.3 Deoxyribo nucleotide triphosphates (dNTPs)

Four dNTPs i.e. dATP, dCTP, dGTP and dTTP are used as precursors by DNA polymerases to extend the growing chain of DNA.

5.2.4 Single strand binding proteins

Single strand binding proteins stabilize the single stranded structure of DNA by preventing the folding of the single strand of DNA on itself. They also protect the single strand DNA from nucleases.

5.3 DNA Replication Process

DNA replication is a bidirectional process that starts from a specific site on the DNA molecule known as origin of replication. There is a single of origin of replication in prokaryotic DNA (Fig. 5.2A) whereas eukaryotic genomes replicate through multiple origins of replications (Fig. 5.2B).

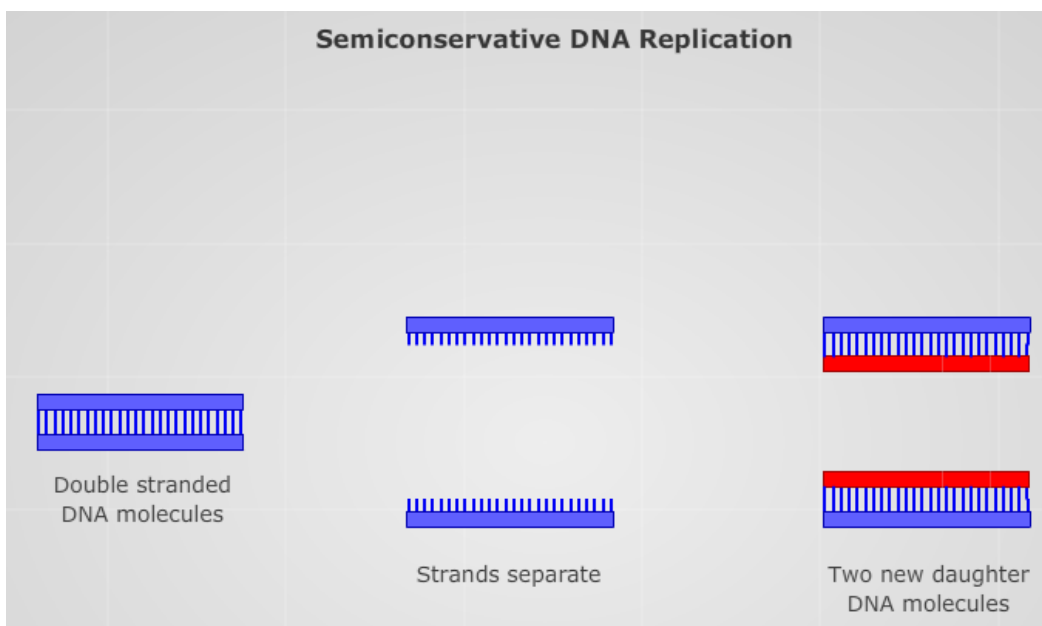


Fig. 5.2 Origin of replication

The process of replication involves the following steps:

DNA helicases bind the DNA duplex at the region of replication and leads to unwinding of DNA molecule and in the process creates replication fork. The replication fork is further extended by DNA helicases in opposite directions resulting in twists of DNA molecule offering resistance to the unwinding process at the unopened regions. This super coiling problem is solved with the help of enzymes called DNA topoisomerases, which create nicks in the DNA molecule to release the twisting or coiling of the DNA molecule as the replication process proceeds forward. The separated single strands have the tendency to fold back and form secondary structures. To prevent this, the single strand DNA binding proteins attach to the single strands and allow the replication process to continue without any constraint.

A short strand of RNA is synthesized by RNA primase using one of the strands as template, which provides a free 3' hydroxyl group for the synthesis of DNA strand. RNA primers are required during replication because DNA polymerases cannot initiate DNA synthesis *de novo* i.e. without a pre formed substrate. Once RNA primers are synthesized DNA polymerase-III is loaded on to the single strand. It extends RNA primers adding nucleotides to the 3' hydroxyl group of growing DNA strand using information available in the form of nucleotide sequence in DNA template. Deoxyribonucleotide triphosphates are first paired with their corresponding, complementary partner on the original, or template strand followed by formation of phosphodiester bond with the previously added nucleotide on the growing DNA strand. The specific base pairing as per the Watson and Crick model is strictly followed during DNA replication. Adenine (A) is always paired with Thymine (T) and Guanine (G) with Cytosine (C).

One strand of DNA is continuously synthesized in 5' to 3' direction using the DNA template having 3' - 5' polarity. This is called leading strand. The other strand being anti-parallel i.e. having 5' - 3' polarity, has to be synthesized in short stretches of ~1000 bases called Okazaki fragments. This discontinuously synthesized strand is called lagging strand (Fig 5.3).

DNA Replication

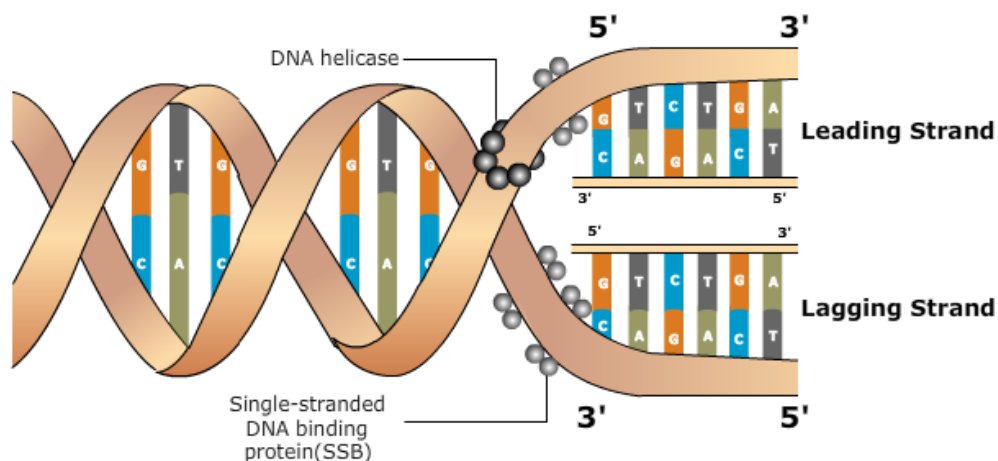


Fig. 5.3 Mechanism of DNA replication at leading and lagging strands

After completion of the replication of the whole genome it is processed to remove RNA primers and replace it with the complimentary missing DNA bases. RNA primers are removed by the 5' to 3' exonuclease activity of DNA Polymerase-I. The gaps thus created are filled by incorporating the deoxyribonucleotides by 5' - 3' polymerization activity of DNA polymerase-I. Finally DNA ligase closes the nicks by the formation of phosphodiester bonds between Okazaki fragments with the hydrolysis of ATP molecule.

5.4 Genetic Code

Genetic code is defined as the set of rules by which information is encoded on the genetic material. Precisely it is ordering of nucleotides in DNA molecules to incorporate amino acids during protein synthesis in correct order to make it biologically active to express a specific function in the cell. It reflects the relationship between the nucleotide triplets called codons located on a mRNA molecule and the 20 amino acids which are the building blocks of proteins. These triplet codons formed from adjacent nucleotides on mRNA molecule represent different amino acids with few exceptions and are made up of 4 different nitrogen bases namely adenine, guanine, cytosine and uracil.

There are 20 amino acids involved in the synthesis of proteins. Each amino acid is coded by one or more codons. Hence, there is a need of at least twenty different codons for synthesis of all the amino acids. Moreover, the start and stop signals in the synthesis of polypeptide chain are also coded by different codons. Since each codon is made of three nucleotides, the minimum possible number of codons by utilizing four available nitrogen bases could be 64 ($4 \times 4 \times 4$).

Salient features of genetic code:

- It is nearly universal code as it is used by all biological systems

- Sixty-one codons code for 20 amino acids
- Genetic code is degenerate due to redundancy of the codons. Many of the amino acids have more than one codon (redundancy) but no single codon codes for more than one amino acid (no ambiguity). For example phenylalanine is coded by two codons (UUU and UUC) while Leucine is coded by six codons (UUA, UUG, CUU, CUC, CUA and CUG). However, tryptophan (UGG) and methionine (AUG) are coded by only a single codon.
- The first codon for initiating the protein synthesis is always AUG (start or initiation codon) and codes for methionine
- UAG, UAA, and UGA are the Stop codons for termination of protein synthesis

All the possible codons constituting the genetic code and their corresponding amino acids are listed in Table 5.1.

Table 5.1 Triplet codons

		2 nd Base							
		U		C		A		G	
1 st Base	U	UUU	Phenyl alanine	UCU	Serine	UAU	Tyrosine	UGU	Cysteine
		UUC		UCC		UAC		UGC	
		UUA	Leucine	UCA		UAA	Stop codon	UGA	Stop codon
		UUG		UCG		UAG	Stop codon	UGG	Tryptophan
	C	CUU	Leucine	CCU	Proline	CAU	Histidine	CGU	Arginine
		CUC		CCC		CAC		CGC	
		CUA		CCA		CAA	Glutamine	CGA	
		CUG		CCG		CAG	CGG		
	A	AUU	Isoleucine	ACU	Threonine	AAU	Asparagine	AGU	Serine
		AUC		ACC		AAC		AGC	
		AUA	ACA	AAA		AGA	Arginine		
		AUG	Start codon & Methionine	ACG		AAG		Lysine	AGG
	G	GUU	Valine	GCU	Alanine	GAU	Aspartic acid	GGU	Glycine
		GUC		GCC		GAC		GGC	
		GUA		GCA		GAA	Glutamic acid	GGA	
		GUG		GCG		GAG	GAG	GGG	

5.4.1 Anticodon

Anti-codon comprises of a sequence of 3 bases that can base pair with a codon sequence in the mRNA. Anticodon is present on one of the four regions of tRNA and help in the incorporation of correct amino acid in the growing polypeptide chain.



Lesson 6 GENE EXPRESSION – TRANSCRIPTION AND TRANSLATION

6.1 Introduction

Gene is defined as a specific nucleotide sequence encoding a particular function through synthesis of a protein. It represents the simplest hereditary unit in a cell which is associated with regulatory, transcriptional and other functional regions. In general these genes hold the information to build and maintain an organism's cells and pass genetic traits to offspring. All the metabolic functions in a cell are performed by expression of Genes. Gene expression is the process by which information from a gene is used in the synthesis of a functional gene product. These products are often proteins which can serve as the structural component of the cell and biological catalysts to facilitate cellular metabolic reactions. rRNA or tRNA constitute the non-protein component of the coding genes. The process of gene expression is used by prokaryotes, eukaryotes and viruses to generate the macromolecular machinery for life. The flow of hereditary information from genes to proteins is dictated by 'Central Dogma of Life'.

6.2 Central Dogma of Life

The central dogma of life was first visualized by Francis Crick during the year 1958 which refers to the fact that hereditary information normally travels in one direction only i.e. from DNA to DNA (replication) to RNA (transcription) and to Protein (translation) as shown in Fig. 6.1. However, the reverse flow of information from RNA to DNA is also possible and the process is called Reverse Genetics.

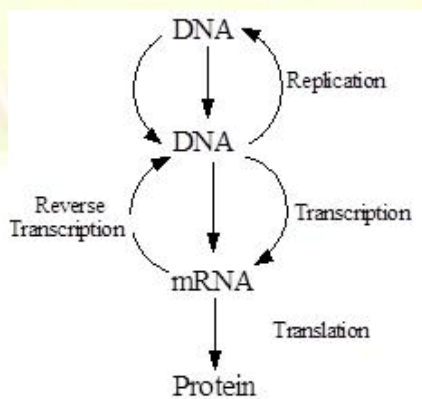


Fig. 6.1 Central dogma of life

The process of gene expression involves two major steps i.e. transcription and translation as defined below:

6.3 Transcription

Transcription is the process by which the genetic information from one strand of DNA is transferred to mRNA which is eventually used as template in the protein synthesis. This is the key step involved in gene expression.

One of the DNA strands which provides information for synthesis of mRNA is called template or anti-sense or non-coding strand. The other strand that matches exactly with the mRNA is called coding strand or sense strand or non-template strand (Fig. 6.2).

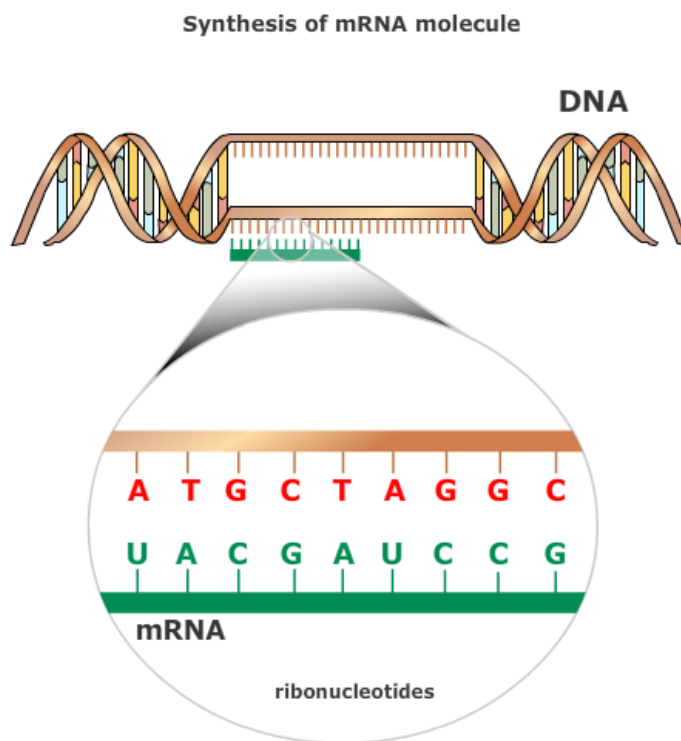


Fig. 6.2 mRNA synthesis

The process of transcription involves the following steps:

6.3.1 Initiation

Initiation begins with the formation of transcription initiation complex with the binding of DNA dependent RNA polymerase (multi subunit complex) at promoter sequence. Promoter sequence is a specific base sequence on a DNA which serves as a binding site for RNA polymerase enzyme. The length of the sequence is typically 20-40 bases. The sequence helps in indicating the correct beginning of a gene which is being transcribed. In bacteria, this region contains two short regions -10 (TATAAT) and -35 (TGTACA). These sequences are recognized by sigma factor of RNA polymerase.

RNA polymerase enzyme unwinds a short section of the DNA double helical structure near the start of the gene and forms a transcription bubble. Once the DNA molecule unwinds, RNA polymerase begins the synthesis of the mRNA using the information of the template strand. Once ~ 9 – 10 bases long mRNA is synthesized, the process enters in the elongation phase.

6.3.2 Elongation

RNA polymerase moves along the DNA in 5' →3' direction adding ribonucleotides to 3'-OH group of growing RNA chain until termination.

6.3.3 Termination

When RNA polymerase reaches a termination sequence, transcription stops either by rho dependent or rho independent process followed by the release of mRNA and RNA polymerase from the DNA molecule.

The newly synthesized mRNA is used as a template for protein synthesis.

6.4 Translation

Translation is the process of decoding the information contained in the form of codon sequences on mRNA and synthesizing a chain of amino acids which finally results into a protein molecule.

The process of translation involves the following steps:

6.4.1 Initiation

Initiation involves the binding of small subunit of ribosome (30S) to a specific sequence called Ribosomal Binding Site (RBS) or Shine-Dalgarno (SD) at the 5' end of mRNA. The larger subunit of ribosome (50S) binds with initiation complex. The structure of prokaryotic ribosome is shown below in Fig. 6.3.

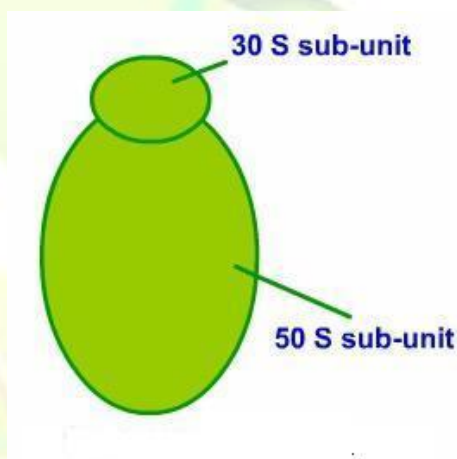


Fig. 6.3 Structure of prokaryotic ribosome

Now, a special acylated tRNA molecule carrying the amino acid methionine binds with pre-initiation complex forming the initiation complex. The cloverleaf structure of tRNA showing acceptor arm for amino acid and the anti codon loop for codon recognition is illustrated in Fig. 6.4.

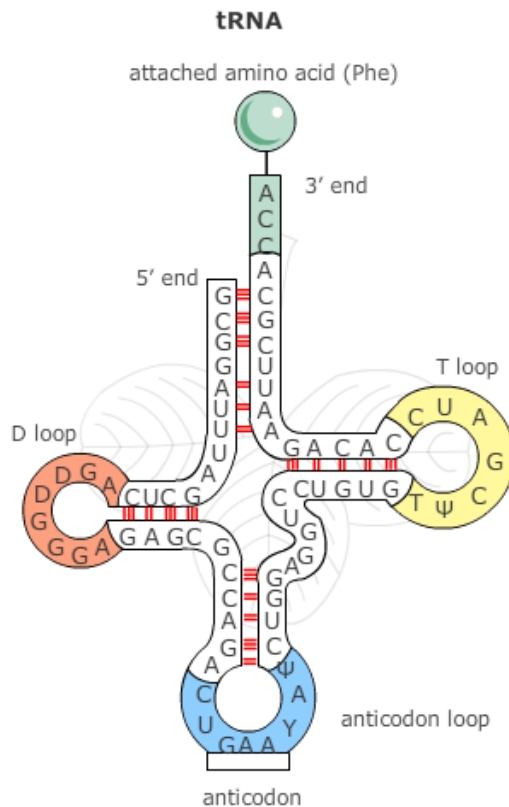


Fig. 6.4 Transfer RNA

The function of tRNA is to transfer the appropriate amino acids from the cytoplasm to the new polypeptide chain, which is being constructed, on the ribosome.

Proper positioning of the mRNA is vital to determine its reading frame, which defines which group of three bases is to be read as codons. For example, **AUG UCC UGG** or **A UGU CCU GG**. Hence, the presence of a start codon AUG coding amino acid methionine signals beginning of genetic message. The translation initiation process is shown in Fig. 6.5.

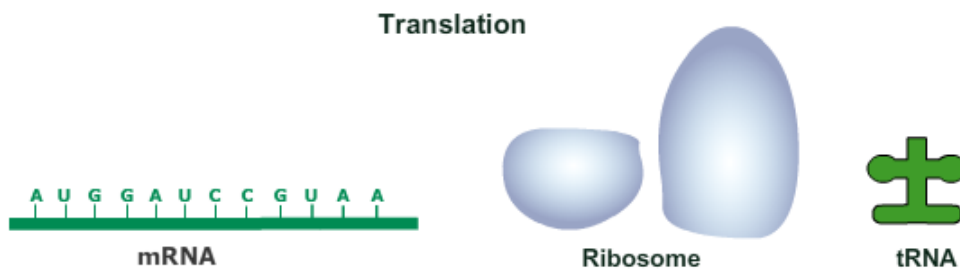


Fig. 6.5 Translation initiation process

6.4.2 Elongation

After completion of the initiation step i.e. 70S initiation complex, ribosome proceeds to the elongation phase of protein synthesis. The methionine containing tRNA occupies directly the Peptidyl “P” site on the ribosome and a new tRNA carrying the next amino acid occupies the Acceptor “A” site. The Second amino acid forms a peptide bond with the previous amino acid with the help of peptidyl transferase. The tRNA carrying the dipeptide moves to the P site emptying the A site in the process, for accommodating the new tRNA molecule carrying the next amino acid. This process is repeated with sliding of mRNA along the ribosome three bases at a time and peptide chain growing with one amino acid at a time. The detailed elongation process has been shown in Fig. 6.6 A & B.

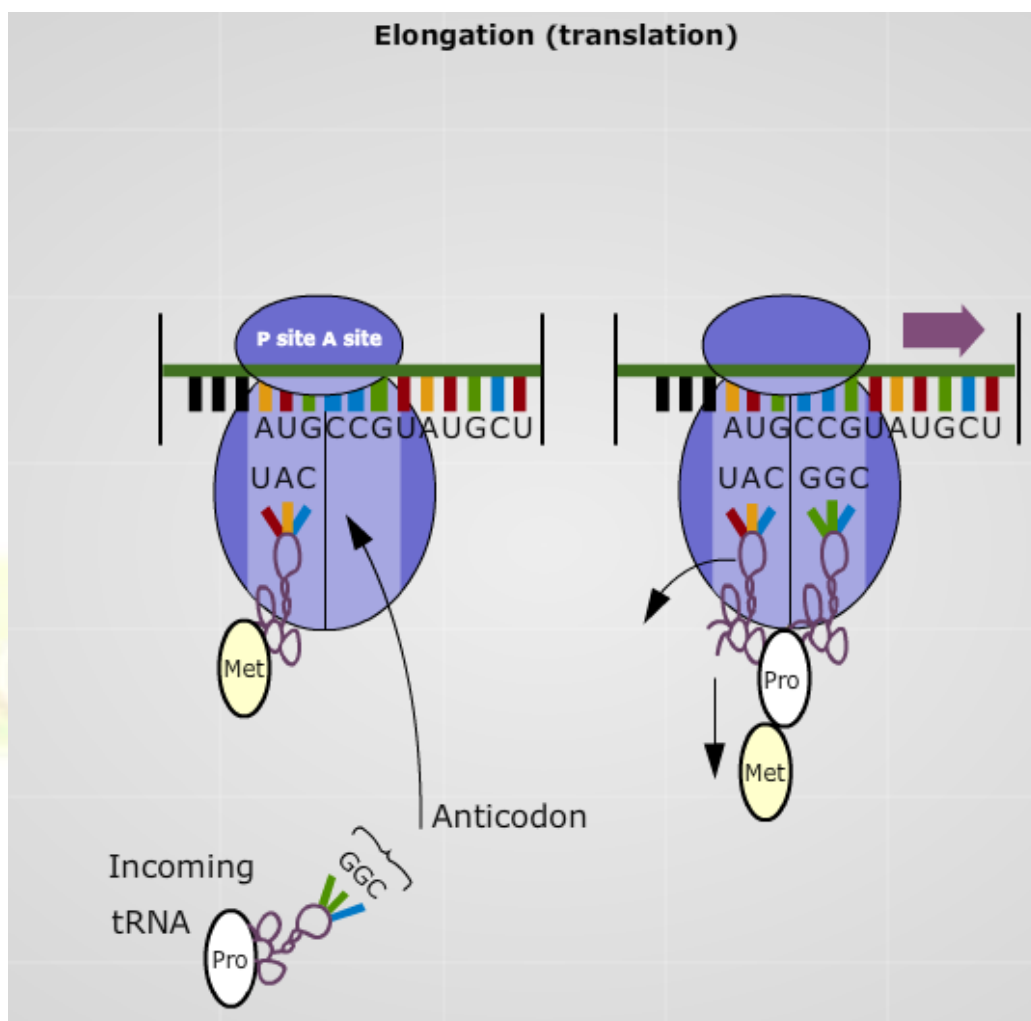


Fig. 6.6 (A) Translation elongation

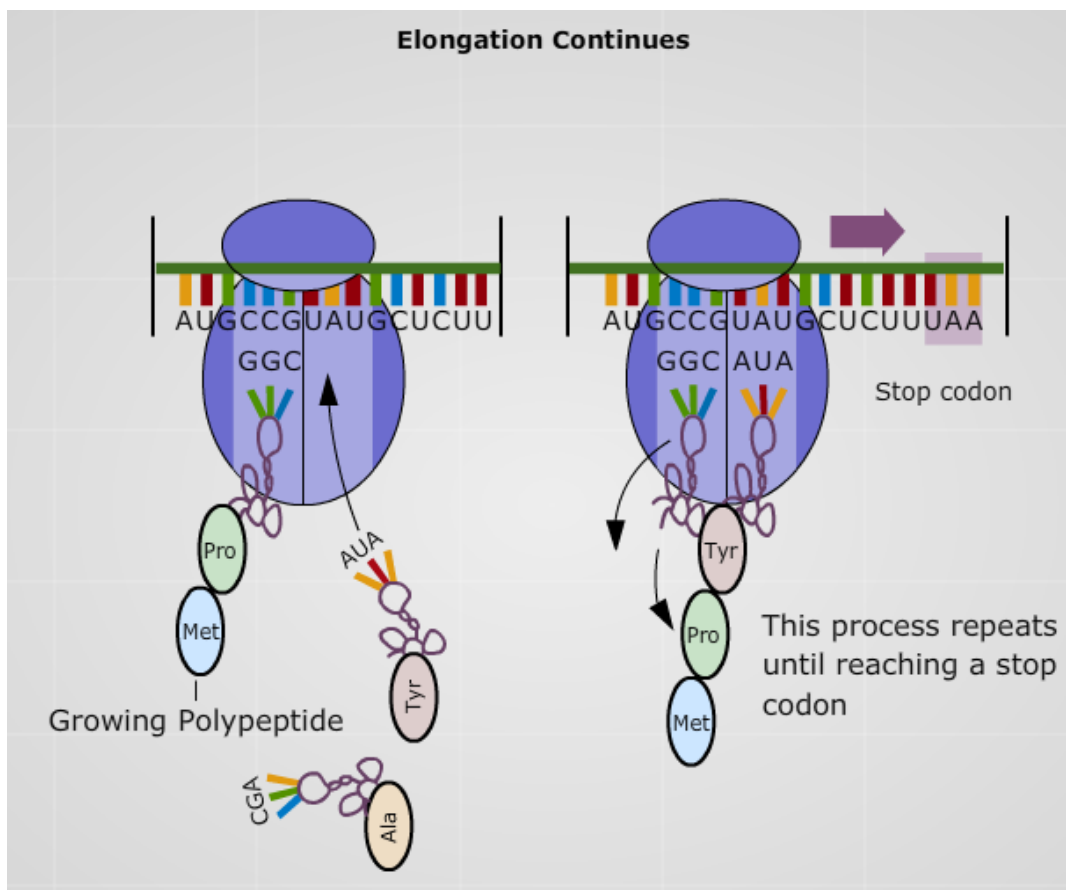


Fig. 6.6 (B) Translation elongation

6.4.3 Termination

There are three termination codons that are employed at the end of a protein-coding sequence in mRNA: UAA, UAG, and UGA. There are no tRNAs that can recognize these codons. In the absence of such specific tRNA, one of several proteins, called release factors, binds and facilitates release of the mRNA from the ribosome and subsequent dissociation of the ribosome. Thus termination codons act as the final punctuation in the message of mRNA by telling cellular machinery that the product is complete and to stop adding amino acids. The process of termination is shown in Fig. 6.7.

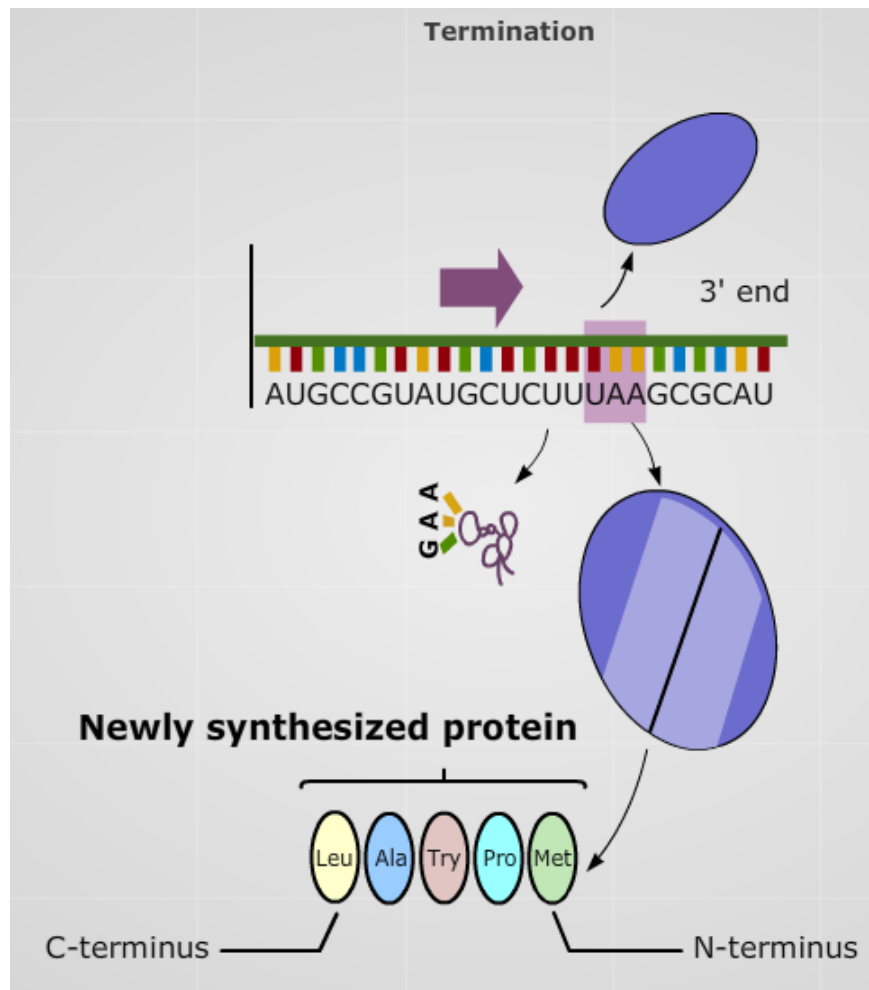


Fig. 6.7. Translation Termination

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Lesson 7

GENE REGULATION - THE 'LAC OPERON'

7.1 Introduction

All the metabolic functions in a cell with regard to both degradative and biosynthetic pathways in prokaryotes and eukaryotes are performed through the expression of specific genes encoded on their DNA for growth and development. However, all the genes present in a cell are not expressed all the time unless their functions are absolutely indispensable. Majority of the genes are turned on only when the products of such genes are needed for the growth in a given environment (signal). Their expression is turned off, when their products are either no longer needed or cell already has adequate amounts of these products. This switching on and off of the gene expression in a cell constitutes a very powerful tightly regulated system for controlling gene functionality. By turning off the expression of genes when their products are not required, an organism can avoid unnecessary wasting of energy since the energy resources available in a cell are limited. The cell has to utilize the conserved energy resources very judiciously to synthesize products that maximize the cellular growth rate. Gene expression in a bacterial population is primarily regulated by three mechanisms i.e. constitutive, inducible and repressible systems operating in these organisms.

7.2 Constitutive Expression

Constitutive gene expression is not regulated which is typical of genes whose products are indispensable for cellular functions. These genes always remain turned on and keep producing the desired enzymes or proteins continuously and stably all the time for the growth and survival of the cell. Such genes are called **house keeping or constitutive genes** that encode RNA and proteins having basal vital functions such as rRNA, ribosomal proteins and glycolytic/respiratory enzymes.

7.3 Inducible and Repressible Genes

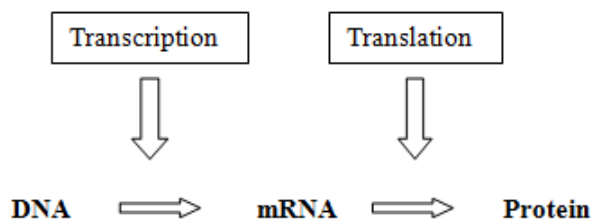
Inducible and repressible gene products are required only under certain circumstances. Inducible genes are 'turned on' in response to the presence of a substrate (inducer) in the environment e.g. lactose in lac operon.

Repressible genes are 'turned off' in response to an environmental signal e.g. the simultaneous presence of lactose/glucose or xylose/glucose which will repress the genes required for utilization of lactose or xylose.

7.4 Negative and Positive Regulation of Gene Expression

Bacteria alter the gene expression by using positive or negative regulation. Fundamental difference between positive and negative regulation is when the regulatory molecule i.e. repressor alone (lac operon) or repressor along with inducer/end product ('trp operon') is binding to the promoter and also whether the molecule is increasing (inducer) or decreasing (repressor) the gene expression.

Prokaryotic gene expression is tightly regulated at the level of transcription, translation and enzyme functions. Since transcription and translation are coupled in prokaryotes, the level of control in gene expression is relatively higher at transcriptional level.



7.5 What is an Operon?

An operon constitutes a genetic switch operating in prokaryotes only to coordinately regulate a cluster of genes involved in metabolic pathways (degradative and biosynthetic) for their functionality. These genes are transcribed together under the control of the same promoter into a single polycistronic mRNA which is eventually translated into their individual polypeptides. Operon consists of three basic elements : The structural genes, the regulatory sequences *viz.* promoter and operator regions and the regulatory gene. The best known example of the operon system is the ‘lac operon’ in *E. coli* which is now used extensively as a model for understanding the mechanism of lactose utilization in bacteria.

Operon is a genetic switch operating in bacteria to coordinately regulate a cluster of genes involved in metabolic pathways both degradative and biosynthetic.

7.6 Lac operon in *E. coli*

An operon is a functional unit of gene expression in bacteria. This represents the unique gene regulatory system operating in prokaryotes only. The ‘lac operon’ model was proposed by Jacob and Monod in 1961 to describe coordinated regulation of genes encoding enzymes required for utilization of lactose in *E. coli*.

7.6.1 Elements of ‘lac operon’

The ‘lac operon’ consists of the following key elements as shown in **Fig.7.1**.

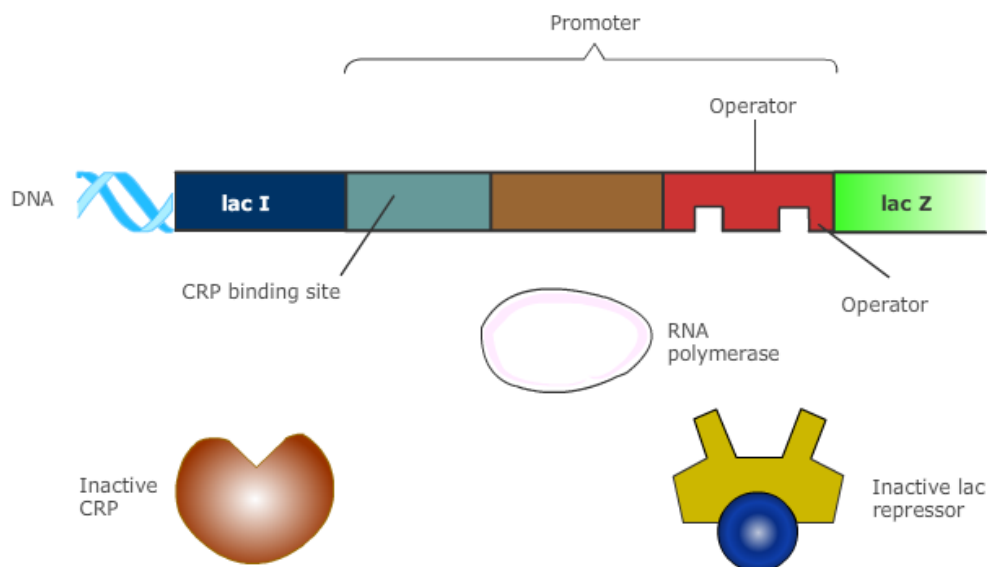


Fig. 7.1 Elements of 'lac operon'

7.6.1.1 Three structural genes

'Lac operon' is comprised of three structural genes viz. *lacZ*, *lacY* and *lacA* encoding enzymes/proteins that are functionally related for bringing about the metabolism of lactose in *E. coli*. All these three genes are under the control of a single promoter and are involved in the breakdown of lactose.

- i) *LacZ* codes for β -galactosidase which breaks down lactose into glucose and galactose.
- ii) *LacY* codes for lactose permease required for the transport of lactose into *E. coli* cells.
- iii) *LacA* codes for thiogalactoside transacetylase whose precise function is not known as yet.

The key elements of 'lac operon' include three structural genes i.e. *LacZ*, *Y* and *A*; Regulatory genes namely *LacI* and *Lac* operator and promoter

7.6.1.2 Regulatory sequences

The regulatory sequences of the 'lac operon' include the following.

- i) *LacO* - the operator region (O) at which the 'lac' repressor binds to block the promoter for RNA polymerase binding, thereby, turning off the 'lac operon'.
- ii) *LacP* - the promoter region (P) at which RNA polymerase binds to initiate the transcription of the structural genes into polycistronic mRNA
- iii) *LacI* - the regulatory gene which encodes the trans acting 'lac' repressor protein that binds at the operator region to block the transcription in the absence of lactose –the inducer of lac operon. In fact, the real inducer of the lac operon is the allo-lactose an isomer of lactose.

iv) *LacI* is not located within the lac operon but upstream several nucleotides away from the lac operon along with its own promoter to synthesize the 'lac' repressor

Apart from these, there are some effector molecules which either activate or deactivate the binding of the repressor or RNA polymerase to their respective sites at the promoter-operator regions of the lac operon. The organization of the 'lac operon' and its functioning in the regulation of the structural genes required for lactose metabolism in *E. coli* are illustrated schematically below

7.7 Working of Lac Operon

The lac operon is regulated differently in presence or absence of the inducer i.e. lactose (allolactose) as described below

7.7.1 In absence of lactose

When lactose is absent from the growth medium inoculated with *E. coli*, lac repressor is synthesized by expressing *lacI* from its promoter with the help of RNA polymerase in active form and diffused into the medium to reach the operator site in the operon. The binding of the repressor at the operator blocks the binding of RNA polymerase at the promoter and thus the structural genes are turned off and hence the polycistronic mRNA is not synthesized by the operon. The regulation of 'lac operon' in absence of inducer i.e. lactose is shown in **Fig. 7.2**.

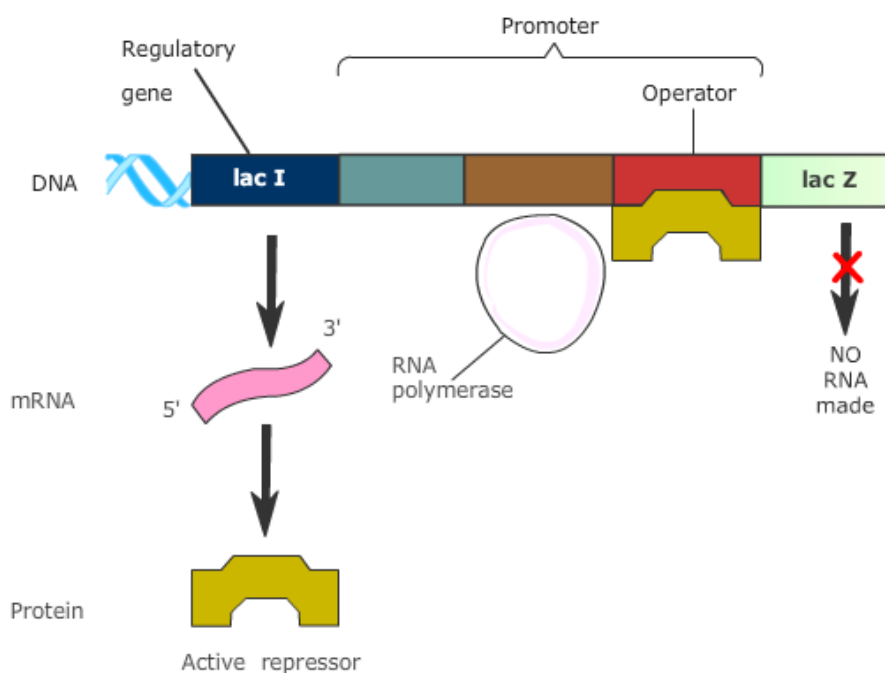


Fig. 7.2. In absence of Lactose

7.7.2 In presence of lactose

When lactose is available in the growth medium, it will bind with the repressor at its inducer binding site and alters the conformation of the repressor which can no longer bind with the operator site on the lac operon. Hence, there is no hindrance in the binding of the RNA polymerase at the promoter and therefore,

all the three structural genes are transcribed into a polycistronic mRNA from which all the three enzymes required for lactose metabolism are translated independently to perform their specific functions. The regulation of 'lac operon' in the presence of inducer i.e. lactose is shown in **Fig. 7.3**.

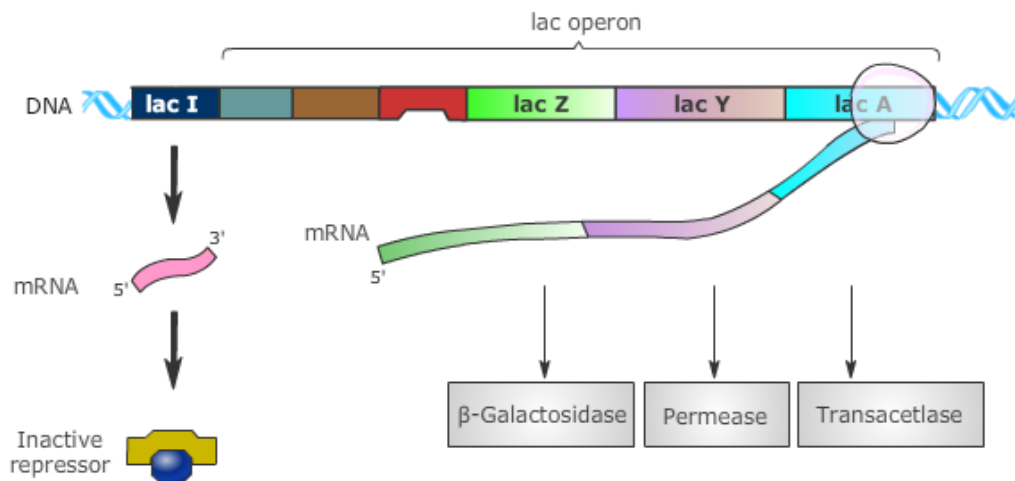


Fig. 7.3. In presence of lactose

In fact, the real inducer of lac operon is not lactose but its isomer allolactose which is produced from lactose with the help of β -galactosidase. However, the major limitation of lactose (Allolactose) serving as the inducer when added in the medium is its constant utilization by *E. coli* during growth and hence needs to be replenished continuously to keep the induction of lac operon get going. This problem has by and large been solved with the synthesis of IPTG (Iso Propyl Thio Galactoside) a structural analogue of lactose -a gratuitous inducer of the lac operon which acts as a an efficient artificial inducer without being metabolized by the bacterium and hence remains there in the growth medium indefinitely. IPTG is extensively used in the laboratory in inducing the expression of heterologous genes in *E. coli* for large scale production of high value recombinant proteins.

7.7.3 Lac operon is under both negative and positive regulation

Lac operon is a classical example of an operon which is subject to both negative and positive regulation. The negative regulation as already mentioned is mediated by the repressor molecule which will bind the operator in the absence of the inducer and thus will turn the structural genes off by inhibiting their transcription. The positive regulation of lac operon is triggered by a signal molecule called cAMP (Cyclic Adenosine monophosphate) which senses the depletion of glucose concentration in the medium (When *E. coli* cells are under starvation conditions). Under high glucose concentration in the medium, the level of cAMP in the cell goes down and conversely, at low glucose concentrations, cAMP level in the cell increases. cAMP activates another protein called CRP (cAMP Receptor Protein) also designated as CAP (Catabolite Activator Protein) which will bind at the CRP site in the lac operon located near the lac promoter only when it is complexed with cAMP. The binding of the active CRP will strengthen the binding of RNA polymerase at the promoter, thereby, increasing the expression of lac operon considerably. The working of positive regulation of 'lac operon' has been demonstrated in **Fig. 7.4**.

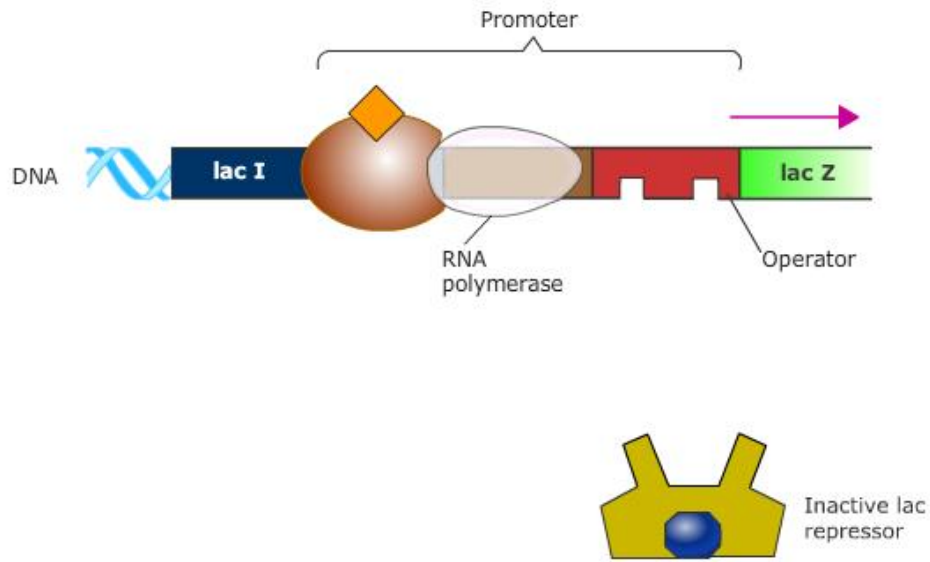


Fig. 7.4. Lac operon is under both negative and positive regulation



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Lesson 8 MUTATIONS

8.1 Definition of Mutation

A mutation is defined as an abrupt qualitative or quantitative permanent change in the DNA sequence of an organism and is one of the major causes of evolution.

Gene mutations occur in two ways *viz.* either they are inherited from a parent (passed from parent to child) designated as 'Hereditary mutations' or acquired some time during a person's life due to replication error or environmental agents. These are rare events because of the very low mutation rates (one out of a million).

8.2 Why are Mutations Important?

Mutations have considerable biological significance and play an important role in biological diversity. The study of mutations is important because of the following reasons.

- i) They may have deleterious or advantageous (rarely) consequences to an organism.
- ii) They are important to geneticists for studying metabolic pathways by making variants (mutants) lacking the ability to perform a process under investigation.
- iii) Mutations are important for evolutionary change as the major source of genetic variations.
- iv) They can also be used as tools for mapping the location of the genes in the host's genome.

8.3 Classification

Mutations can be classified based on their effect on phenotype and the kind of alterations they make in the DNA sequence (genotype).

Genotype is the genetic make up of an organism in terms of gene sequence.

Phenotype is the outward physical manifestation of an organism based on the expression of a particular gene.

8.3.1 Effects on phenotype

Based on their effect on phenotype, they are classified as

a) Lethal mutations

The mutations which result in the death of the cells are called as lethal mutations.

b) Subvital Mutations

The mutations which reduce the chances of survival are called as subvital mutations.

c) Supervital Mutations

The mutations which result in the improvement of biological fitness under certain conditions are called as supervital mutations.

8.3.2 Kinds of alterations in DNA

Based on their effect on alteration in their DNA, mutations are classified as

Chromosomal Mutations (Intergenic mutations): These are the mutations which occur in different genes.

Point Mutations (Intragenic mutations): These are the mutations which occur within the same gene and affect the same. These include base pair substitutions and frame shift mutations.

i) Base Pair Substitutions – Base pair mutations result from single base substitution leading to either transitions, transversions or both. e.g.

Wild Type : GCT GAT CTT GAT CAT

Mutant : GCT GAT **CGT** GAT CAT

Mutations brought about by base pair substitutions are of the following types.

a) Transitions: Transitions are known to occur when there is replacement of one base with a different base of the same chemical category e.g. purine to purine or pyrimidine to pyrimidine.

ACT**G**CTA → ACT**A**CTA

b) Transversions: Transversions occur when there is replacement of one base with a different base of other chemical category e.g. purine to pyrimidine or pyrimidine to purine.

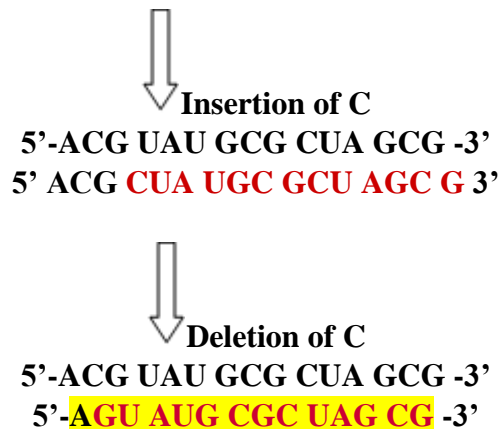
ACT**G**CTA → ACT**T**CTA

c) Inversions: Inversions occur when a segment of DNA is removed and reinserted in a reverse direction.

CGT GCT **TGC** GCT CGT

CGT GCT **CGT** GCT CGT

ii) Frame Shift Mutations – Frame shift mutations cause either deletions or insertions of one or more nucleotides leading to shift in the reading frame. Such mutations cause changes in all the amino-acids downstream of the place of mutation.



8.3.3 Qualitative effect on gene product

Based on qualitative effect on gene product, mutations can be classified as:

8.3.3.1 *Mis-sense mutations*

Mis-sense mutation is a point mutation that results from substitution of one nucleotide leading to change in codon and the proteins that differ only in single amino-acid. e. g. UUU codes for Phenyl alanine. When UUU is changed to UGU by substitution of second U with G, it codes for cysteine. Protein may or may not have normal biological activity or may have partial loss of function. An example of such mutations is sickle cell anemia wherein sixth amino-acid of hemoglobin beta chain becomes valine instead of glutamic acid.

8.3.3.2 *Non-sense mutations*

Non sense mutations occur due to change of codon to one of the three stop / termination codons (UGA, UAG, UAA) resulting into premature termination and partial or complete loss of function e.g. cystic fibrosis.

8.3.3.3 *Silent mutations*

A change in nucleotide does not cause any change in amino-acid sequence and hence does not lead to any phenotypic change. As genetic code is degenerate, most amino-acids are encoded by several different codons. Such mutations may not be able to modify the function of protein and as such can not be detected without sequencing of the gene.

8.3.3.4 *Mutations in termination codons*

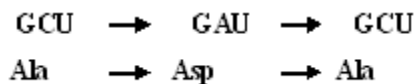
These mutations are just reverse of the non-sense mutations. In this type of mutations, termination codon is changed to sense codon which may then produce longer polypeptides by incorporating specific amino-acids in the protein.

8.3.3.5 Mutations in non-coding sequences

Mutations which occur in introns, promoters, regulatory sequences and origin of replication etc. fall in this category. These may or may not influence the function of a gene.

8.3.3.6 Reverse mutations

It is also known as back mutation or true reversion and changes the mutation back to wild type. Such mutations lead to restoring the function of protein.



8.3.3.7 Suppressor mutations

Suppressor mutations occur at sites away from the original mutation and mask or compensate for the initial mutation without reversing it.

8.3.4 Effect of mutational events

On the basis of mutational events that take place in an organism, mutations can be classified as

8.3.4.1 Spontaneous mutations

Spontaneous mutations are the ones which occur without any known cause/agent and are random. These mutations arise due to errors in replication, replication slippage, mismatches, instability of bases, oxidative damage, tautomeric shift and deamination of bases. The mutations that occur due to an error in replication or as a result of mismatches are shown in **Fig. 8.1**.

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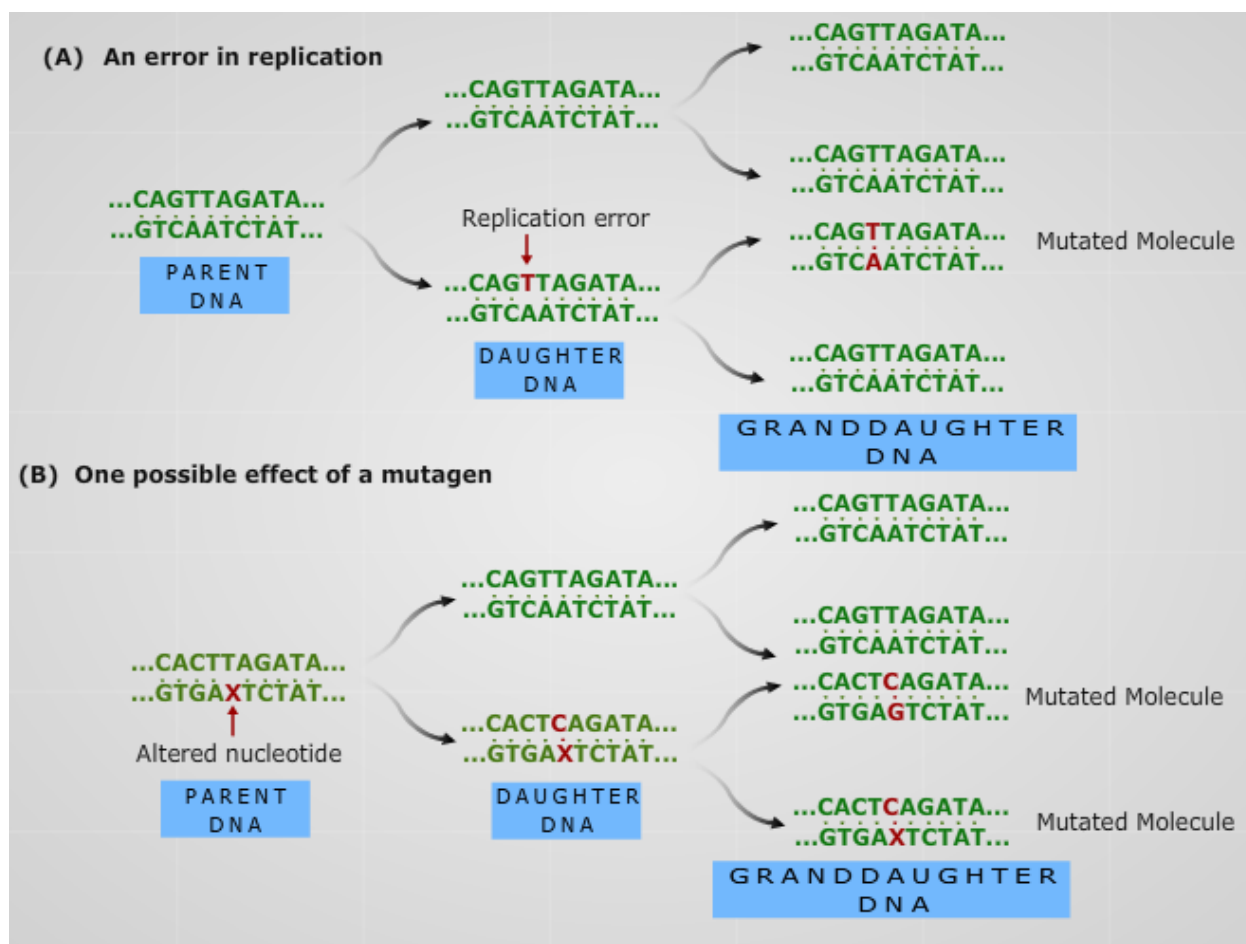


Fig. 8.1 Error in Replication

The DNA polymerase may incorporate a wrong base during synthesis and if proof reading activity of polymerase has not removed the wrong base before synthesis, then mutation occurs as has been shown in **Fig. 8.2**.



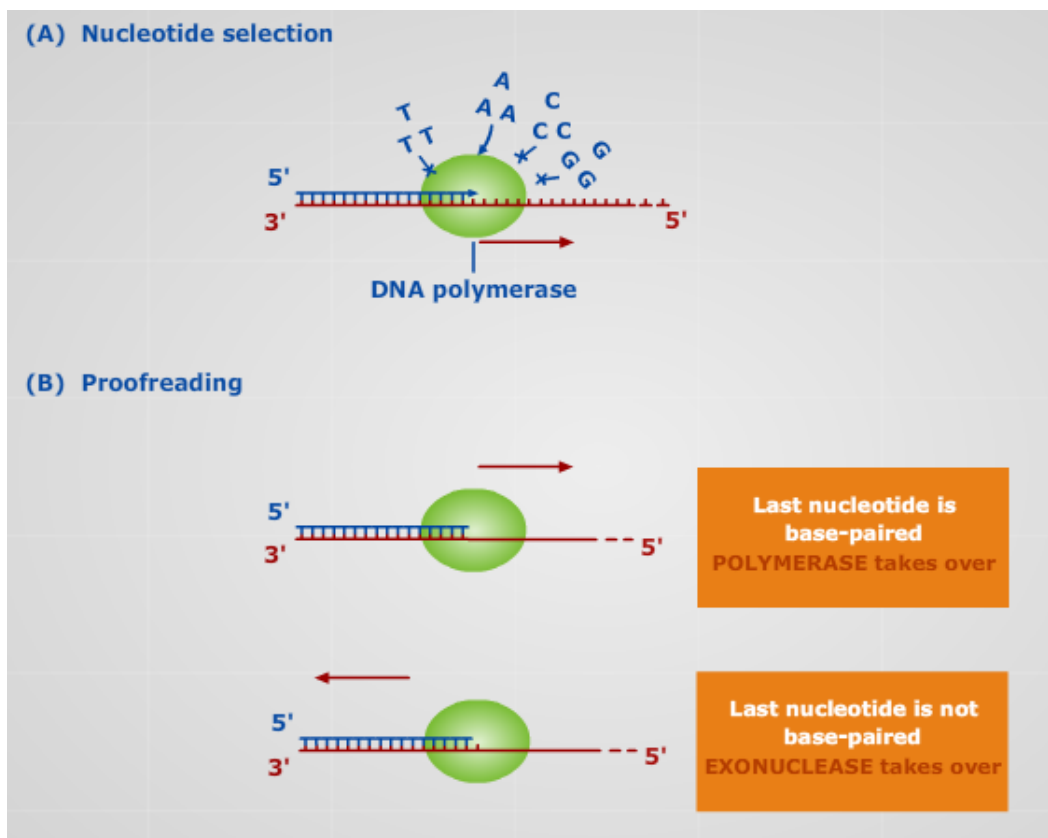


Fig. 8.2 Nucleotide selection and Proofreading

8.3.4.2 Induced mutations

Induced mutations are the ones which are induced by mutagenic agents and include physical and chemical agents. These mutagens increase the frequency of mutations.

8.3.4.2.1 Physical agents

Physical agents are of two types.

i) Ionizing Radiations (X-rays, cosmic rays, gamma rays etc.) – Ionizing radiations can randomly cause damage to all the cellular components by direct or indirect interaction. The reactive oxygen species formed by radiations can also cause damage to DNA and lead to several mutagenic and carcinogenic effects. The major effect is due to strand breaks. These radiations are widely used in tumor therapy.

ii) Non-ionizing Radiations (UV rays) - These are low energy radiations and hence do not cause any ionization. UV rays cause formation of pyrimidine dimers and most common are thymine dimers (**Fig. 8.3**) as well as thymine cytidine dimers. Adjacent pyrimidines are covalently linked by the formation of a four membered ring due to saturation of 5, 6 double bonds. Pyrimidine dimers may also occur in adjacent strands and lead to distortions.

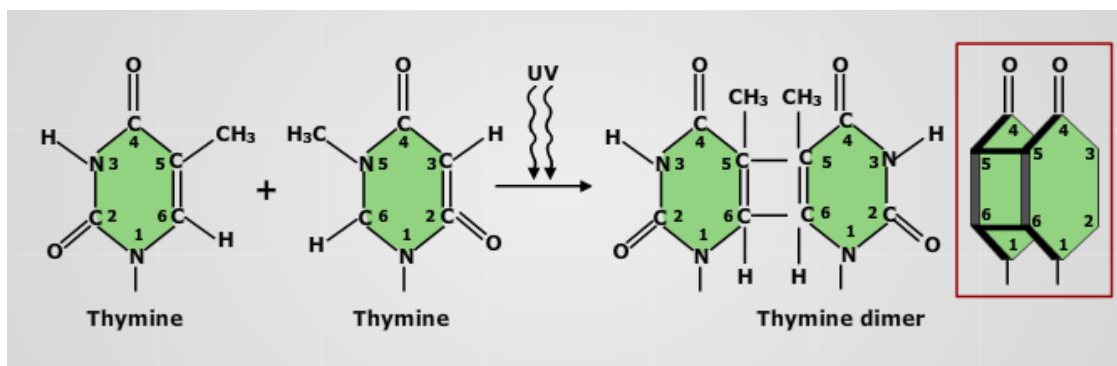


Fig. 8.3 Thymine Dimers

8.3.4.2.2 Chemical Agents

A number of chemical agents which are frequently used for studying or producing mutations are listed below.

a. Base analogues

The structure of base analogues are similar to the nitrogenous bases and hence are incorporated in DNA during its replication. The examples are 5 – bromouracil and 2 – aminopurine (Fig. 8.4). Base analogues mainly cause transitions. When 5 – bromouracil is incorporated in DNA, it pairs with adenine. It can spontaneously shift into another isomer which then pairs with guanine. On the other hand, 2-amino-purine pairs with thymine since it is an analogue of adenine. It also spontaneously shifts into another isomer which can then pair with cytosine.

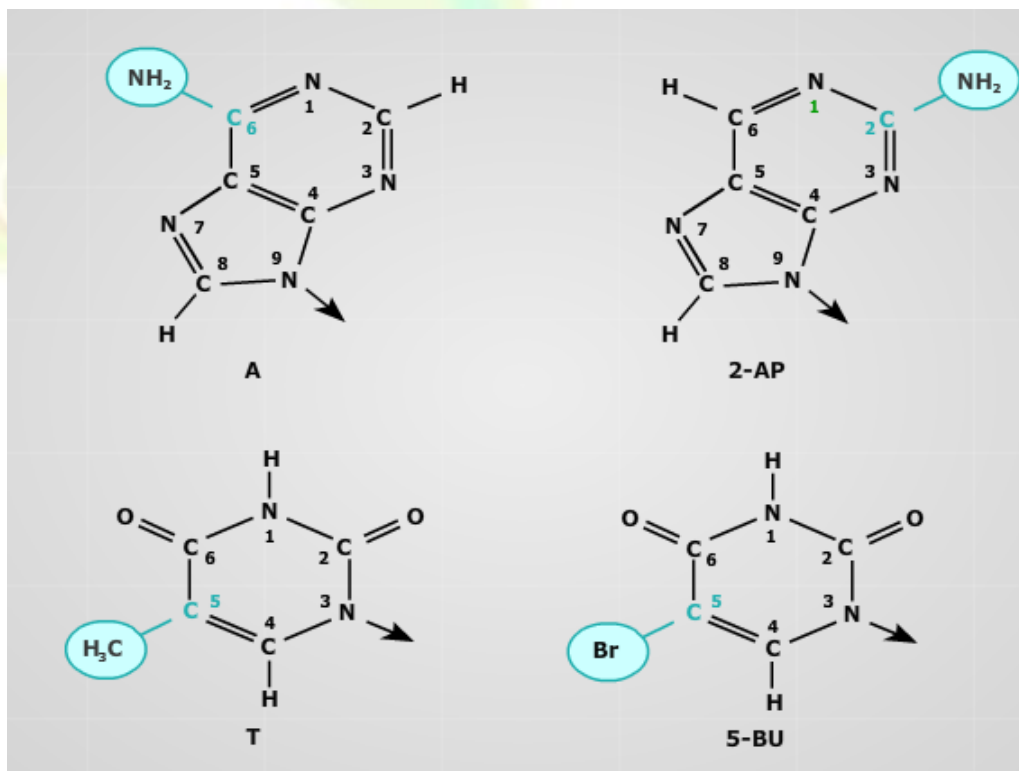


Fig 8.4 Base Analogues

b. Alkylating agents

The alkylating agents cause alkylation at the N7 position of guanine and the N3 of adenine by adding either methyl or ethyl groups. These agents act both on replicating and non-replicating DNA. The examples are mustard gas i.e. Di (2-chloroethyl) sulphide; Ethyl methane sulfonate, EMS; Ethyl ethane sulfonate, EES and N-methyl-N'-nitro-N-nirtosoguanidine (NTG). They cause both transitions and transversions. The action of EMS has been shown in **Fig. 8.5**.

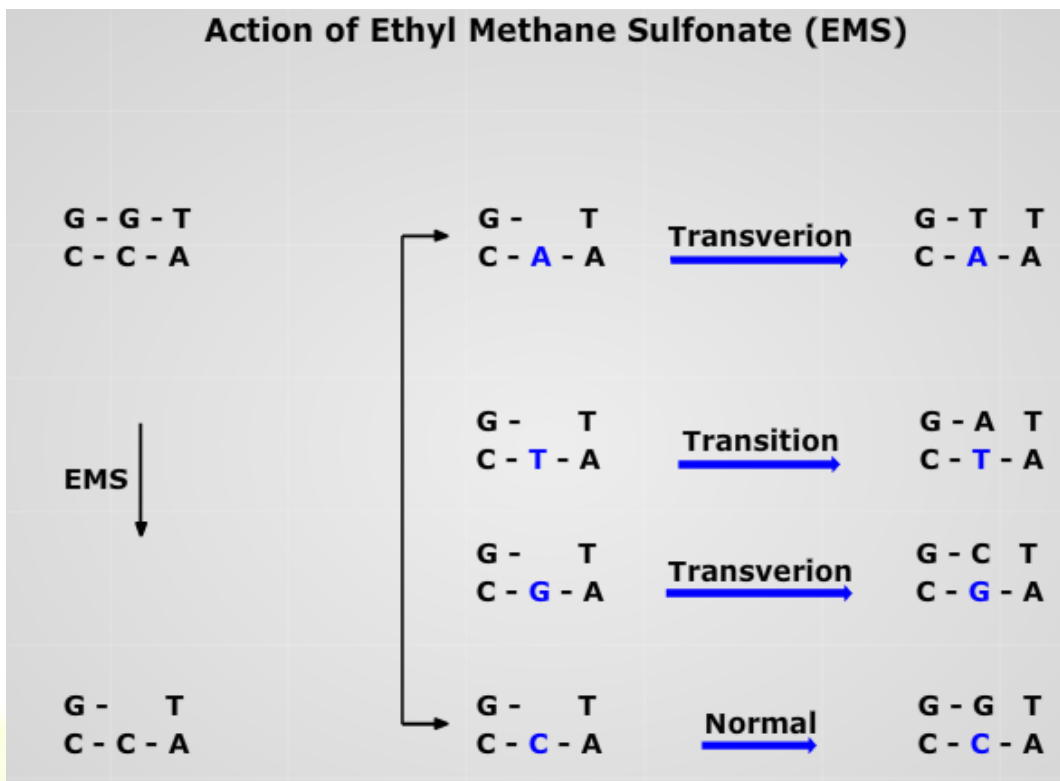


Fig. 8.5 Action of Ethyl Methane Sulfonate (EMS)

c. Deaminating agents

These agents cause deamination of bases and do not require replication. The examples are nitrous acid and hydroxylamine. They cause transitions.

d. Intercalating dyes

Intercalating dyes intercalate (insert) between base pairs in a double helix or between ring-stacked bases in a polynucleotide chain thus distorting the structure of DNA. They can cause insertions as well as deletions, thus, leading to frame shift mutations (**Fig.8.6**). The examples of intercalating dyes are acridine orange, ethidium bromide and proflavin etc. (**Fig. 8.7**).

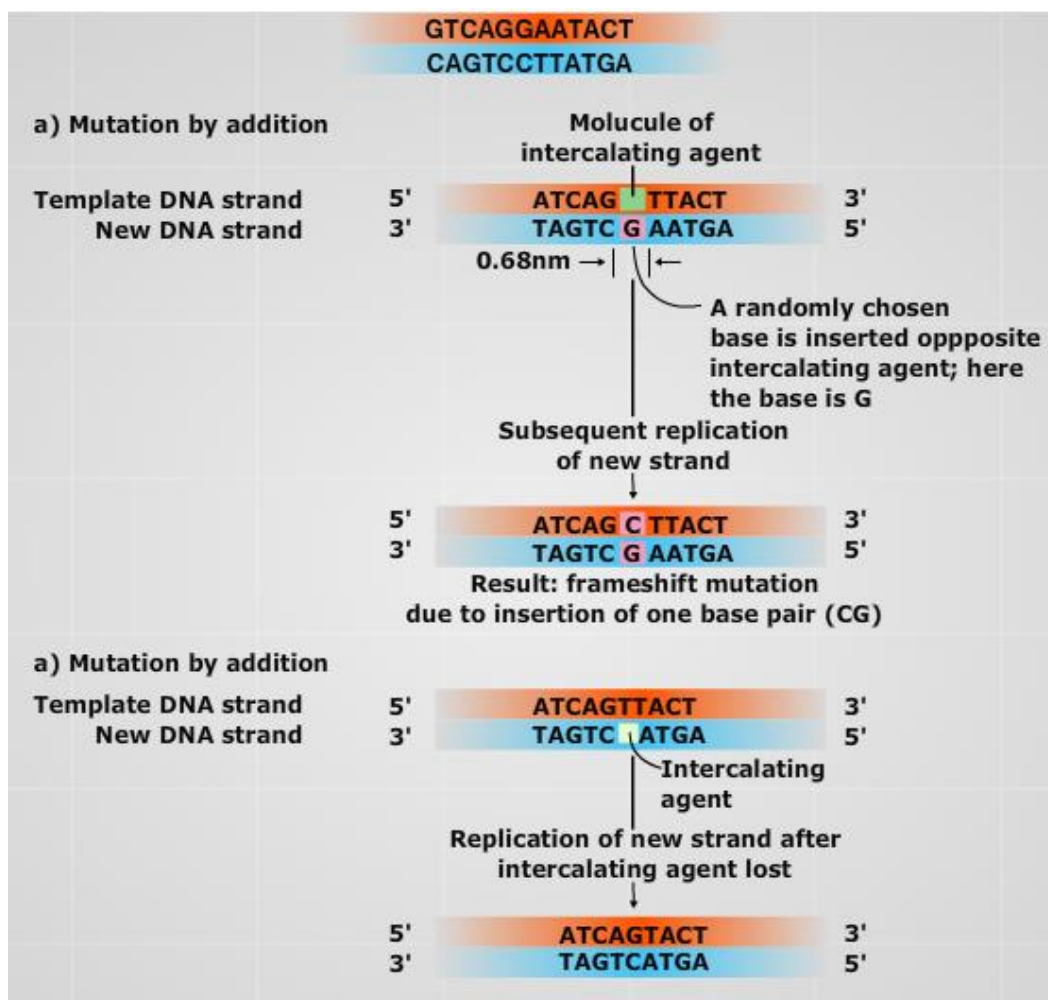


Fig. 8.6 Intercalating Dyes

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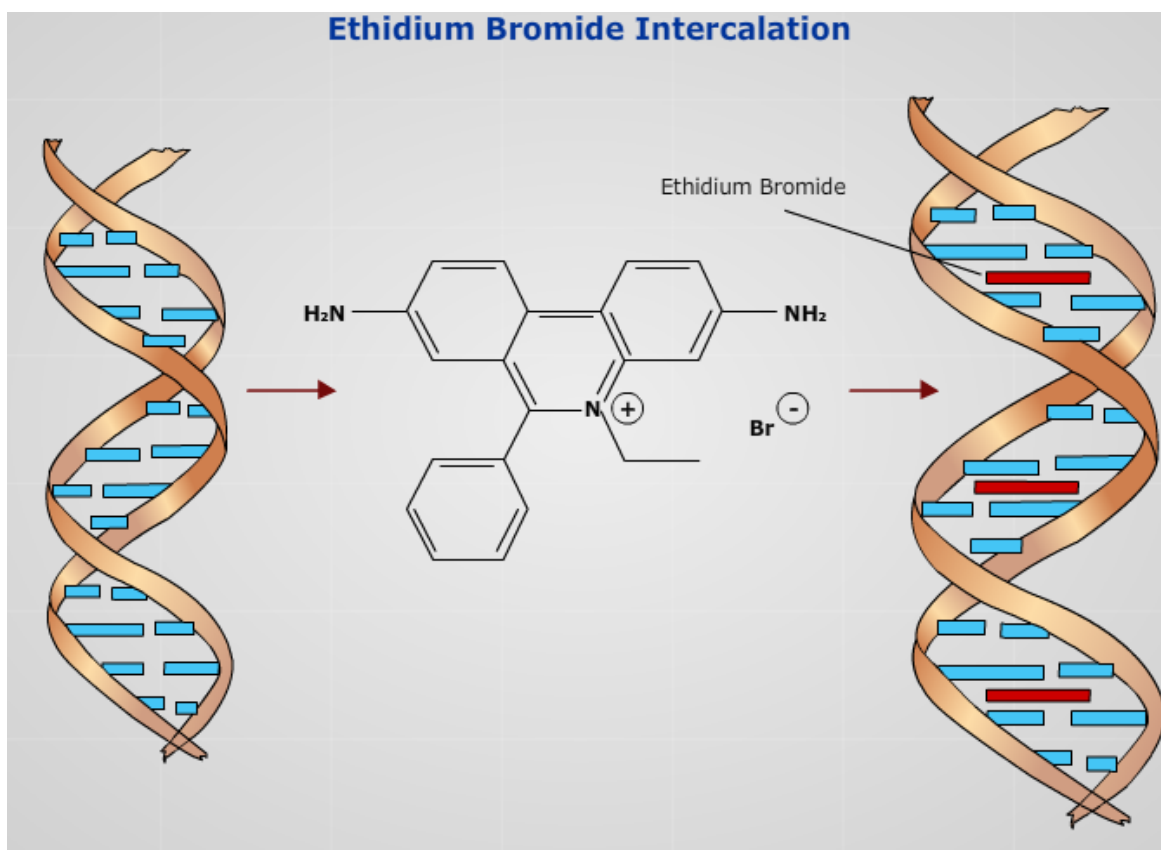


Fig. 8.7 Ethidium Bromide Intercalation

8.4 DNA Repair and Its Importance

DNA is the repository of hereditary information and nearly all the DNA damage is harmful. Therefore, it is essential to reduce this damage to a minimum tolerable level. There are a number of repair mechanisms which operate in prokaryotes and eukaryotes.

8.4.1 Direct reversal of damage or photoreactivation

This repair system acts on exposure to light and is mainly responsible for repair of thymine thymine dimers. It was discovered by Albert Kelner in 1949. UV damage is reversed if exposed to light. The enzyme photolyase encoded by *phrA* and *phrB* genes of *E. coli* is responsible for repair of the damage. The best studied example is that of Cyclobutane pyrimidine dimer (CPD) photolyase. This enzyme binds to pyrimidine dimers and use energy from visible light to split the dimers apart. This enzyme contains two chromophores i.e. Flavin adenine dinucleotide (FADH⁻) and the other is either methenyl-tetrahydrofolate (MTHF) or 8-hydroxy-5-deazaflavin (8-HDF). The latter gathers the light and transfer energy to FADH which then splits the dimer. The detailed mechanism has been illustrated in **Fig. 8.8**.

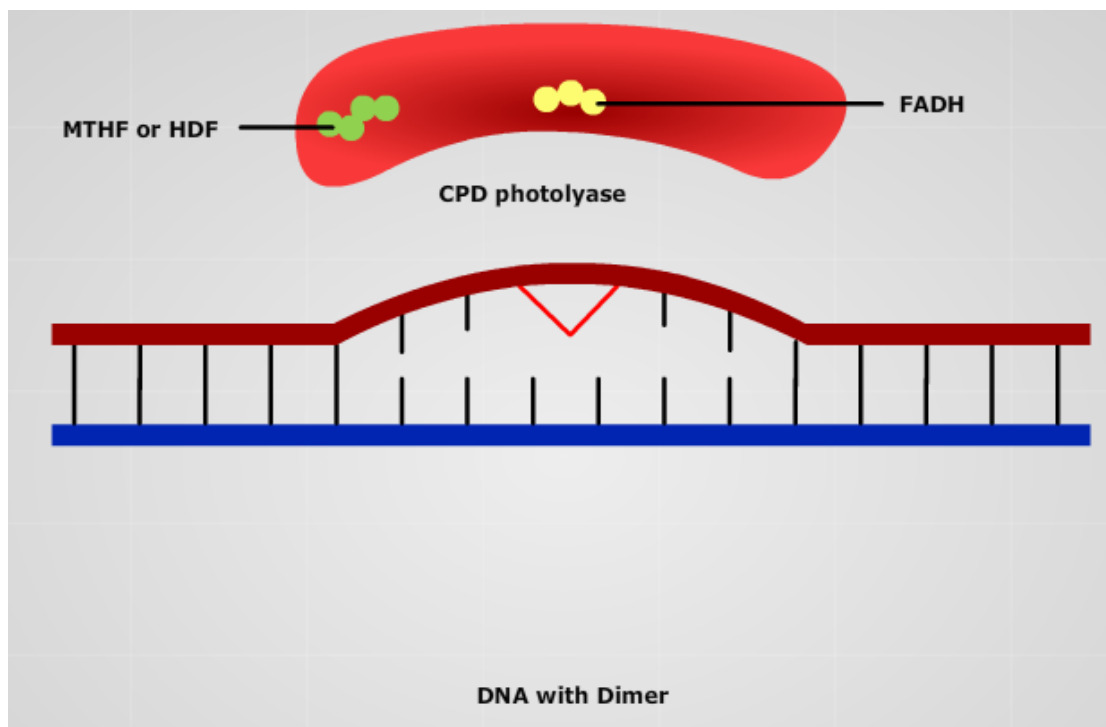


Fig. 8.8 Direct reversal of damage or photoreactivation

8.4.2 Dark repair or light independent systems

The following three mechanisms act independent of light and repair several types of damage that occur due to mutagenic agents.

i) Excision of damaged region, followed by precise replacement

Base excision repair, Nucleotide excision repair, Mismatch repair

Base excision repair (BER)

It is defined as a process of DNA repair wherein the altered base is excised by DNA glycosylase followed by excision of the resulting sugar phosphate. The gap is then filled in by the DNA polymerase and ligase.

Nucleotide excision repair (NER)

Nucleotide excision repair is the process wherein UV induced pyrimidine dimers or other unwanted mutations are removed by excision with uvrABC exinuclease. The gap is then filled in with the DNA polymerase.

Mismatch repair

Mismatch repair system corrects errors in DNA. The mismatch correction enzyme detects the wrong bases and removes the segment of the DNA strand containing the mismatched bases. The gap is then filled by DNA polymerase.

ii) Tolerance of DNA damage

Replicative bypass with gap formation/Recombinational repair - using other duplexes for repair.

When the template DNA strand is not available for repair such as when a replication fork meets a lesion (thymine dimer) and separates the strands before excision repair can take place, then the information lost at that site of damage is recovered by taking a corresponding segment from a separate but identical DNA molecule is called recombinational repair.

iii) SOS error-prone 'repair'

When the cell is severely damaged, cell engages in SOS repair in order to salvage a functioning set of genetic information. It is the last resort for the cell to survive even by incorporating a wrong base.



Module 3. Genetic engineering technology / recombinant DNA technology

Lesson 9

MOLECULAR CLONING - TOOLS

9.1 Introduction

Molecular cloning also referred to as ‘Gene cloning’, ‘Gene Manipulation’, ‘Genetic Engineering’ or ‘Recombinant DNA technology’ involves construction, creation or designing of a recombinant DNA molecule or a new combination of genetic material by constructing in laboratory under controlled conditions of digestion and ligation. DNA from two sources is digested and ligated together to generate a recombinant as shown in Fig. 9.1

Construction of a recombinant DNA molecule

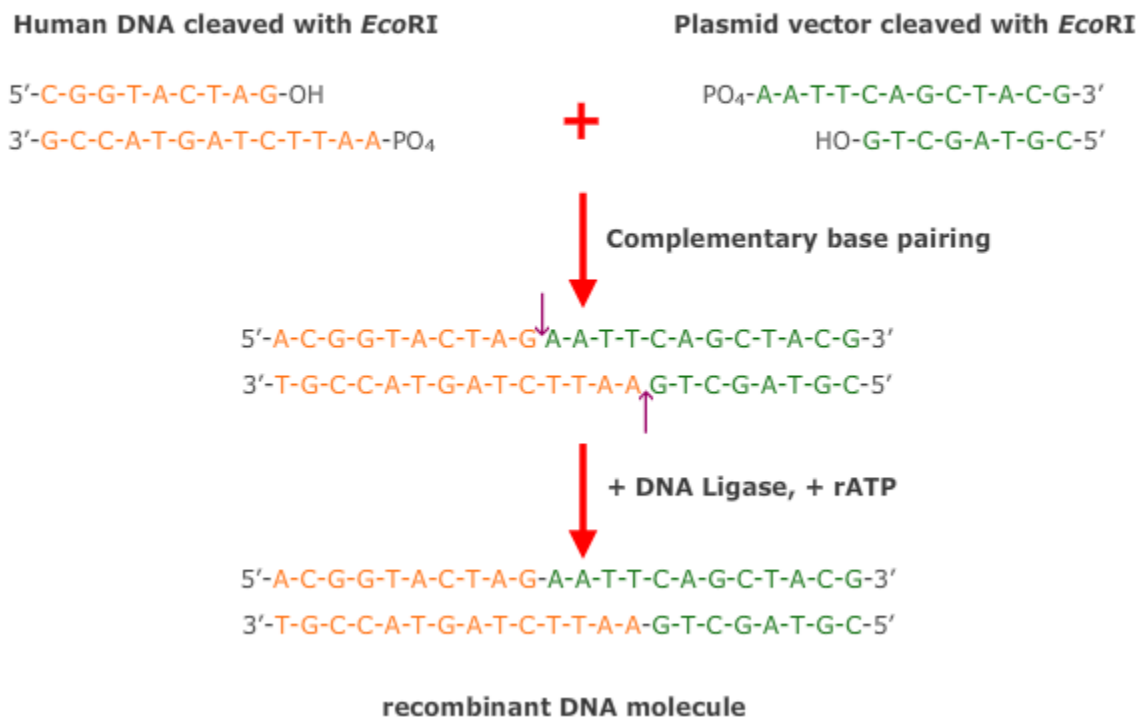


Fig. 9.1 Construction of a recombinant DNA molecule

Initially, before the invention of PCR, the genomic libraries were prepared to fish out the gene of interest based on functional assays from the resource DNA material (chromosomal or plasmid) using restriction endonucleases either frequent cutters or rare cutters. Currently, the protocol used for gene cloning in vogue is based on amplification of the target gene. This can be brought about by designing suitable primers, if gene sequences are already known and available in the NCBI database. The amplified gene product then can be digested with the appropriate restriction endonucleases and ligated in a vector for propagation in the desired host.

Molecular cloning is a technique for creating a recombinant molecule wherein the target gene is inserted into a carrier vector for its stable maintenance and replication in an appropriate host.

9.2 Basic Steps involved in Molecular Cloning

Basic components involved in molecular cloning are as follows:

1. DNA to be cloned i.e. foreign or insert or passenger or target DNA
2. Vector DNA (Vehicle / Carrier for transmission) which includes
 - Plasmids
 - Phages like lambda and M-13 viruses
 - Cosmids
3. Restriction digestion of both target and vector DNA
4. Construction of recombinant DNA (Chimera) i.e. ligation of foreign DNA with vector DNA
5. Introduction of recombinant DNA into host cells of E. coli or yeast or mammalian system by transformation/electroporation
6. Screening and selection of recombinants on the basis of antibiotic resistance or by expression of the target gene

The strategy used to produce recombinant DNA is shown in Fig. 9.2

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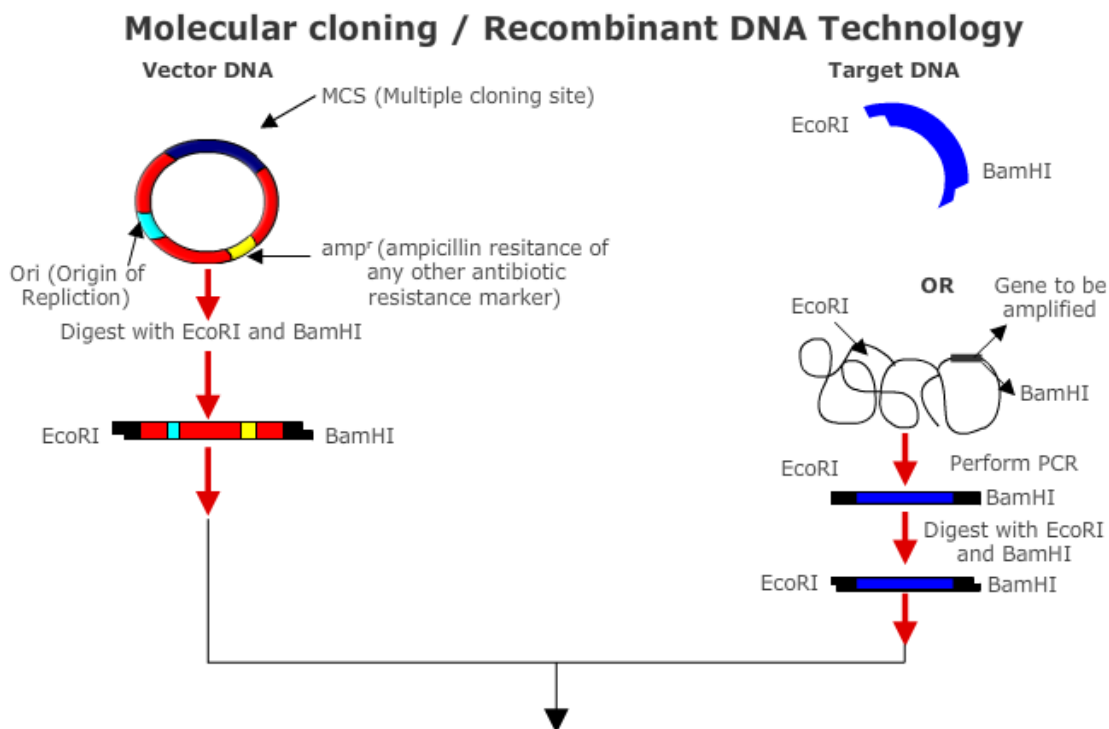


Fig. 9.2 Molecular cloning/Recombinant DNA Technology

The following tools are required for carrying out the above steps for construction of a recombinant DNA molecule.

Biological Scissors - Restriction Endonucleases

Vehicle - Plasmid, Phage or cosmid

Glue - DNA Ligase

Sieve - Agarose Gel electrophoresis

Ligase is a sealing enzyme that fills the gaps or the nicks left in the recombinant molecule during its construction.

9.2.1 DNA to be cloned i.e. foreign or insert or passenger or target DNA

The DNA to be cloned is known as target or foreign or passenger or insert DNA which is obtained from the prokaryotic or eukaryotic source either by digesting with restriction enzymes or by PCR amplification of the target gene using primers designed against the target gene. The PCR amplified product is then digested with the specific restriction enzyme/s to generate compatible ends as that of the vector DNA. The restriction endonucleases will be discussed in the next chapter.

9.2.2 Vector DNA (Vehicle for transmission)

Vector DNA is the vehicle / carrier which is used for carrying the insert / target DNA into the host cell. Vectors will be dealt in detail in a subsequent section.

9.2.3 Construction of recombinant DNA

The recombinant DNA can be constructed using DNA ligase. DNA ligase is an enzyme that joins two DNA fragments by formation of phosphodiester bond between 5' phosphate and 3' hydroxyl groups in duplex DNA as previously shown in Fig. 9.1. DNA ligase seals the nicks produced in single stranded DNA and can also join cohesive or blunt ends. DNA ligases are of the following two types.

The term 'Chimera' is derived from the ancient Greek mythology and refers to a creature with head of a Lion and tail of a Serpentine.

9.2.3.1 *E. coli* DNA Ligase

E. coli DNA ligase is obtained from an over producing strain of *E. coli* in which 'lig' gene has been cloned. Generally, it is used for joining of cohesive or staggered ends. This enzyme uses NAD as a cofactor.

The term 'clone' is commonly referred to as the pure and homogenous colony of the transformant and represents multiple copies of the same.

9.2.3.2 T4 DNA Ligase

T4 DNA ligase was initially purified from phage infected cells of *E. coli* and is the product of gene 30 of phage T4. Now, it is produced from an over producing strain of *E. coli* wherein phage T4 gene 30 has been cloned. It uses ATP as a cofactor. T4 ligase can join both cohesive as well as blunt ended DNA fragments.

9.2.4 Introduction of recombinant DNA into host cells of *E. coli* or yeast or mammalian system by transformation/electroporation

The recombinant DNA is introduced into an appropriate host such as *E. coli* by calcium chloride induced transformation or electroporation. Both the processes will be discussed in the next sections.

9.2.5 Screening and selection of recombinants on the basis of antibiotic resistance or by expression of foreign DNA

The recombinants / transformants in *E. coli* can be selected on Luria Bernati (LB) agar supplemented with ampicillin on the basis of ampicillin resistance / inactivation in the vector DNA or any other antibiotic resistance or phenotypic marker. Another way of selecting recombinants is on the basis of detection of the recombinant protein expressed in the transformants or the recombinant clones.

The presence of the recombinant vector in the transformants can be monitored on agarose gel after its isolation from the recombinant clones by electrophoresing them on running agarose gel.

9.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis is a widely used method that separates molecules based upon charge, size and shape and is used for separating charged biomolecules such as DNA, and RNA. Agarose is a polysaccharide extracted from a seaweed (*Gelidium* genera of seaweed). It is a linear polymer made up of repeated subunits of D-galactose and 3,6, anhydro-L-galactose. Most agarose gels are made using 0.7% (good for separation of large size DNA fragments of 5–10 kb) and 1- 2% (for small 0.1–1.0 kb fragments). The commonly used buffers used in the agarose gel electrophoresis for separation of the recombinant DNA molecules include TAE (Tris Acetate EDTA), TBE (Tris Borate EDTA) and TPE (Tris Phosphoric acid EDTA). However, the most extensively used buffer in majority of laboratories is TAE. Prior to gel electrophoresis, the DNA samples are mixed with xylene cyanol and bromophenol blue which are the most commonly used DNA loading buffers / dyes in order to monitor the movement of DNA samples.

9.3.1 Procedure

Generally, agarose of 0.7 – 2.0 % is prepared depending on size of DNA by dissolving the appropriate quantities of agarose in 1 X TAE buffer. Ethidium bromide stock solution (10 mg/ml) is added directly to molten agarose solution at the rate of 0.5 µg/ml before casting the gel. Molten agarose is then cooled to 50°C and poured on to respective moulds (mini or maxi) with appropriate combs. The series of steps are illustrated in Fig. 9.3. However, before pouring the gel, the surface of the mould should be leveled properly. After complete setting of the gel, the comb is removed carefully and gel plate is mounted on to the respective electrophoresis apparatus filled with 1 X TAE buffer. The DNA samples are mixed with tracking dye and loaded slowly into the wells using a micropipette. Electrophoresis is carried out at 80 V till the tracking dye reaches one third of the length of the gel. After completion of the electrophoresis, the gel is examined under UV trans-illuminator and photographed using Gel documentation systems (Bio-Rad, Minibis, G-box etc.).

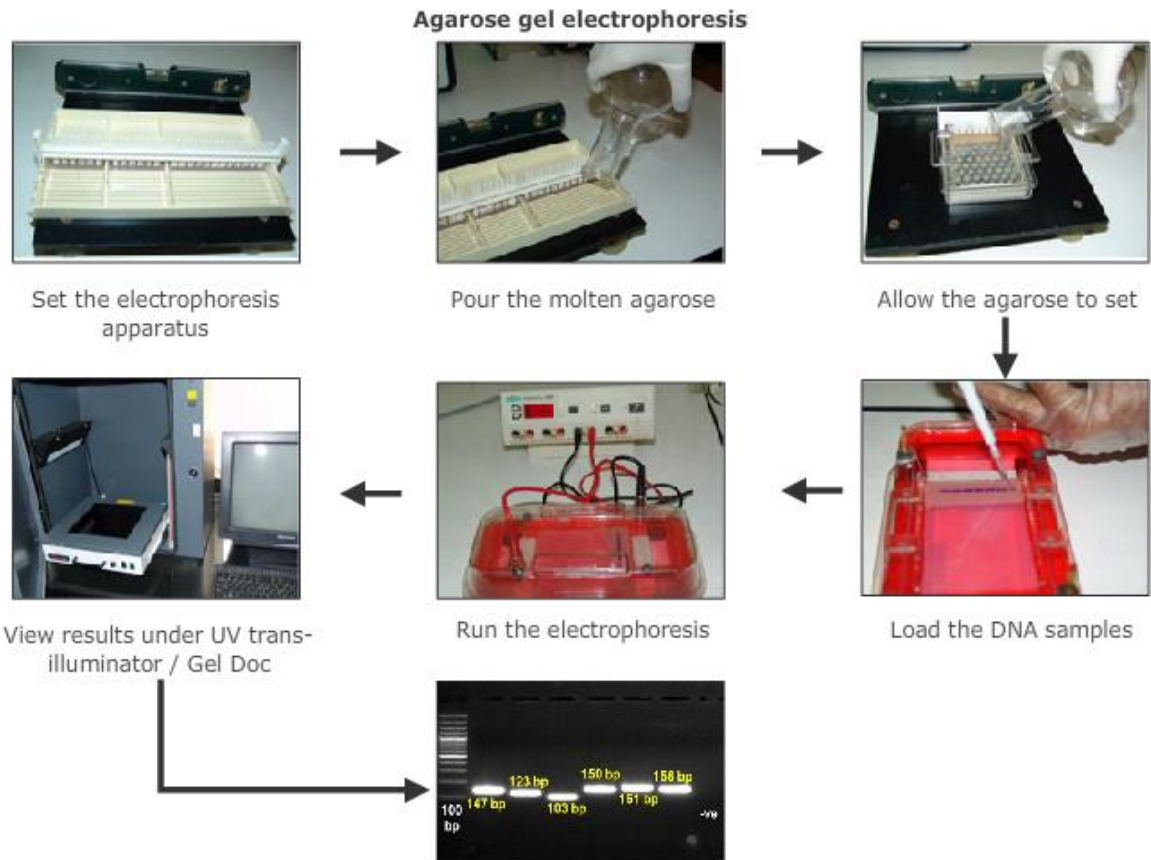


Fig. 9.3 Agarose gel electrophoresis



All About Agriculture...

Module 3. Genetic engineering technology / recombinant DNA technology

Lesson 10
RESTRICTION ENZYMES

10.1 Restriction Modification (RM) System in Bacteria

Like the immune system which operates in eukaryotes for their protection against foreign agents /antigens, Restriction Modification (called RM) system operates in bacteria for destroying foreign DNA. This phenomenon was first discovered when bacteriophages were transferred from one bacterium to another. Bacteria have developed inbuilt defense mechanisms to protect themselves from foreign invasion. The RM system comprises of a restriction endonuclease and a methylase as shown in Fig. 10.1. Restriction enzymes are unable to digest the host DNA since they methylate it.

Restriction and modification system in bacteria

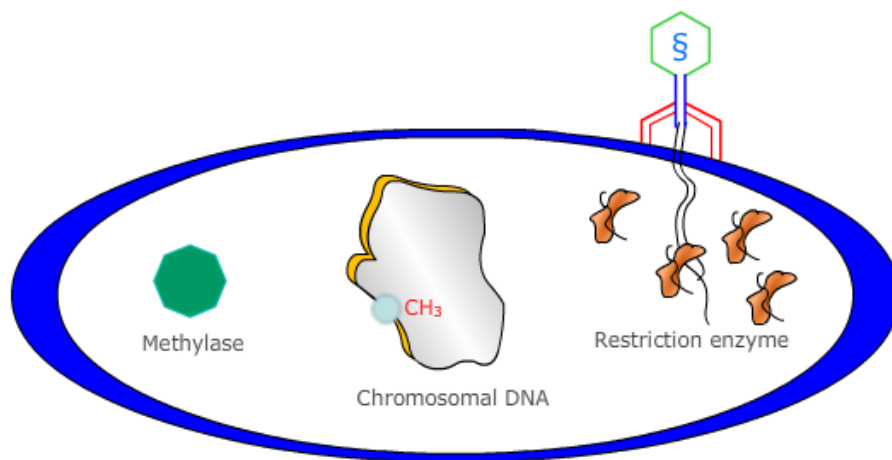


Fig. 10.1 Restriction and modification system in bacteria

Restriction enzymes cut the DNA sequence at a particular site whereas methylases add up a methyl group at that site in the DNA sequence recognized by the restriction enzymes and thus, that methylated DNA sequence can not be cleaved by the restriction endonucleases. The RM phenomenon is elaborated in Fig. 10.2. If a bacteriophage is grown in an *E. coli* strain B and then titered on *E. coli* B and K strains, the phage will be restricted by strain K. However, any particle of bacteriophage which results from infection of *E. coli* strain K, is retitered on *E. coli* strain K, it will not be restricted as it has been methylated by *E. coli* K. The restrictive host strain protects its own DNA from restriction endonuclease by modifying the same appropriately.

Restriction and modification system in bacteria

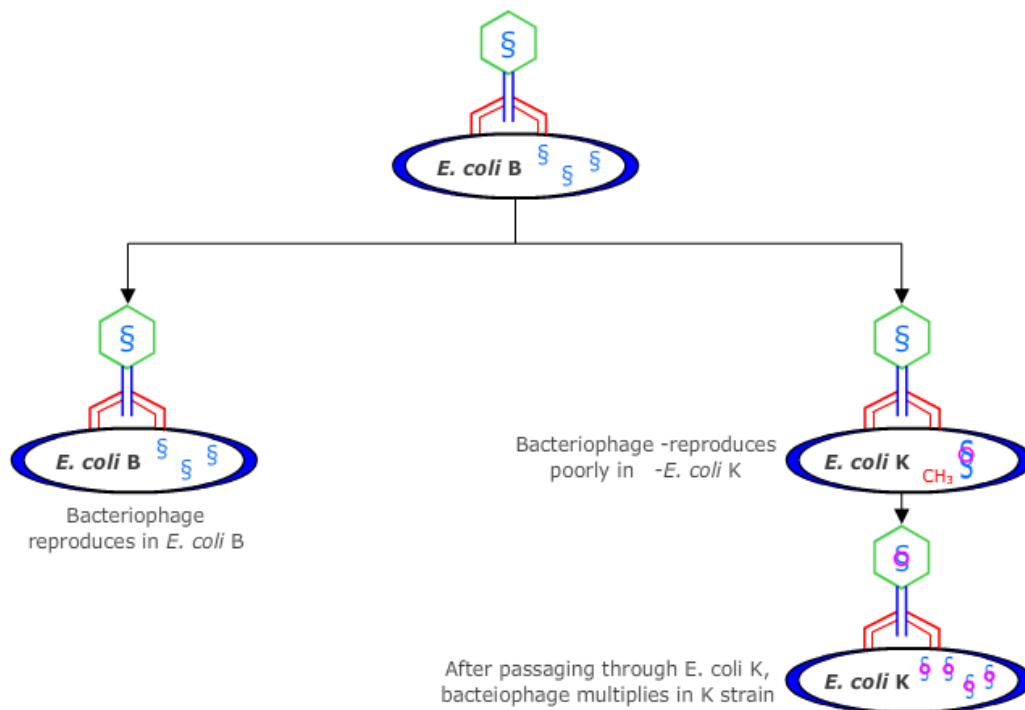


Fig. 10.2 Restriction and modification system in bacteria

10.2 Restriction Enzymes

Restriction endonucleases (RE) are the biological scissors naturally present in bacteria that cleave the incoming foreign DNA entering therein. The breakthrough in restriction endonucleases came in 1970 with the discovery of the first RE enzyme HindII from *Haemophilus influenzae* for which Daniel Nathans, Werner Arber and Hamilton Smith got nobel prize in 1978. The discovery of Restriction enzymes was the turning point in Molecular Biology that led to the development of Recombinant DNA technology and the very first use of RE was made in the production of human insulin in *E. coli*. Restriction endonucleases are DNA cutting enzymes which recognize and cut DNA only at specific sequence of nucleotides. These are four types of restriction endonucleases namely Type I, II, III and IV as described below:

Restriction endonucleases are biological scissors which recognize and cut DNA only at specific sequence of nucleotides.

10.2.1 Type I enzymes

Type I restriction enzymes are REs whose cleavage site is 1000 bp away from the 5' end of "TCA" located in recognition site and the recognition site is 15 bp in length. These enzymes require magnesium

ions (Mg^{2+}), S-adenosyl methionine (S-adomet) and hydrolyzed adenosine triphosphate (ATP) for restriction. The examples are EcoB and EcoK. Their recognition sequences are given below:

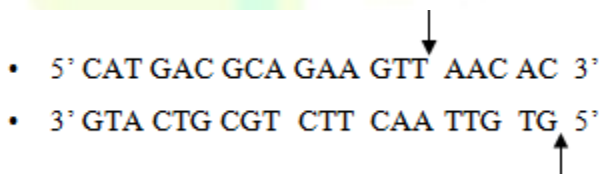
- EcoK : 5' AAC(N)₈GTGC 3'
- EcoB : 5' TGA(N)₈TGCT 3'

10.2.2 Type II enzymes

Type II REs recognize a particular (specific) target sequence in a duplex DNA molecule and cut polynucleotide chains within that sequence to give rise to discrete staggered / cohesive or blunt DNA fragments of defined length and sequence. Type II REs are the most extensively used endonucleases and constitute the most ideal tools for molecular cloning and require only Mg^{2+} ions as co-factor. Examples include EcoRI, BamHI, HindIII, PstI, NotI etc.

10.2.3 Type III enzymes

Like Type I REs, recognition site for type III is specific but not always at a symmetric sequence and cleavage site is at a specific distance from recognition site i.e. in the immediate vicinity of recognition site. These enzymes require magnesium ions (Mg^{2+}), S-adenosyl methionine (S-adomet) and hydrolyzed adenosine triphosphate (ATP) for restriction. The example is of HgaI whose recognition sequence is 5'GACGC3' but cleavage site is 5-10 bases away from it.



10.2.4 Type IV enzymes

These REs are modification dependent restriction enzymes and are composed of proteins that cut only methylated DNA.

The differences between three types of restriction enzymes Type I, II and III are given in Table 10.1.

Out of the four types of restriction enzymes, the type II restriction enzymes are useful for molecular cloning and hence will be dealt in detail in the following section:

Table 10.1. Differences between Type I, II and III Restriction Endonucleases

Property	Type I	Type II	Type III
Restriction and modification activities	Single multifunctional enzyme	Separate endonuclease and methylase	Separate enzymes
Protein structure	3 subunits	1 subunit	2 subunits
Requirement for restriction	ATP, Mg ²⁺ and S-adenosyl methionine	Mg ²⁺	ATP, Mg ²⁺ and S-adenosyl methionine
Cleavage Sites	1000 base pair away from host specificity site	Recognition and cleavage site at same place	Cleavage site is in the vicinity of host specificity site (5-10 bases away from the recognition sequences)

10.3 Type II Restriction Enzymes

Type II restriction endonucleases are extensively used biological tools in molecular cloning experiments. Type II restriction endonucleases have been divided into two categories i.e. Type IIa and Type IIb as per their end specificity.

10.3.1 Type IIa

The type IIa REs cleave the DNA sequence within the recognition site in a staggered pattern generating specific fragments with identical single stranded cohesive / sticky / staggered 5' to 3' (EcoRI) as shown in Fig. 10.3 and 3' to 5' ends (PstI) as follows in Fig. 10.4.

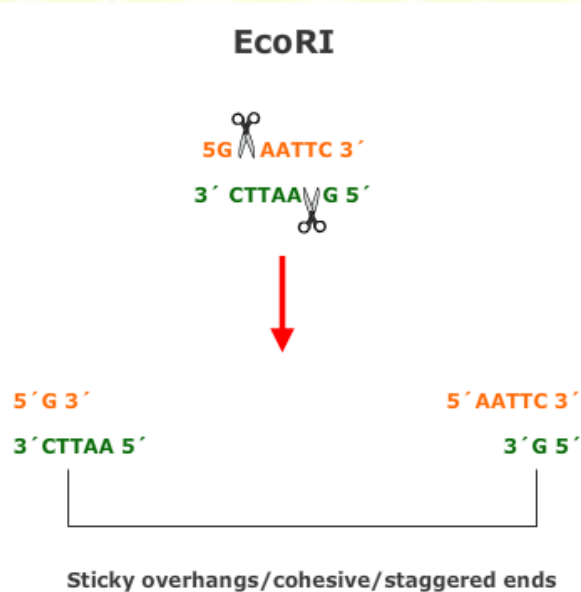


Fig. 10.3 Type IIa

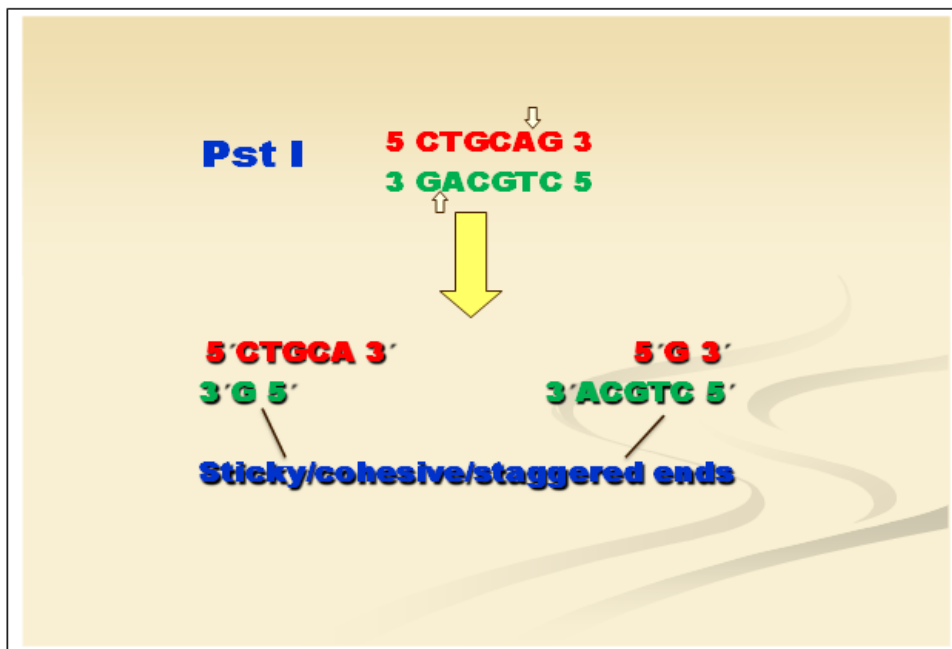


Fig. 10.4 Recognition sequence and cleavage of DNA with PstI

10.3.2 Type IIb (Blunt ends)

The type IIa REs generates flush / blunt ends e.g. HindII, SmaI as shown in Fig. 10.5.

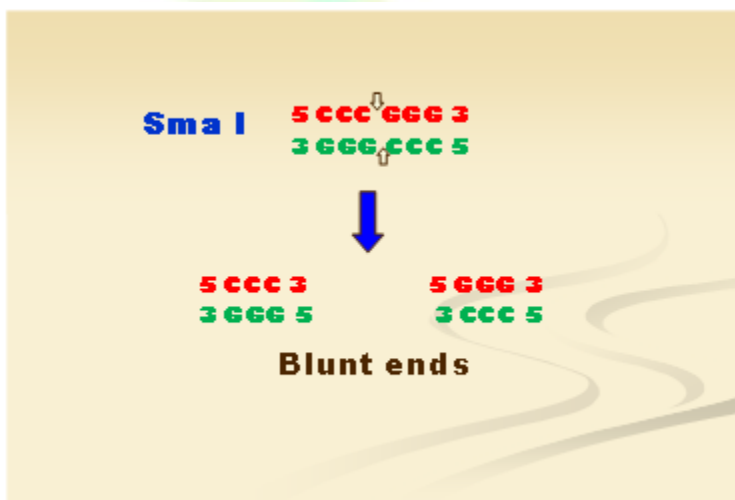


Fig. 10.5 Recognition sequence and cleavage of DNA with SmaI

10.4 Restriction Enzyme Sites for Some Common REs

The following Table 10.2 gives recognition sequences and cleavage sites for some commonly used REs.

Table 10.2 Restriction enzyme sites for some common type II enzymes

Enzyme	Name of the organism	Target site 5' - 3'
BamHI	<i>Bacillus amyloliquifaciens</i>	G ⁺ GATCC CCTAG ₃ G
Hind III	<i>Haemophilus influenzae</i>	A ⁺ AGCCT TTCGGA ₃
Hae III	<i>Haemophilus aegyptius</i>	GG ⁺ CC
Sal I	<i>Streptomyces albus</i>	G ⁺ TCGAC CAGCT ₃ G
Hpa I	<i>Haemophilus parainfluenzae</i>	G ⁺ TAAAC CAATT ₃ G
Sma I	<i>Serratia marcescens</i>	CCC ⁺ GGG GGG ₃ .CCC
Mbo I	<i>Moraxella bovis</i>	⁺ GATC CTAG ₃ ,

10.5 Frequency of Cutting of Recognition Enzymes

Based on cleavage, enzymes are recognized as Tetra, Penta, Hexa, Octa cutters. The frequency of cutting is calculated as $1/4^n$ (n = no. of nucleotides). Some examples are:

Sau 3A (GATC) cuts $1/4^4$ i.e. $(1/4)(1/4)(1/4)(1/4) =$ once every 256 bp

BamHI (GGATCC) cuts $1/4^6$ i.e. $(1/4)(1/4)(1/4)(1/4)(1/4)(1/4) =$ once every 4096 bp

10.6 Nomenclature of Restriction Enzymes

The nomenclature of restriction enzymes was given by Smith and Nathans in 1973. The first three letters of REs represent first letter of genus and two letters of species name of the organism from which these enzymes were isolated e.g. Eco from *Escherichia coli*, Hin from *Haemophilus influenzae*. Fourth letter represents serotype of the organism e.g. EcoR where R is serotype and Hinf where f is the serotype. The last Roman letter is for number of enzymes recovered from the same organism e.g. EcoRI, EcoRII means two different REs from *E. coli* were isolated.

Example of EcoRI

E *Escherichia* (genus)

co *coli* (species)

R RY13 (strain)

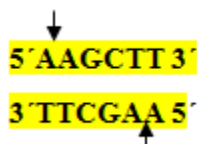
I First identified in *E. coli*

10.7 Isoschizomers

The following types of enzymes are known as Isochizomers.

i) REs which share the same recognition and cleavage sites

-**HindIII and HsuI** where HindIII is from *Haemophilus influenzae* Rd and HsuI is from *Haemophilus suis*



MboI also cleaves at /GATC, and so is an isoschizomer of *Sau3A* (/GATC)

ii) REs which share same recognition sequence but differ in cleavage sites

- **SmaI and XmaI**



iii) Differ in recognition sequences but still generate identical overlapping termini

BamHI

BglII

BclI

Sau3A

MboI

5' GGATCC 3'

5' AGATCT 3'

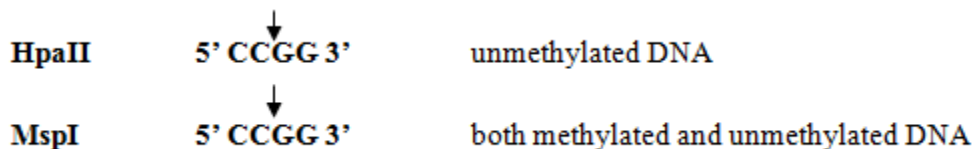
5' TGATCA 3'

5' GATC 3'

5' GATC 3'

All these generate cohesive 5'GATC 3' ends

iv) Some REs recognize only unmethylated sequences, their isoschizomers cleave both methylated and unmethylated sites



10.8 Modification Enzymes

The modification enzymes are designated as Methylases and they recognize the same DNA sequences as REs. However, the modification enzymes are differentiated from REs by writing letter M before RE. They modify the DNA by methylating either adenine at ⁶N position or cytosine at ⁵C position and require Mg²⁺, ATP and S-adenosylmethionine (as methyl donor). Based on methylation pattern, they are designated as 'dam' and 'dcm' methylases. The 'dam' methylase adds methyl group at 6N adenine e.g. 5'G*ATC3'. The susceptible RE sites include DpnI, PvuI, BclI, BglIII, Sau3A, MboI etc. On the other hand, 'dcm' methylase adds methyl groups at ⁵C cytosine 5'C*CAGG or C*CTGG 3' (EcoRII). The susceptible RE sites include BamHI, BclI, KpnI, NarI etc.

10.9 Application of Restriction Enzymes

10.9.1 Molecular cloning/ recombinant DNA technology

Molecular cloning involves amplification of a gene of interest from the target and digesting it with restriction enzymes for ligation into vector cut with same enzymes to generate recombinant gene for introduction into prokaryotic or eukaryotic host. It has already been discussed in detail in previous section.

10.9.2 Restriction digestion

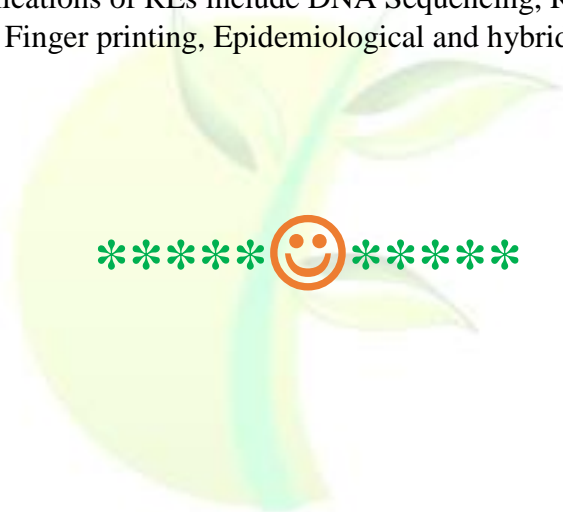
The process of cutting/digesting DNA molecules into smaller pieces with REs is known as restriction digestion. Reactions are set up and samples are electrophoresed to check for the generation of restriction fragments. Some of the REs are influenced by reaction conditions and exhibit star activity e. g **Eco Star Activity**. The star activity is exhibited due to deviations from optimal reaction conditions which alter the cleavage specificity. In buffers containing 100 mM NaCl, 5mM MgCl₂, pH 7.3, EcoRI recognizes and cleaves the sequence GAATTC. However, increasing the pH, lowering salt concentration, replacing magnesium by manganese etc. alter the specificity of EcoRI. EcoRI then recognizes AATT which occurs more frequently and starts cutting non-specifically. This new specificity is called EcoRI* i.e. star activity.

Star activity : Star activity is the erroneous activity exhibited by some restriction endonucleases due to abnormal assay conditions.

10.9.3 Restriction mapping

Restriction Enzymes are also used to map the location of restriction enzyme sites on vector or any gene.

Besides above, the other applications of REs include DNA Sequencing, RFLP (Restriction fragment length polymorphism), DNA Finger printing, Epidemiological and hybridization studies.



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**Lesson 11
VECTORS**

11.1 What is a Vector?

A vector (a carrier, a Latin word meaning bearer) is a DNA molecule having its own origin of replication that carries foreign DNA into a host cell and produces many copies of itself and that of foreign DNA. They are categorized into following types.

11.1.1 Plasmids

Plasmid vectors can be used for cloning of DNA fragments of size generally ranging from 0.1 to 10 kb. The most common examples of plasmid cloning vectors are pUC18/19, pBluescript, pGEX, pET series etc.

11.1.2 Lambda phage

Lambda phage can also be used as vector for cloning gene of interest of size ranging from 8-25 kb fragments. This is based on lambda phage genome which is 48,502 bp with a central 33% (stuffer region) which is not critical for lytic growth and can be replaced with 8-25 kb gene inserts e.g. gt11, charon phages, lambda ZAP vectors. Lambda vectors can be introduced into cells at a very high efficiency.

11.1.3 Cosmids

Cosmids vectors (5-7 kb) are hybrid between plasmids and phages. They contain antibiotic genes and replication origin from plasmids and 'cos' sites from phages which are necessary for packaging of DNA. These vectors can clone larger fragments of 35 size – 50 kb.

In simple language, cosmids are the plasmids with lambda 'cos' sites and they have the option to exist in the host as a plasmid or lambda phage.

11.1.4 BAC vectors (Bacterial artificial chromosomes)

BAC vectors contain sequences from the *E. coli* F plasmid and they have the ability to clone up to 75 - 200 kb fragments.

11.1.5 YAC vectors (Yeast artificial chromosomes)

YAC vectors contain sequences required to replicate and maintain chromosome in budding yeast (like 1,

end up as a linear molecule) and contain a yeast origin of replication, a centromere, and a telomere at each end. These vectors are able to clone very large fragments of >2,000 kb inserts (2 Mb).

Out of the above mentioned vectors, we shall study plasmid cloning vectors in detail as the plasmids are the most commonly used vectors in majority of the laboratories.

A replicon is any piece of DNA having its own origin of replication that can replicate autonomously in a bacterial host.

11.2 Definition of Plasmids

Plasmids are small extra-chromosomal circular DNA molecules (replicons) that can replicate independently in bacteria (i.e. they have their own origin of replication) and are present in one or multiple copies in a bacterial cell (Fig. 11.1). Plasmids naturally occur in different types of bacterial cells. However, most of the plasmids are cryptic in nature i.e. their functions remain by and large unknown.

Genomic and plasmid DNA in a bacterial cell

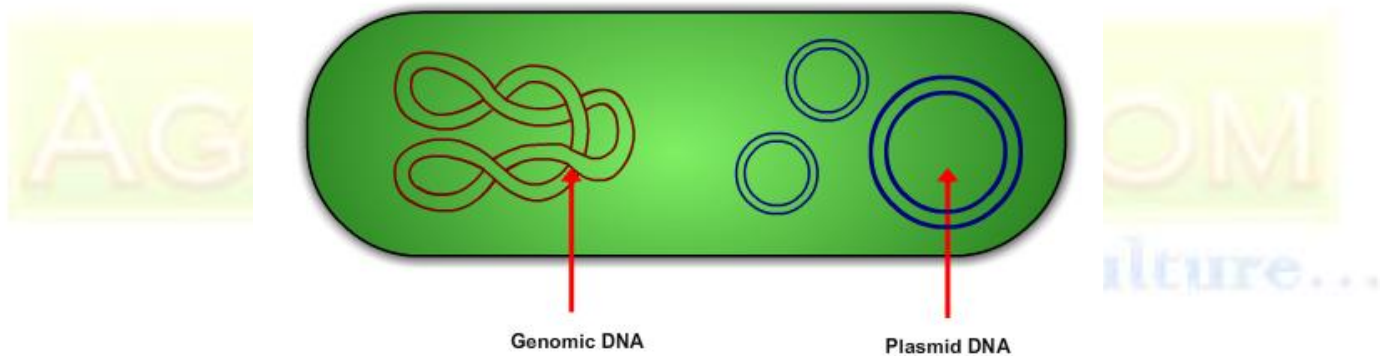


Fig. 11.1 Genomic and plasmid DNA in bacterial cell

11.3 Classification of Plasmids

Plasmids can be classified differently based on their conformation, copy number and ability to transfer.

11.3.1 Conformation/forms of plasmids

Plasmids can exist in four forms as shown in Fig. 11.2.

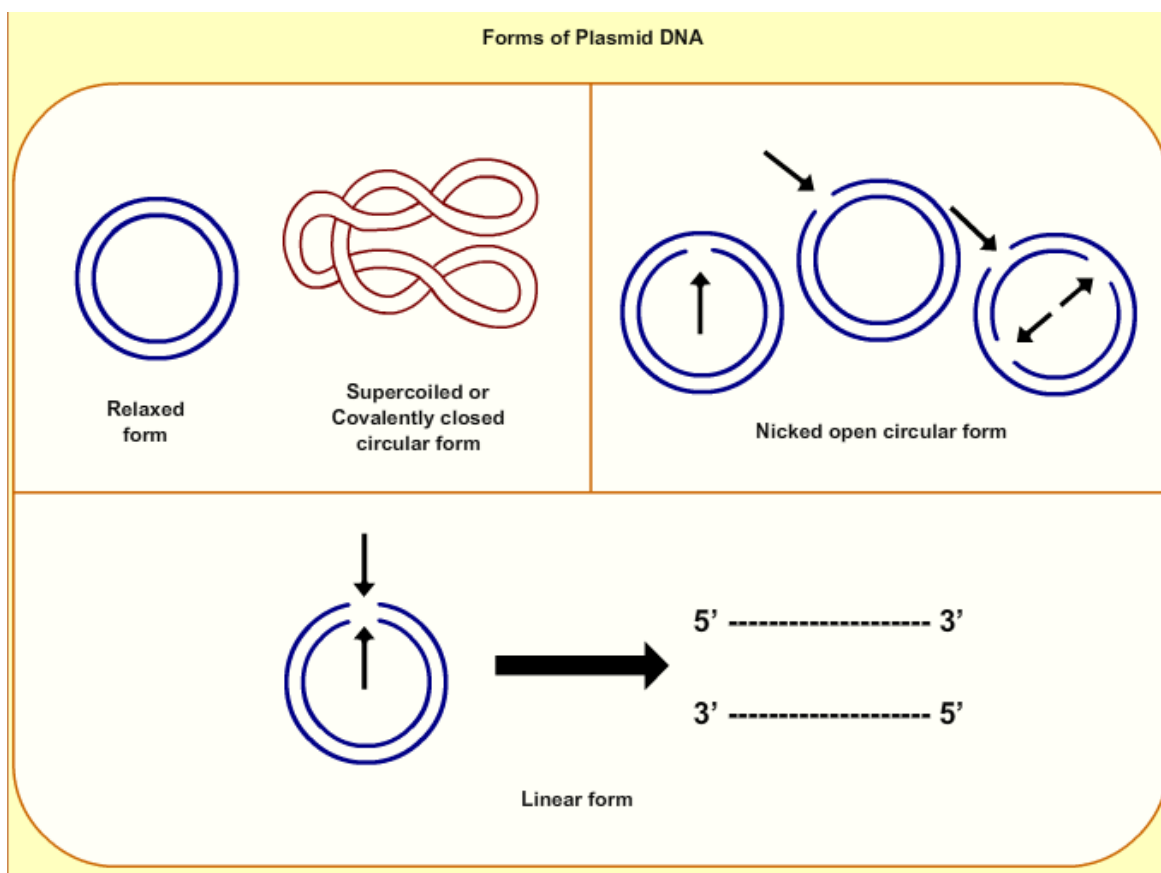


Fig. 11.2 Forms of Plasmid DNA

- i) Relaxed form – In this state, plasmid does not possess super coils and both the strands remain intact without any stress / torsion
- ii) Super coiled form or covalently closed circular form (CCC form) – In this form, plasmid is twisted enzymatically and is highly compact
- iii) Nicked open circular form – the form of plasmid in which one of the two strands is nicked or cut as a result of which it loses super coiled configuration and remain in circular form
- iv) Linear form – As both the strands of DNA are cut at the same site, the plasmid DNA becomes linear.

Out of the aforesaid forms, super coiled or CCC form is the most prevalent form that occurs in the bacterial host under natural conditions.

11.3.2 Based on copy number

Based on the copy number, plasmids are classified as

11.3.2.1 Stringent plasmids

Stringent plasmids are low copy number plasmids of relatively larger size and exist in 1-3 per cell. These plasmids replicate along with the main bacterial chromosome.

11.3.2.2 Relaxed plasmids

These are high copy number plasmids of relatively small size that can exist in > 25 copies per cell. These are the preferred type of vectors used extensively in molecular cloning and recombinant DNA experiments where high level of expression of the recombinant protein is required for commercial application.

11.3.3 Based on ability to transfer

11.3.3.1 Conjugative plasmids

The conjugative plasmids contain 'tra' genes (transfer genes) as well as 'mob' genes (mobility genes) which enable them to be transferred to other bacteria through conjugation.

11.3.3.2 Non-conjugative plasmids

The non-conjugative plasmids lack the ability to be transferred to other bacteria as they do not possess 'tra' genes. However, they can be transferred with the help of other conjugative plasmids if they harbor 'mob' gene.

11.4 Properties of Plasmid Cloning Vectors

Artificially constructed plasmids used for cloning of DNA / genes are called Plasmid Cloning Vectors e.g. pBR322 and its derivatives which were the first plasmids to be constructed and used in Molecular Biology. With the advancements in molecular biology, many more efficient plasmid vectors are now available commercially and used world wide for cloning and expression of heterologous proteins in prokaryotic and eukaryotic host systems. Some of the examples are pBR322, pUC18/19, pBluescript KS+ / SK+, pGEM series etc. (Fig. 11.3)

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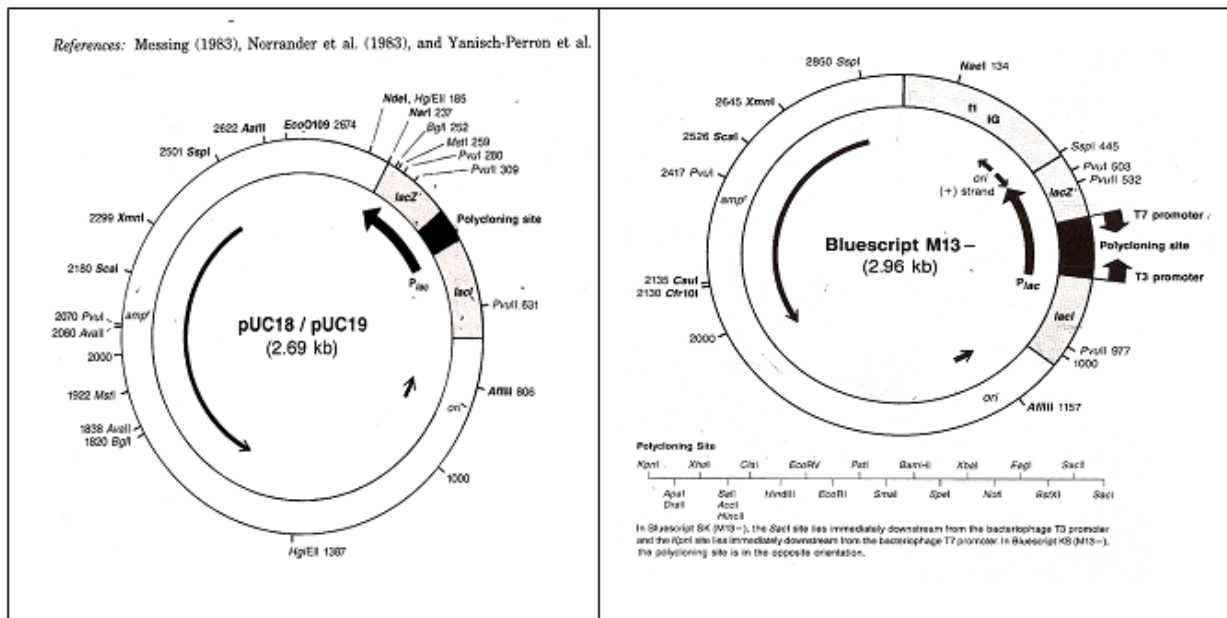
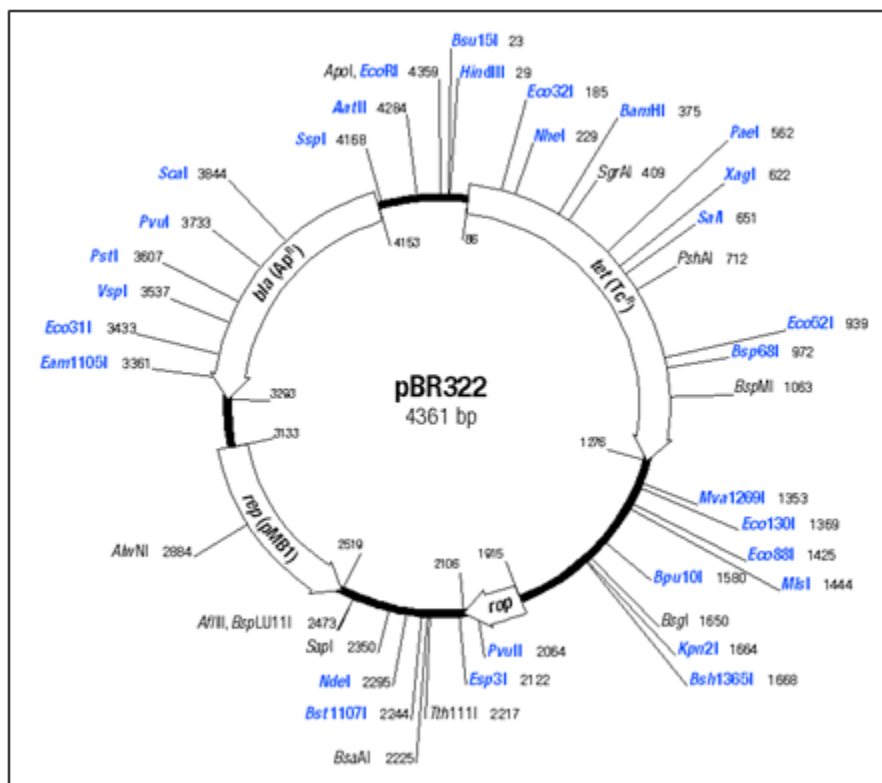


Fig. 11.3 Genetic map of pBR322, pUC18/19 and pBluescript

The plasmid cloning vectors possess the following properties as shown in the Fig. 11.4.

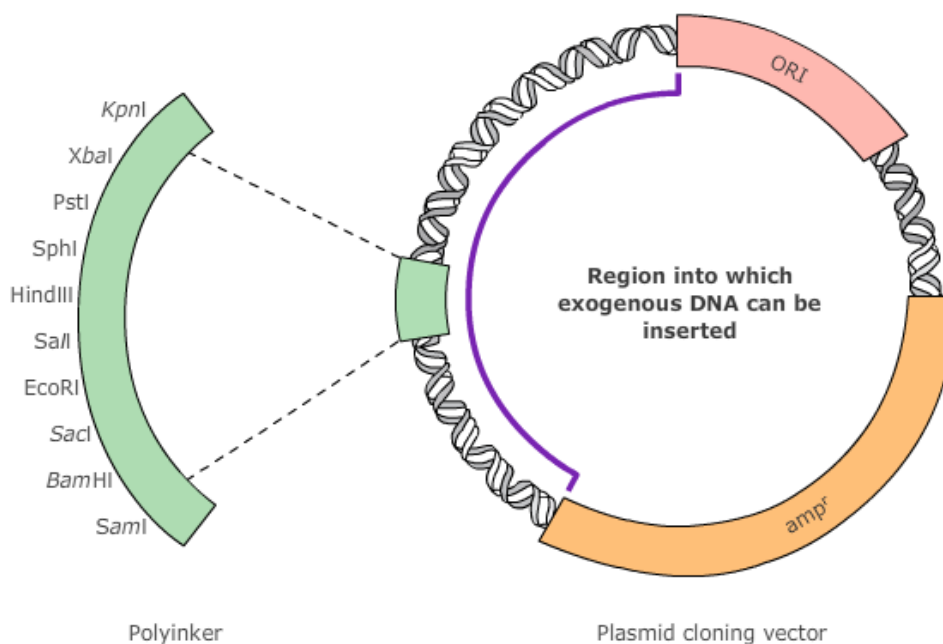


Fig. 11.4 Plasmid cloning vector

11.4.1 Origin of replication (Ori site)

Plasmid vectors contain sequences that permit propagation of vectors in bacteria or yeast i.e origin of replication. Origin of replication helps in replication of DNA and is recognized by DNA polymerase for plasmid replication.

11.4.2 Multiple cloning or Poly cloning site (MCS or PCS)

Plasmid vectors must possess cloning site to insert foreign DNA. Most of the commercially available vectors are designed in such a way that they have a multiple cloning site or poly cloning site by introducing a large number of restriction sites for different restriction endonucleases to widen their cloning prospects. However, restriction enzyme sites present in MCS /PCS should not be present anywhere else in the rest of the plasmid sequence. Genes of interest to be cloned or expressed are introduced into the cloning vector at restriction enzyme sites by using MCS /PCS.

11.4.3 Selection marker

Plasmid vectors possess selection markers e.g. antibiotic resistance markers (amp^r, tetr, chl^r) or histochemical markers e.g. lacZ – such that selection of recombinants is based on insertional inactivation (selection marker i.e. antibiotic resistance gets inactivated when a foreign gene is inserted) or alpha complementation such as using lacZ gene for selection of recombinant clones based on blue white selection.

11.4.3.1 Inactivation of Antibiotic resistance

The incorporation of foreign DNA fragment into the antibiotic resistance gene resulting into inactivation

of that gene is called insertional inactivation. Insertional inactivation strategy is based on using two antibiotic resistance marker genes in the vector (e.g. ampicillin and tetracycline), one of which is inactivated due to insertion of foreign gene and the other remains intact and thus helps in the selection of recombinants as has been shown in Fig. 11.5.

Insertional inactivation of antibiotic resistance gene

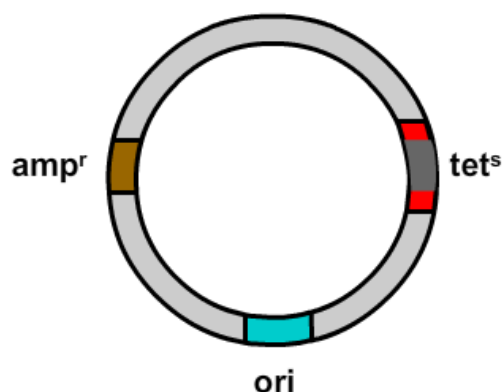


Fig. 11.5 Insertional inactivation of antibiotic resistance gene

11.4.3.2 Alpha complementation

Alpha complementation is a commonly used process wherein alpha portion of '*lacZ*' gene (that encodes β -galactosidase) is incorporated into the vector and *E. coli* host contains ω portion of '*lacZ*' gene. Neither of them alone is able to express functional β -galactosidase. However, when both the fragments are present simultaneously in the host, they complement each other and produce a functional β -galactosidase. The insertion of foreign gene at the multiple cloning site located in '*lacZ*' region of the vector results into inactivation of alpha-complementation by disrupting the open reading frame of '*lacZ*' gene. This criteria forms the basis of blue white screening for selection of recombinant clones by adding substrate X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactoside) into the medium. If '*lacZ*' gene is not inactivated, the colonies give blue colour otherwise white colour due to insertion of the foreign DNA (insert). This alpha complementation phenomenon has been illustrated in Fig. 11.6. IPTG (Iso-propyl-thio- β -galactoside) is also used as gratuitous inducer if the promoter is inducible.

Selection markers are also required for maintenance of plasmids in the bacterial cells e.g. plasmid containing ampicillin or tetracycline markers will be lost if the medium will be devoid of these antibiotics which are used as positive selective pressure for their maintenance.

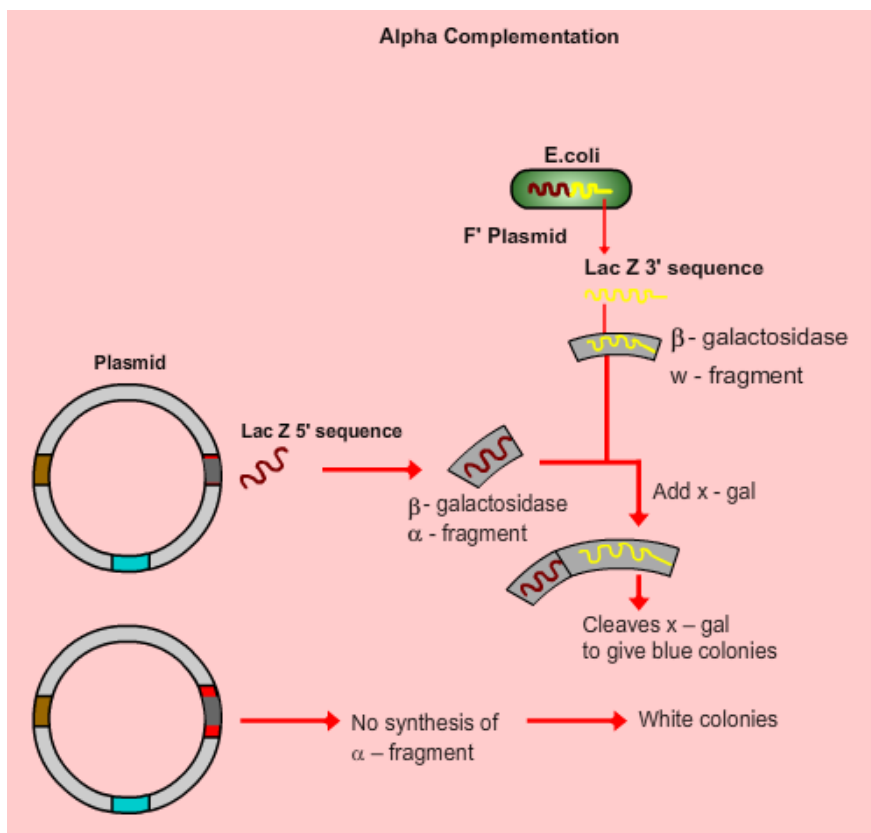


Fig. 11.6 Alpha Complementation

11.5 Plasmid as Cloning and Expression Vectors

Plasmids can be explored both as cloning and expression vectors as described below.

11.5.1 Cloning vectors

Vector that is used for reproducing the insert DNA For example pBR322 and pUC18/19. PCR products can be directly cloned into PCR cloning vectors known as TA cloning vectors e.g. pGEM-T, pGEM-TEasy, pDrive (Fig. 11.7). These vectors use the terminal transferase activity of some DNA polymerases. Taq polymerase adds additional A at 3' end to each end of the PCR product which is exploited for cloning of PCR products in pGEMT vector since T is incorporated at the 3' end of the vector. TA Cloning makes it possible to clone the PCR product into a cloning vector with 3'-T overhangs.

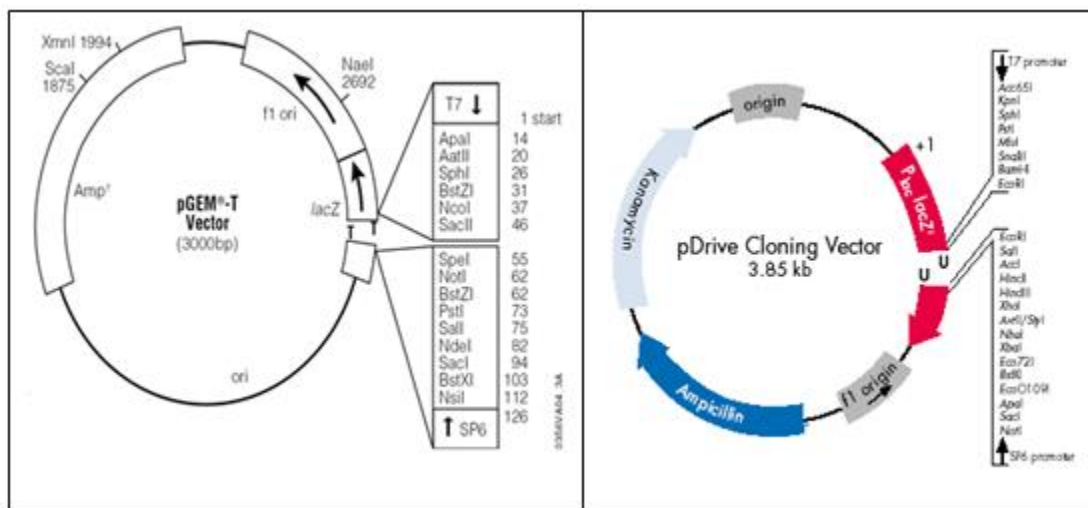


Fig. 11.7 Genetic map of pGEMT-easy and pdrive vectors

11.5.2 Expression vectors

Vector that allow the exogenous DNA to be inserted, stored, and expressed in an appropriate host are called expression vectors. Some of the expression vectors available commercially include pUC, pBluescript, pET series, pGEX series (Fig. 11.8) etc. These vectors also contain Ribosome binding sites (RBS), strong promoters (T3, T7, SP6) and transcription termination sequences along with fusion tags for protein purification as well as other components of cloning vectors. Most of the extensively used plasmid expression vectors use T7 promoter which is considered as a strong promoter for enhancing the expression of heterologous proteins for large scale production. Expression vectors are basic tools for the production of heterologous proteins of food, feed and pharmaceutical interest e.g. Insulin, Hepatitis B vaccine, Chymosin, Human lactoferrin and Phytase etc.

		2 nd Base							
		U		C		A		G	
1 st Base	U	UUU	Phenyl alanine	UCU	Serine	UAU	Tyrosine	UGU	Cysteine
		UUC		UCC		UAC		UGC	
		UUA	Leucine	UCA		UAA	Stop codon	UGA	Stop codon
		UUG		UCG		UAG	Stop codon	UGG	Tryptophan
	C	CUU	Leucine	CCU	Proline	CAU	Histidine	CGU	Arginine
		CUC		CCC		CAC		CGC	
		CUA		CCA		CAA	CGA		
		CUG		CCG		CAG	CGG		
	A	AUU	Isoleucine	ACU	Threonine	AAU	Asparagine	AGU	Serine
		AUC		ACC		AAC		AGC	
		AUA		ACA		AAA	AGA		
		AUG	Start codon & Methionine	ACG		AAG	Lysine	AGG	Arginine
	G	GUU	Valine	GCU	Alanine	GAU	Aspartic acid	GGU	Glycine
		GUC		GCC		GAC		GGC	
		GUA		GCA		GAA	GGA		
		GUG		GCG		GAG	GGG		

Fig. 11.8 Genetic map of expression vector pET

Module 3. Genetic engineering technology / recombinant DNA technology

Lesson 12

INTRODUCTION OF RECOMBINANT DNA INTO HOST CELLS

12.1 Introduction

Till now, we have already discussed restriction enzymes used for cutting/digesting of DNA fragments, vectors for acting as carriers of DNA fragments and insertion/ligation of DNA fragments into vector. The next step in this process is to introduce recombinant vectors carrying the cloned gene into appropriate host including bacteria and animals which will be the subject of this chapter.

12.2 Cloning in Bacteria

The most common model organism used as a potential host for cloning is *E. coli* since it is very convenient to use. Besides, a large number of *E. coli* vectors are available. Moreover, the recombinant DNA can be easily introduced into *E. coli* by transformation and a high level expression of recombinant proteins can be obtained with commercially available vectors. However, sometimes some important functional genes need to be expressed in other organisms like lactic acid bacteria including *Lactobacillus* spp., *Pseudomonas* spp., *Bacillus* spp. besides yeast and fungal spp. In this chapter, we will confine to cloning in *E. coli*. One of the pre-requisites is the need for a protocol to introduce the recombinant DNA in bacteria and we will concentrate on the methods used in *E. coli* since all the prokaryotic or eukaryotic genes need to be sub cloned into *E. coli* before they can be cloned and expressed in other hosts.

12.2.1 Introduction of recombinant DNA in *E. coli*

The recombinant/vector DNA can be introduced in *E. coli* using three gene transfer systems namely Transformation. However, before we proceed to methods of introduction of recombinant DNA into *E. coli*, we will discuss the gene transfer systems operating naturally in bacteria in brief so that the students can get adequate insight into these systems operating in bacteria.

12.2.1.1 Transformation

Transformation is defined as the process of taking up of naked DNA from the surroundings into the host cell (Fig. 12.1). The process of transformation was first demonstrated in *Streptococcus pneumoniae* by Frederick Griffith in 1928 who named the active principle as the transforming principle which converted rough cells into smooth cells in the presence of heat killed smooth cells of *S. pneumoniae* leading to death of mice. The experiment performed by Griffith is illustrated in Fig. 12.2. Later, Avery, MacCleod, McCarty in 1944 named this transforming principle as DNA. Transformation can be brought about by the following methods.

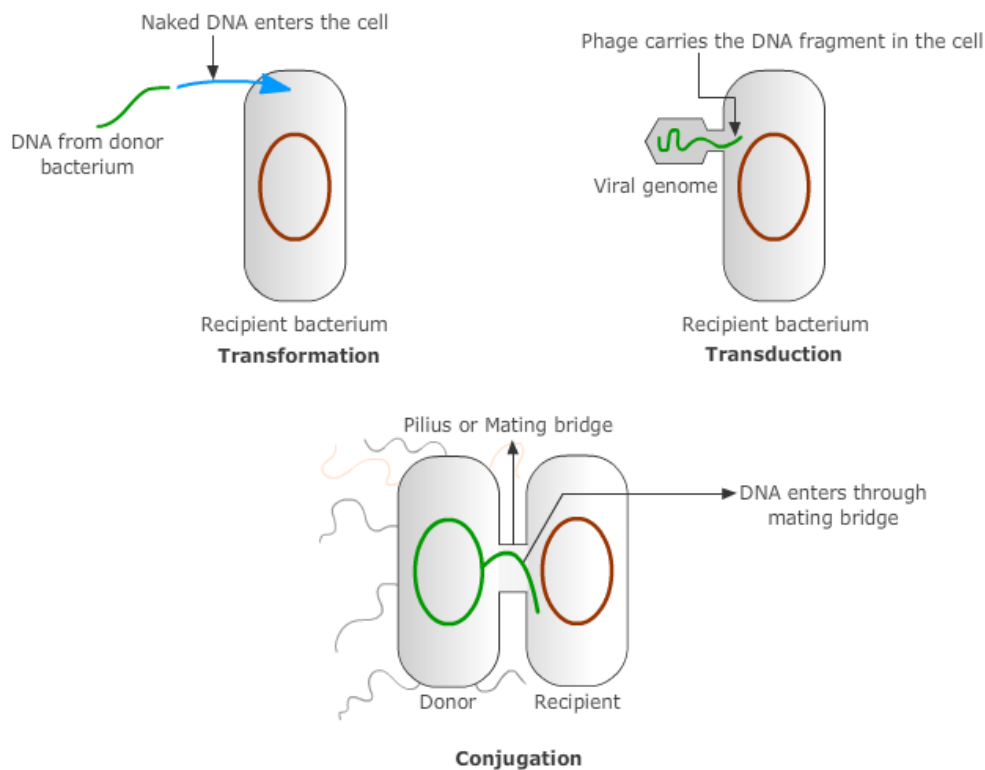


Fig. 12.1 Transformation, Transduction and Conjugation

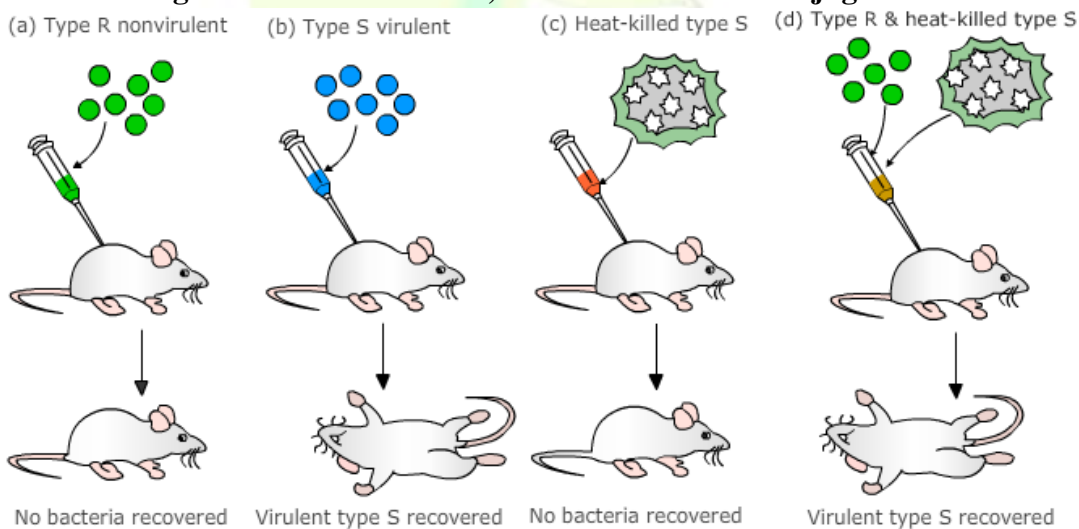


Fig. 12.2 Experiment of Griffith

1. Transformation by Natural competence
2. Transformation by Induced competence
3. Protoplast Transformation and Protoplast Fusion
4. Electroporation

1. Transformation by Natural competence

Bacteria belonging to *Pneumococcus*, *Bacillus* and *Haemophilus* spp. etc. are naturally competent and can take up naked DNA.

2. Transformation by Induced competence

Calcium chloride induced transformation

Calcium chloride induced transformation involves making the *E. coli* cells competent to take up the plasmid DNA. Since DNA is a very hydrophilic molecule, it won't normally pass through a bacterial cell's membrane. In order to make bacteria take up the plasmid, they must first be made "competent" to take up DNA. This involves addition of calcium chloride to the cell suspension which enhances the binding of plasmid DNA to lipopolysaccharide (LPS). Positively charged calcium ions attract both the negatively charged DNA and the negatively charged groups in the LPS inner core. The plasmid DNA can then pass into the cell upon heat shock, wherein cells are cooled to a low temperature (4°C) and then heated to a high temperature (42°C) for a short time. This causes the bacteria to take up the DNA. The cells are then plated on LB agar (Luria Bertani) or SOC (Super Optimal broth with catabolite repression is SOB with added glucose) medium containing appropriate antibiotics after incubation at 37°C for one hr. to enable the transferred genes to express in *E. coli* on recovery of cells. It is one of the most common method used for transformation in *E. coli* since this is simple and competent cells can be prepared in laboratory and are also commercially available from a number of suppliers. Transformation efficiencies of 10^8 to 10^9 transformants/ μg of DNA can easily be achieved. Generally, for the construction of genomic libraries, transformation efficiencies of 10^{11} are desirable which also can be obtained with good competent cells.

Electroporation

Electroporation is yet another versatile method of transformation of bacteria including Gram negative and Gram positive bacteria, yeasts like *Saccharomyces cerevisiae*, *Pichia pastoris* and even the mammalian cells. Bacterial cells mixed with plasmid DNA are subjected to a brief pulse of high voltage electricity using electroporator (Bio-Rad, BTX, Fig. 12.7). The membrane of the cells exposed to high intensity electric pulse is temporarily destabilized in specific regions of cell. Cells become permeable and DNA enters the cells. Cells are then incubated for one hour and plated on LB agar with selection marker as described above. High efficiencies of transformation i.e. 10^{11} – 10^{12} transformants/mg of DNA are usually obtained.



Fig 12.2a BTX Electroporation system

Selection of recombinants

Selection of recombinants is made on the basis of Inactivation of Antibiotic resistance and Alpha complementation which have been explained in the previous chapter on Vectors.

3. Protoplast transformation/fusion

Protoplast transformation/fusion is also used in bacteria such as *Bacillus* spp., *Lactococcus* spp. and *Lactobacillus* spp. etc. during their genetic manipulation. Initially, protoplasts are generated by enzymatic removal of cell walls of bacteria which are then transformed using polyethylene glycol (PEG) along with the DNA. PEG is then removed and protoplasts are allowed to regenerate on an osmotically stabilised medium. The process wherein protoplasts from two different cells fuse together in PEG is called protoplast fusion.

12.2.1.2 Transduction

Transduction is the process of transfer of bacterial genes into a host bacterium with the help/aid of phages. Transduction is also known as phage mediated genetic transfer (Fig. 12.1). Transduction is of two types:

a) Generalized transduction

When a phage particle attacks a bacterial cell and during its assembly, fragments of host DNA are packaged into phage particles in place of its own DNA. When such a phage/virus particle infects a new host cell, DNA is injected into the host which can recombine with the host genome (Fig. 12.3).

b) Specialized transduction

A lysogenic phage undergoes recombination with the host genome at a particular site e.g. gal and bio as shown in Fig. 12.4a and later when it is excised to become an independent phage genome, it carries one or more host genes with it (Fig. 12.4b). These genes can be transduced into a new host cell which recombine into the genome of that cell.

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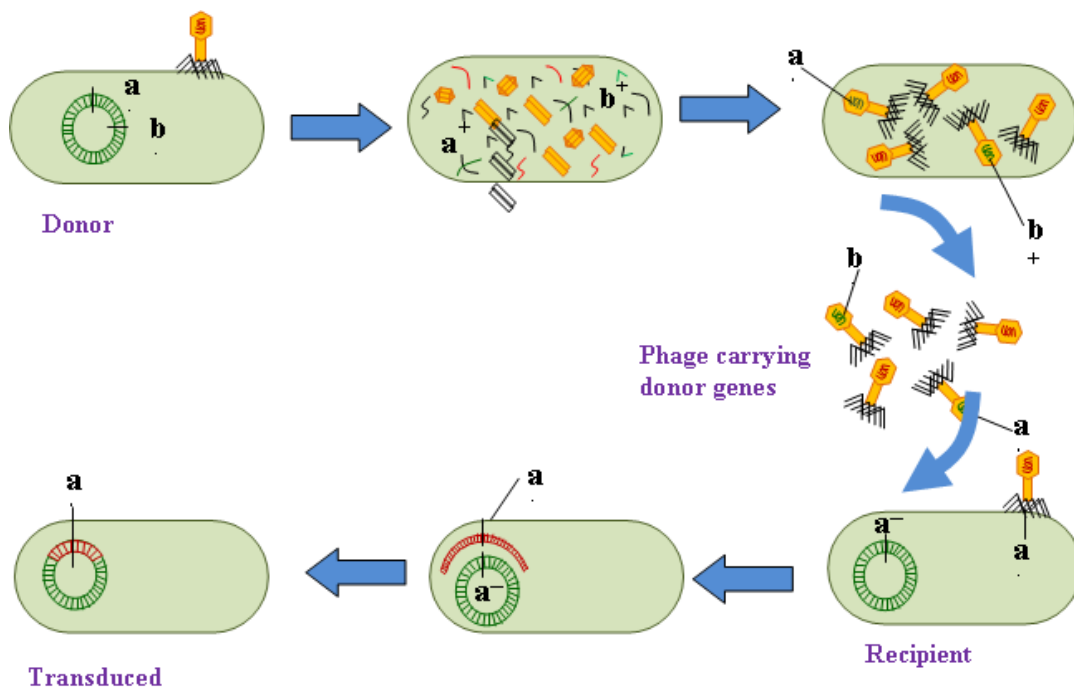


Fig. 12.3 Generalized transduction

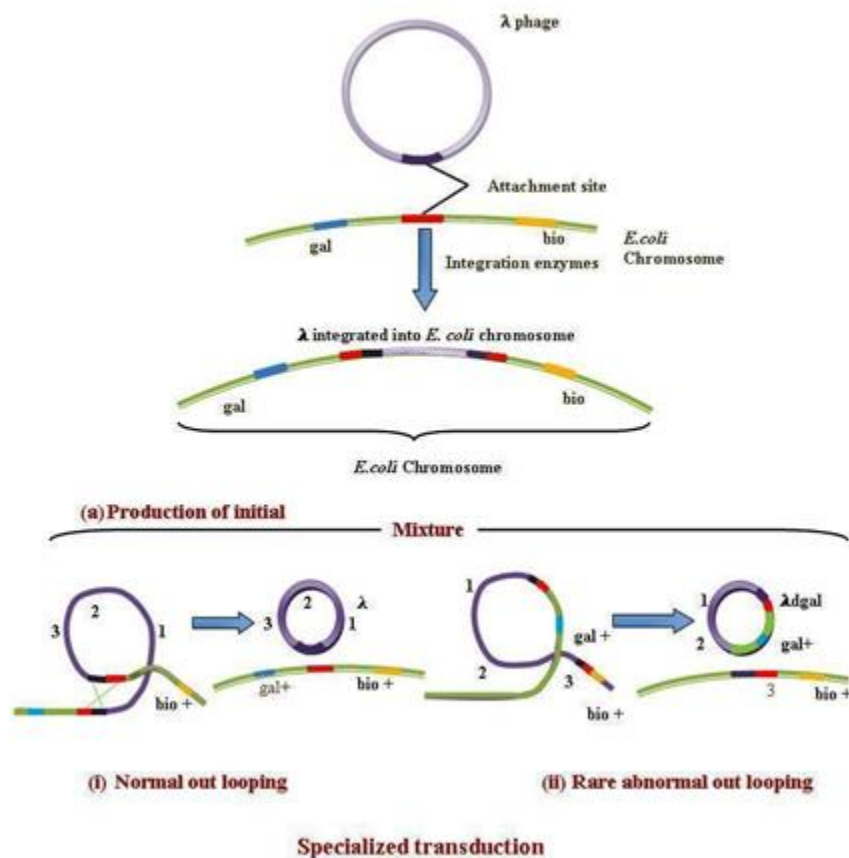


Fig. 12.4 a. A lysogenic phage with *gal* and *bio* genes b. phage after excision

12.2.1.3 Conjugation

Conjugation is defined as the process of transfer of genetic material from a donor cell to a recipient cell through a mating bridge (Fig. 12.1). It was discovered by Lederberg and Tatum in 1946 who showed that two different strains of bacteria with different growth requirements could exchange genes (Fig. 12.5). They surmised that the bacterial cells must interact with each other in order to transfer the genetic material and the process is now known as sexual conjugation by direct contact. A segment (rarely all) of the donor's chromosome recombines with the homologous recipient chromosome. Recipients containing donor DNA are called transconjugants. Bernard Davis demonstrated that the physical contact is essential for transfer of genetic material (Fig. 12.6). William Hayes (1953) demonstrated that genetic exchange in *E. coli* occurs in only one direction i.e. unidirectional and is mediated by sex factor F. Donor cell is F+ and the recipient cell is F- Demonstration of conjugal transfer of marker genes form donor to recepient strains of *E. coli* when grown together in a mixture and plated on minimal medium.

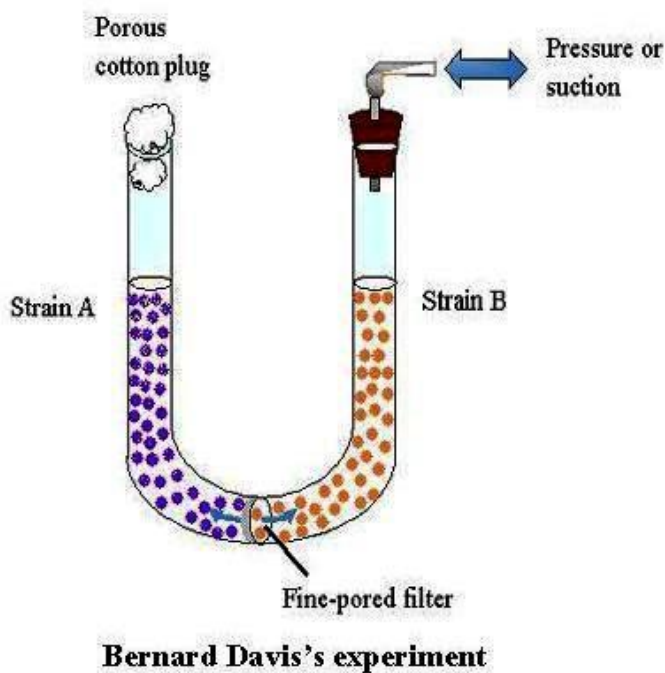


Fig. 12.6. Bernard and Davis experiment

Fig. 12.6 Benard Davis's U tube experiment to demonstrate transfer of F factor from donor to recepient strains of *E. coli* by conjugation

The transferred DNA then gets integrated into the host cell through a process called recombination.

12.2.2 What is recombination?

Genetic recombination is defined as the process of transfer of DNA from one organism to another. The transferred donor DNA may then be integrated into the recipient's DNA by various mechanisms. Now, we will discuss here the techniques used for introduction of recombinant DNA into *E.coli* and other bacteria. One of the most commonly used technique is transformation.

12.3 Cloning in Animal Cells/Cultured Cells

Cloning of DNA into animal cells has been practiced since long. Animal cells have the ability to bring about post translational modifications which can not be performed by prokaryotic expression systems. Gene transfer in cultured cells can be achieved by direct DNA transfer (physical introduction of DNA in cells) e.g. microinjection whereas for cells *in vivo* transfer is by bombardment with tiny DNA coated metal particles i.e. transfection involving physical and chemical techniques facilitating transfer of DNA (similar to transformation in bacteria) and viral mediated transfer.

12.4 Cloning in Animals

Gene cloning techniques in animals can be used to produce transgenic animals. The term transgenic animal refers to an animal in which there has been a deliberate modification of the genome. Foreign / exogenous / target DNA is introduced into the animal, using recombinant DNA technology, and then must be transmitted through the germ line so that every cell, including germ cells of the animal contain the same modified genetic material. Genetically modified mammals have been used as bioreactors for the production of recombinant proteins in their milk by introduction of transgene in mammary gland of the animal. The method of cloning i.e. nuclear cloning will be discussed in module 4.0.

The term transgenic was first used by Gordon and Ruddle in 1981 and since then there has been rapid development in the use of genetically engineered animals. Mice have become the main model species used in the field of transgenics because of their small size, low cost housing in comparison to that of larger mammals / invertebrates, their short generation time and their fairly well defined genetics.

The three principal methods used for the creation of transgenic animals are

- DNA microinjection
- Embryonic stem cell-mediated gene transfer
- Retrovirus-mediated gene transfer

The improvement in livestock through transgenesis has led to very encouraging results including enhanced milk production in cattle, increased growth rate of livestock and fish as well as large scale production of valuable proteins in milk, urine and blood of livestock enabling the use of transgenic animals as 'bioreactors' for 'molecular farming / pharming' as well as improvement of wool production through production of transgenic sheep.



Module 3. Genetic engineering technology / recombinant DNA technology

**Lesson 13
DNA SEQUENCING**

13.1 Definition

DNA sequencing is a process that deciphers the arrangement of nucleotides (adenine, guanine, cytosine and thymine) in a strand of DNA. The order of nucleotides provides the understanding of gene function. Hence, the application of DNA sequencing in medicine, agriculture, food and nutrition, and all branches of life sciences has become very important.

13.2 History

Maxam and Gilbert's method was the first method of DNA sequencing developed in 1976-1977 by Allan Maxam and Walter Gilbert. The method involved chemical modification of DNA and subsequent cleavage at specific bases. The method was laborious and time consuming. Subsequent to this, in 1955, Frederick Sanger determined the first complete amino acid sequence of the two polypeptide chains of bovine insulin and was awarded Noble Prize. In 1977, Sanger developed the "dideoxy chain termination method" for sequencing DNA and he again was awarded a second Nobel Prize in 1980. Sanger's method based on dideoxy chain termination subsequently became the method of choice in almost all the laboratories world wide. The protocol requires a primer to initiate DNA synthesis, DNA polymerase that lacks proof reading activity, deoxynucleotidephosphates (dNTPS i.e dATP, dTTP, dCTP and dGTP) besides dideoxynucleotidephosphates (ddNTPs, lack 3' hydroxyl group which leads to chain termination) and DNA template. The ddNTPs when incorporated in the newly synthesized DNA strand terminate the reaction. These ddNTPs are either radioactively labeled or fluorescently labeled. The steps involved in dideoxy DNA sequencing reaction have been shown in Fig. 13.1. The reaction is divided into four separate tubes each comprising of template, all the four dNTPs as well as one of the ddNTP. Since ddNTPs are unable to form phosphodiester bond between two nucleotides as they lack 3' OH group, they lead to chain termination and hence results into DNA fragments of varied lengths which are run on a polyacrylamide urea gel. Urea minimizes the secondary structures. In original Sanger's method, dNTPs were used to be labeled with ³²P and then after gel electrophoresis, auto-radiography was carried out. However, now there are several modifications to Sanger's method using fluorescent dyes which are read at their respective absorption spectra.

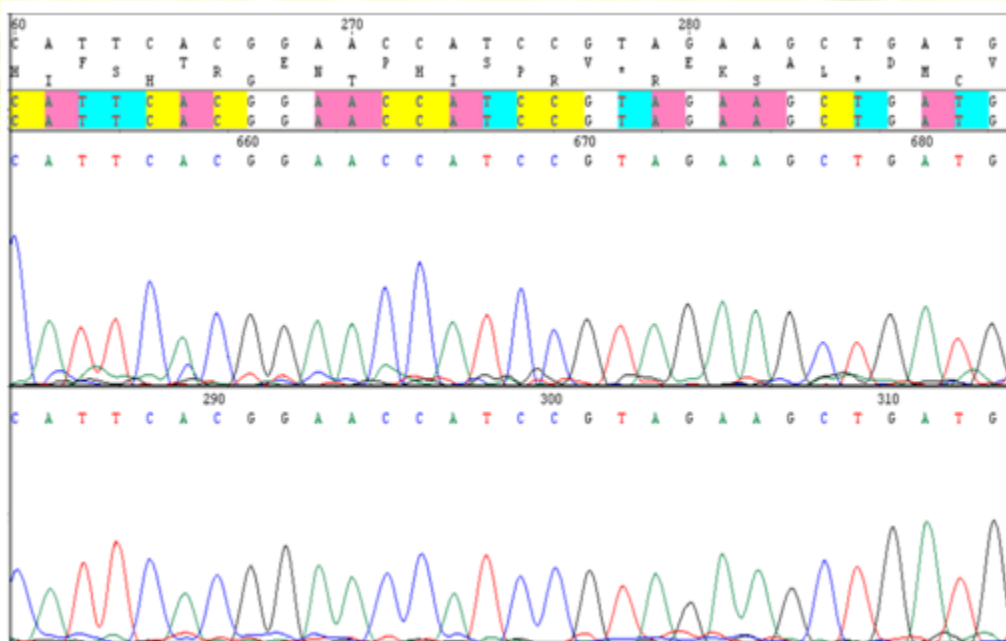
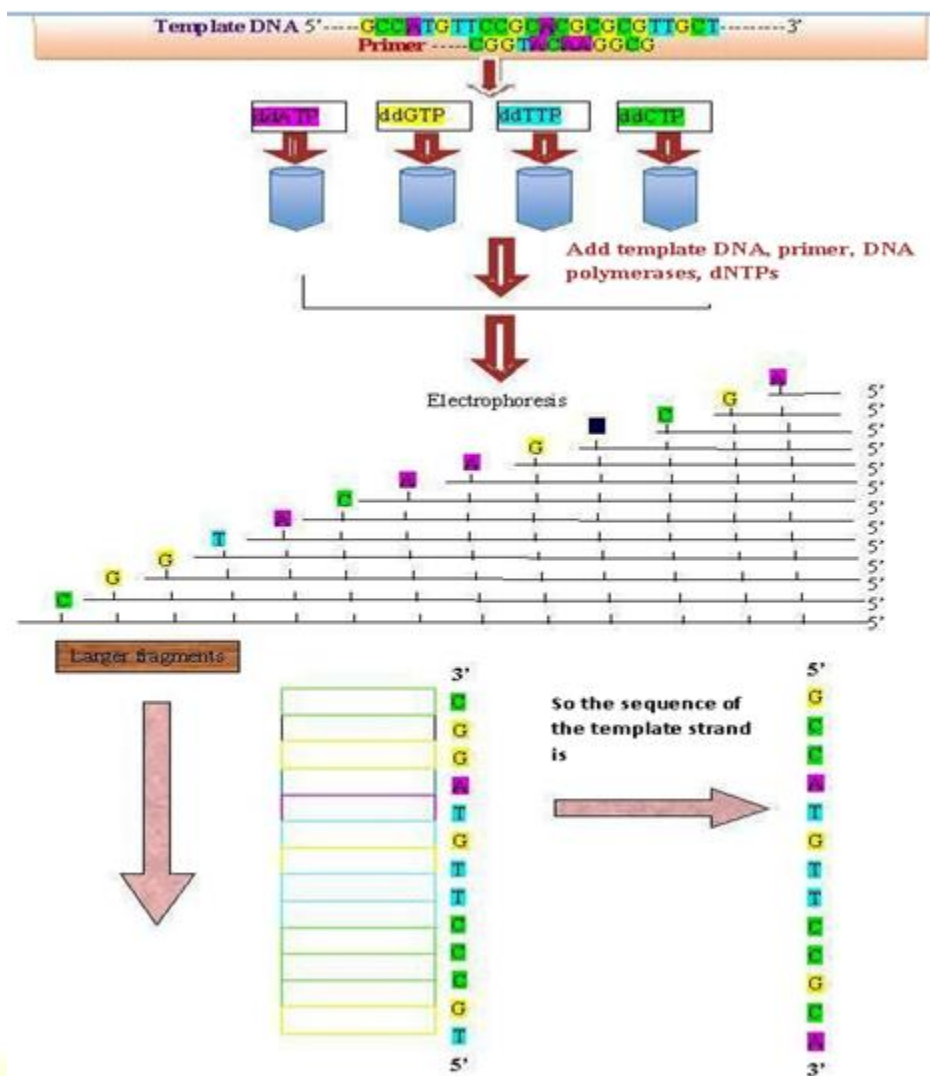


Fig. 13.1 The DNA sequencing reaction

However, Sanger's sequencing method had certain limitations which include i) application of toxic chemicals ii) use of radioactivity iii) difficulty in reading auto-radiographs iv) tedious method prone to errors and v) lack of automation.

13.3 Advancements in Sequencing Techniques

The Sanger's method now has been improved considerably in terms of use of fluorescent labeling, DNA polymerase enzymes (thermostable polymerases) to improve overall read length and accuracy. All these changes including ready to use kits containing all the requisite reagents led to the development of automated sequencing. Dye terminator approach using cycle sequencing by PCR is mainly used in automated sequencing. It involves four dideoxynucleotide chain terminators each labeled with a different fluorescent dye which emits light at different wavelengths. The incorporation of fluorescently tagged ddNTP leads to termination of synthesis alongwith attachment of fluorescent tag. Cycle sequencing that involves the use of fluorescent dye-tagged terminators or primers is the standard protocol used in automated sequencing equipments. The fluorescent tags are read by lasers.

Several automated high through put sequencers are now available from different companies e.g. Applied Biosystems (ABI), GE Healthcare Life Sciences etc. as shown in the following Figs. 13.2 and 13.3.



Fig. 13.2 MegaBACE 4000 capillary array GE Healthcare Life Sciences



Fig. 13.3 Applied Biosystems ABI Sequencer

These sequencers have the capacity of sequencing 384 samples in a single run and upto 24 runs in a day. Automated DNA sequencers generate excellent data and eliminates any errors which can arise by reading manually as was initially done in Sanger's original method. Automated sequencers involve capillary electrophoresis for separation of bands and detection and recording of fluorescence. As DNA fragments pass the detector, a signal is generated and the nucleotide is identified based on the fluorescent base.

13.4 Next Generation or Second Generation Sequencing Techniques (High through put sequencing)

Next generation sequencing techniques make use of several platforms which include Pyrosequencing (454 pyrosequencer from Roche Applied Sciences, Basel); Solexa (Illumina, San Diego); SOLiD (Applied Biosystems, CA, USA) and the HeliScope Single molecule sequencer (Helicos, Cambridge, MA, USA) as shown below. Next is the arrival of third-generation systems. Recently, Indian scientists Vinod Scaria and Sridhar Sivasubbu — the two young scientists from the Council of Scientific and Industrial Research (CSIR) have successfully sequenced the human genome. Earlier, they decoded the genome of a zebrafish in April, 2009.



Fig. 13.4 (a) Genetic Analyzer IIX Illumina FLX Roche (Solexa)



Fig. 13.4 (b) Genome Sequencer (454 pyrosequencer)



Fig 13.4 (c) 5500 SOLiD system (Applied Biosystems)

13.5 Applications of DNA Sequencing

1. Sequencing of cloned genes and PCR products
2. Sequencing of promoter and regulatory regions
3. Sequencing of complete genomes and resequencing
4. Sequencing of short regions to identify mutations or SNPs (single nucleotide polymorphism)
5. High-throughput DNA sequencing technologies have opened the way for large-scale sequencing of humans and other model organisms revealing a wealth of information on genomic variability.
6. High throughput sequencing technologies are being extensively used for *de novo* sequencing, re-sequencing of whole genomes and target DNA regions, metagenomics, RNA analysis, ultra-deep amplicon sequencing.
7. Assembly of genomes *de novo*.



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Module 3. Genetic engineering technology / recombinant DNA technology

Lesson 14
PCR AND REAL TIME PCR

14.1 Polymerase Chain Reaction (PCR)

PCR is one of the most powerful tools in Molecular Biology. It is defined as a technique used for *in vitro* amplification of specific target DNA sequence using a set of specific and unique primers for primer extension of complementary strands by Taq Polymerase. PCR acts like a ‘copying or xeroxing machine’ creating a large number of duplicated copies of DNA molecules from a minute amount of starting material. It is a rapid, inexpensive, extraordinarily powerful and most commonly used versatile technique in all day to day lab experiments. Kary Mullis from Cetus Corporation was awarded Nobel Prize in chemistry in 1993 for his invention of PCR in 1983.

PCR reaction consists of the following components as depicted in Fig. 14.1.

The basic protocol—what’s in the tube

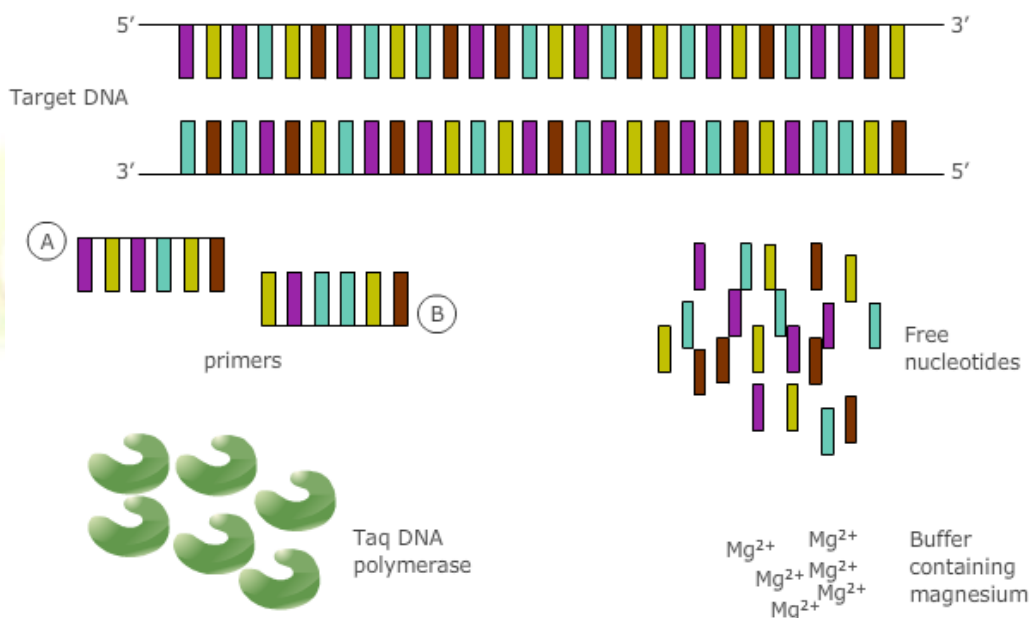


Fig. 14.1 The basic protocol - what is required for a PCR reaction

These are briefly described as follows:

14.1.1 Template / Target DNA

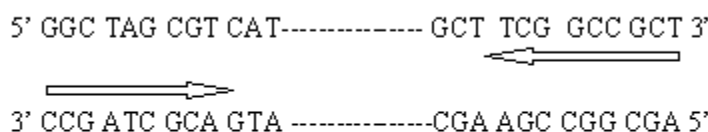
Template DNA is the resource material for the amplification of the target gene. It is extracted by following different DNA extraction protocols. It could be either genomic DNA, plasmid DNA or cDNA prepared from RNA from any source like microbes, viruses, animals and plants. The beauty of this technique is such that because of its high specificity, it can pick up the target amongst the large background of the non-specific DNA also and hence template DNA need not be absolutely pure for PCR amplification.

14.1.2 Deoxynucleotide triphosphates (dNTPs)

The deoxynucleotide triphosphates (dNTPs) are the nucleotide building blocks (adenine, guanine, cytosine and thymine) which need to be present for incorporation during amplification of the target gene.

14.1.3 Primers

A pair of primers (Forward and Reverse) is required for amplification of the target DNA from both the strands i.e. 5'---3' and 3'----5'. In brief, primer is a short single-stranded oligonucleotide sequence of DNA that is required to initiate the synthesis of new strand of DNA in a polymerase chain reaction. Generally, these are about 20 to 22 bp in length for general PCR reaction but may vary according to the designed experiment (upto even 50 bp). The primers are identical to the 5' ends of sense and antisense strands of DNA. These are designed to flank the terminal regions of gene to be amplified as shown below also:



Forward primer : 5' GGC TAG CGT CAT ---- 3'

Reverse primer : 5' AGC GGC CGA AGC -----3'

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Melting temperature (T_m) is defined as the minimal temperature at which two strands separate as a result of denaturation

Melting and annealing temperatures are the two most important parameters which need to be looked into while designing a primer for their optimal usage.

Melting and annealing temperatures of primers can be determined by the following formulae :

$$T_m (\text{°C}) = 4(G + C) + 2(A + T)$$

$$\text{Annealing temperature i.e. } T_{\text{anneal}} = T_m - 4\text{°C}$$

Annealing temperature (T_{anneal}) is the temperature at which primers anneal optimally to the template DNA.

14.1.4 Taq DNA polymerase

Most of the enzymes/proteins get inactivated at higher temperature but Taq DNA polymerase withstands high temperature of denaturation. Taq DNA polymerase was isolated from a thermophilic organism *Thermus aquaticus*, which normally lives in hot springs at temperatures close to 100°C. Hence, this enzyme can remain stable at high temperatures as close as 100°C i.e. it is stable under the extreme temperature conditions of PCR and hence does not need to be supplied afresh in the PCR reaction mix. Taq DNA polymerase catalyses DNA polymerization i.e. incorporates / adds nucleotides during the synthesis of new strands of DNA during extension at 72°C. However, Taq DNA polymerase requires the use of a buffer containing MgCl₂ for its optimal functionality. Similar enzymes have been isolated from other thermophilic bacteria which even possess proof reading activity (Pfu polymerase) and are used in cloning of genes to avoid any errors. Many companies are now selling recombinant Taq polymerase which is very cheap and considerably economical.

Primers



- Usually about 20 nucleotides in length
- Designed to flank the region to be amplified
- Melting point determined by G-C and A-T content
 - $T_m = 4\text{°C}(G+C) + 2\text{°C}(A+T)$
 - Ex: a primer with 10 G/C and 10 A/T would have a T_m of 60°C $\rightarrow 4(10) + 2(10) = 60\text{°C}$

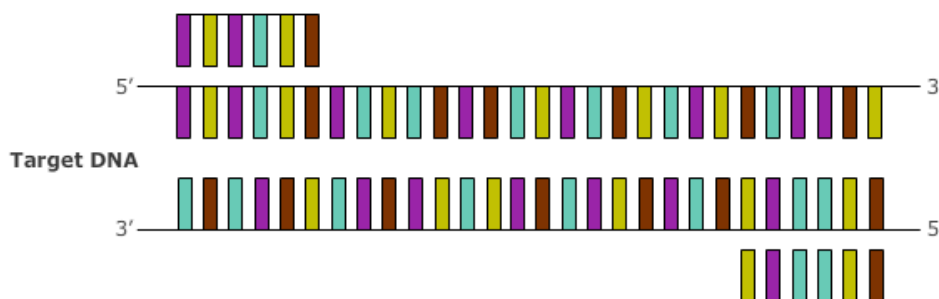


Fig. 14.2 Primers

14.1.5 Thermal cycler

The Thermal cycler also known as PCR machine or DNA amplifier is an equipment which is used for amplification of a gene using polymerase chain reaction. These thermal cyclers are available from a number of companies viz. Applied Biosystems, Bio-Rad, Eppendorf, Perkin Elmer, MJ Research Inc. etc.

The machine consists of a thermal block whose temperature is raised or lowered according to the programmed steps during cycling parameters. These machines also come with heated lid to prevent condensation of water from reaction mix. Some of the machines have silver blocks which can achieve fast temperature changes and uniform temperature through out the block.

14.1.6 Cycling parameters

PCR cycling parameters involve several cycles (25 – 40) of denaturation, annealing and extension in order to amplify a desired gene.

14.1.6.1 Denaturation

Template DNA is heated at high temperatures (94°C) at which the duplex DNA gets depolymerized to single strands. These strands then become accessible to primers annealing for synthesis of new strands during amplification.

14.1.6.2 Annealing

The reaction mixture is cooled down to the specific temperature (~ 55°C) at which the primers anneal to the complementary regions in the template DNA.

14.1.6.3 Extension

Taq DNA polymerase then starts adding nucleotides to the respective primer using information from the DNA template strand at 72°C.

These steps are followed again and again to generate multiple copies of the DNA template i.e amplified PCR product or amplicons (Fig. 14.3).

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The basic protocol--extension

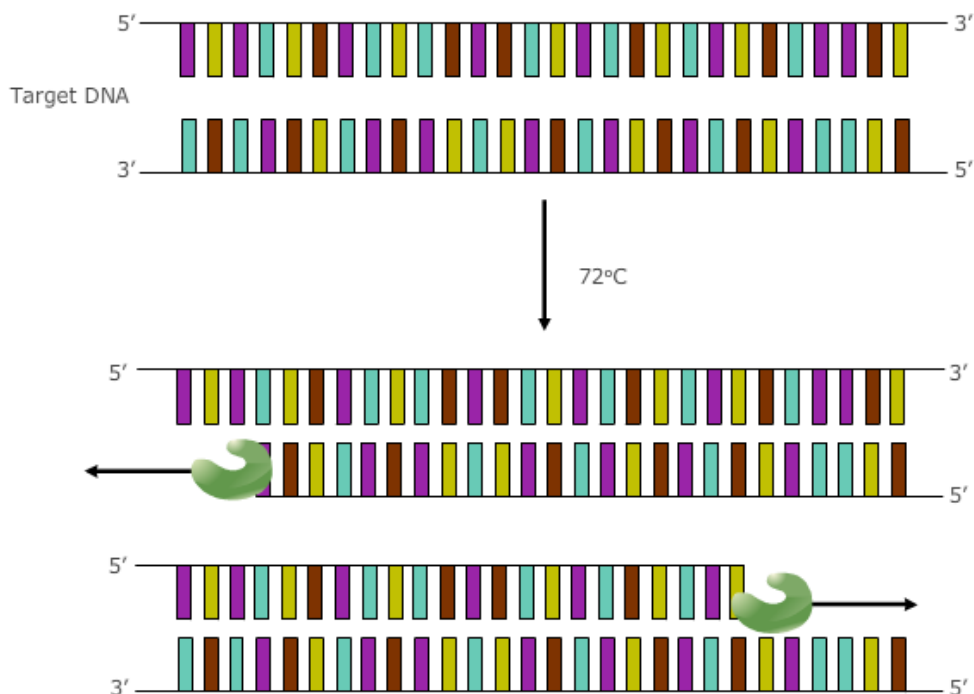


Fig. 14.3 The basic protocol of PCR reaction

Initially, an initial denaturation step at 94°C for 2-5 min. is given followed by 30-40 cycles of denaturation at 95°C/30sec to 1 min.; annealing at 45-65°C/30 sec to 1 min. and extension at 72°C for 30 sec to 2 min. depending on the length of fragment to be completed and final extension step at 72°C for 5-10 min. The amount of amplified product at the end of PCR cycles is $2^{n\# \text{ of cycles}}$ e.g. if we start with a single molecule of DNA, after 25 cycles, the amount will be 2^{25} i.e. 3.4×10^7 molecules of DNA.

14.2 Analysis of PCR products

The specificity of PCR products is ascertained by any of the following protocols:

14.2.1 Agarose gel electrophoresis

The PCR products are electrophoresed along with molecular size markers i.e. 100 bp, 250 bp, 500 or 1 kb ladder or any other desired size marker to check the specificity of the desired amplicon. The presence of a PCR product specific / unique for a particular target gene indicates the presence or detection of that gene in an organism e.g. specific sizes of 147 for *gapB*, 123 for *ldhD* etc. are detected as shown in the Fig. 14.4.



Fig. 14.4. Agarose gel electrophoresis of PCR products

14.2.2 Restriction analysis

The PCR products are digested with the restriction enzymes to yield specific fragments of desired length for confirmation of the amplified gene.

14.2.3 Southern hybridization

It involves the application of a radio-labeled or non-radio-labeled DNA probes (nucleotide sequence of upto 50 bases for the target gene) to check for the specificity of the PCR product.

14.3 Applications of PCR

PCR finds applications in almost all the branches of science and areas and in a variety of other fields such as

- Diagnostics

Detection of food borne pathogens from dairy foods, water and other food products as well as clinical samples

- Forensic Labs – paternity testing
- *In vitro* mutagenesis (Site directed mutagenesis)
- Molecular Evolution
- Sequencing
- Cloning of genes
- Monitor cancer therapy
- To detect mutations (genetic disorders)
- Metagenomics

14.4 Reverse Transcriptase –PCR (RT-PCR)

RT-PCR, the Reverse Transcriptase PCR involves two steps *viz.* Reverse Transcriptase step and PCR amplification. RNA is extracted and is reverse transcribed into cDNA (complementary DNA) using the enzyme reverse transcriptase and polyT oligo or random or sequence specific primers. The cDNA is then used as a template for PCR amplification using specific sets of primers for amplification of the desired gene of interest. RT-PCR is a sensitive technique for detection of low copy number of mRNA molecules (the functional part of DNA). Detection of mRNA is used for gene expression studies and cloning of eukaryotic genes which possess introns.

14.5 Real Time PCR or qRT-PCR (quantitative RT-PCR or RT-qPCR) or qPCR or RTi-PCR

Real Time PCR was first reported by Higuchi *et al* (1992). It is also known as kinetic PCR or qPCR. Real Time PCR is based on detection of fluorescent signal. The Real Time PCR machine incorporates an optical module to detect fluorescence. Furthermore, compared to PCR, it is quantitative i.e. copy number of the gene can be determined. RT-PCR monitors the amplicon in real time by making use of reporter and quencher dyes. The most common reporter dyes are FAM, TAMRA, TET, HEX, Cy5, Cy3 etc. and

Quencher dyes are TAMRA, DABCYL, BHQ-1 and BHQ-2 etc. The Real Time PCR machines available in market are from Applied Biosystems (ABI 7500, 7700, 9700 etc.); Bio-Rad (iCycler IQ); Stratagene (Mx 4000); Smartcycler II (Cepheid); Roche (Lightcycler 480) etc. All the machines incorporates a thermal cycler, a computer, optics for fluorescence excitation, data acquisition and analysis software. The most common Real Time PCR used all over the world are shown in the Fig. 14.5.

Reporter dyes also called fluorophores are the dyes which emit fluorescence when excited.

Real Time PCR works on two types of chemistries viz. non-specific and specific which are discussed in the following section:

Quencher dyes are the dyes which quench fluorescence before it is emitted.

Range of Real Time -PCR Instruments



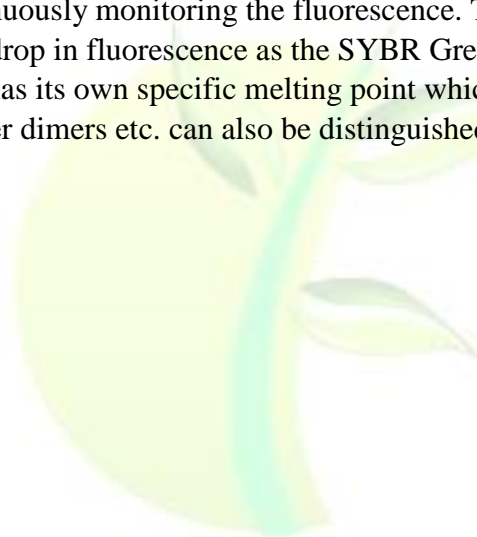
Fig. 14.5 Range of Real time – PCR Instruments

14.5.1 Non-specific chemistry

Non-specific chemistry involves using intercalating dyes which bind to all double stranded DNA produced during PCR reaction.

14.5.1.1 Intercalating dyes

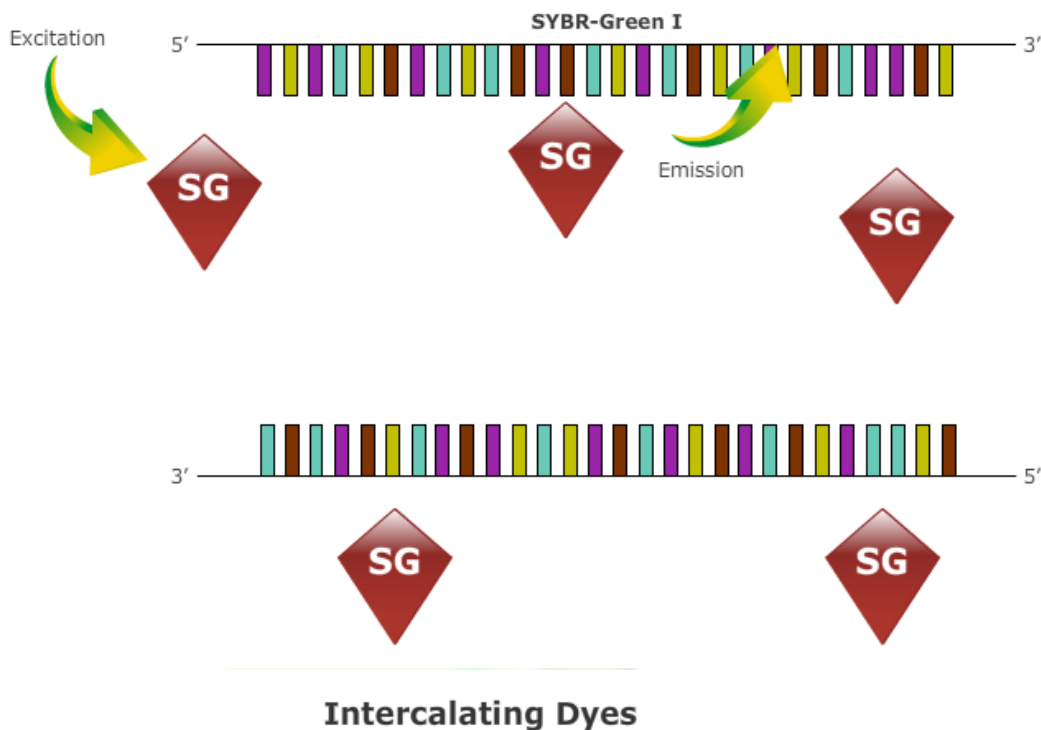
Intercalating dyes are the ones which bind to any double stranded DNA generated during any PCR or Real Time PCR reactions. These dyes fluoresce once bound to the DNA. The most common dye is Syber Green (SYBR Green) which gets excited at 497 nm and emits fluorescence at 520 nm. The specificity of products can be determined by melt curve / dissociation curve analysis. The reaction mix is slowly heated from 40 to 95°C while continuously monitoring the fluorescence. The point at which double stranded DNA melts is recorded as a drop in fluorescence as the SYBR Green dissociates from ds DNA molecules. Every target gene sequence has its own specific melting point which can help in distinguishing different targets in one reaction. Primer dimers etc. can also be distinguished. Fig. 14.6 gives an outline of the intercalating chemistry.



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Intercalating Dyes



Intercalating Dyes

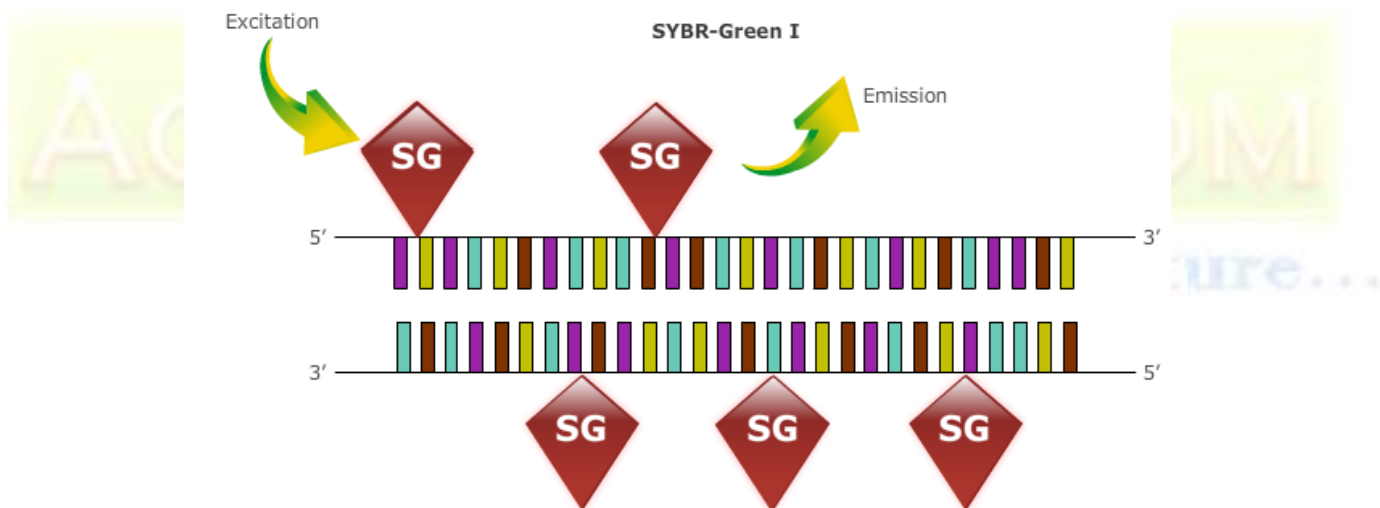


Fig. 14.6 Intercalating dyes (SYBR Green)

14.5.2 Specific detection chemistries

There are a number of chemistries used in Real Time PCR which involve use of probes labeled with fluorescent / reporter and quencher dyes. Specific chemistries include Molecular Beacons, Scorpion

probes, TaqMan probes, FRET probes, Amplifluor, Dual labeled probes etc. which discriminate between specific and non-specific DNA sequences. The commonly used chemistries are shown in Fig. 14.7.

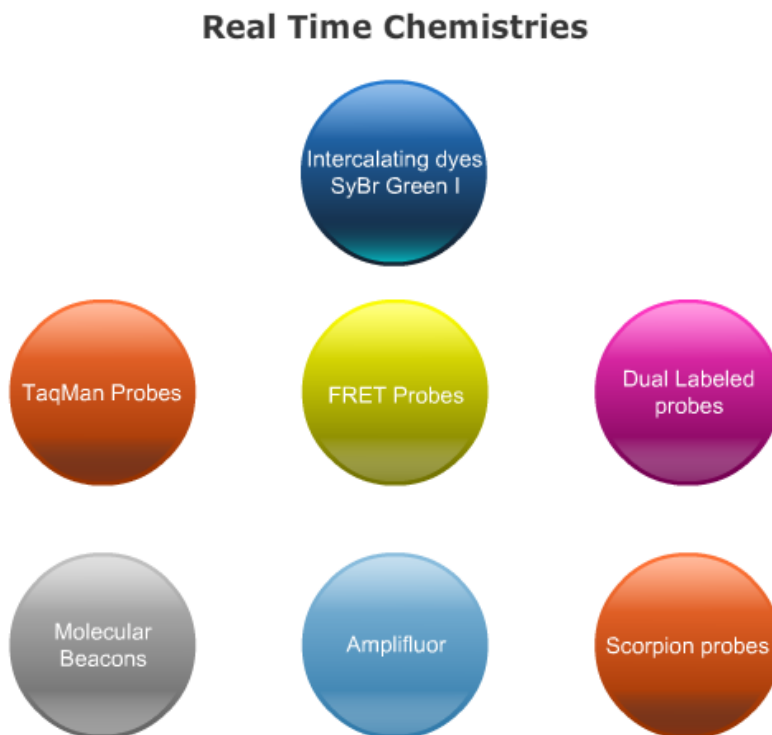


Fig. 14.7 Real Time PCR chemistries

Two of the Real Time PCR chemistries will be discussed here.

14.5.2.1 TaqMan / Hydrolysis probes

TaqMan probes also called hydrolysis probes or dual labeled probes are the most widely used probes. These were developed by Roche and ABI and consist of single stranded probe sequence that is complementary to one of the strands of amplicons. A fluorophore is attached at one end and quencher at the other end as shown in the following Fig. 14.8. The probe binds to amplicon during each annealing step of the PCR reaction. When Taq polymerase extends the target gene from the primer bound to the amplicon, it displaces the 5' end of the probe which is then degraded by 5' to 3' exonuclease activity of Taq polymerase. This process separates the fluorophore and quencher and leads to irreversible increase in fluorescence which is being read in every extension step.

Taqman probes are “hydrolysis” probes

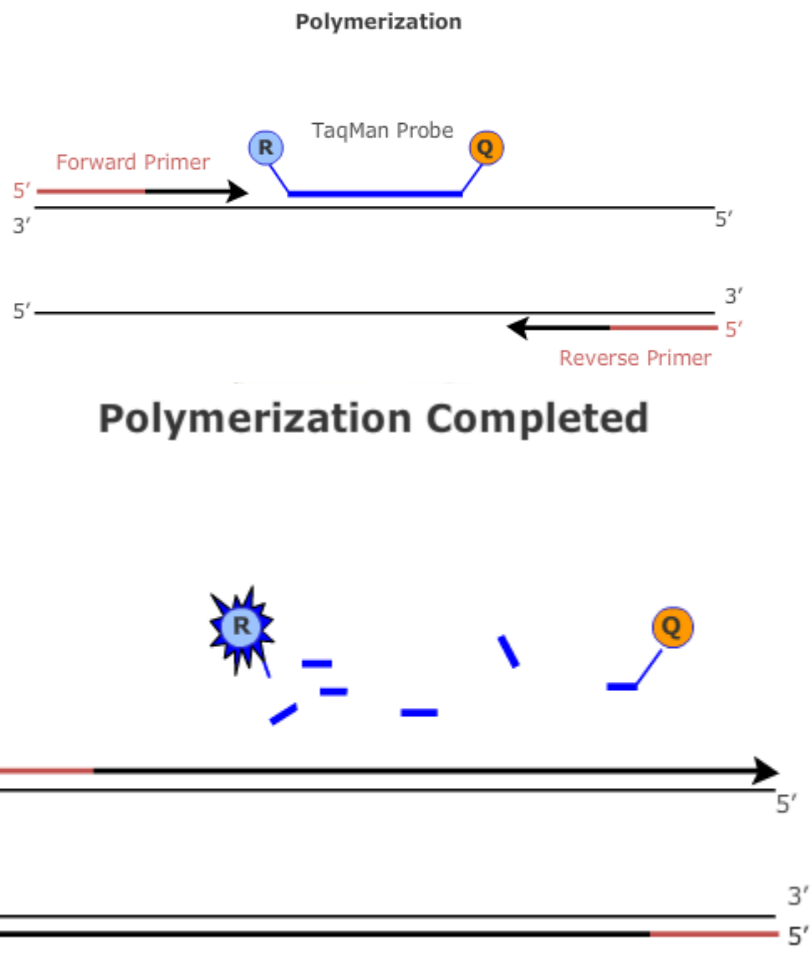


Fig. 14.8 TaqMan Probe

14.5.2.2 Molecular beacons

Molecular beacons (MB) were invented by Tyagi and Kramer in 1996 and differ from the TaqMan probes. MBs consist of single stranded DNA with hairpin loop structure (Fig. 14.9). The loop which is single stranded bears complementarity to the amplicon. The stem is approximately six to eight bases long and mainly consists of G and C which can hold the probe in hairpin structure. The stem bears fluorophore at one end and quencher at another. The probe opens up at high temperature and binds to specific sequence in the target DNA. Binding of probe to amplicon disrupts the stem loop structure leading to the separation of fluorophore and quencher resulting in fluorescence which can be read during annealing step.

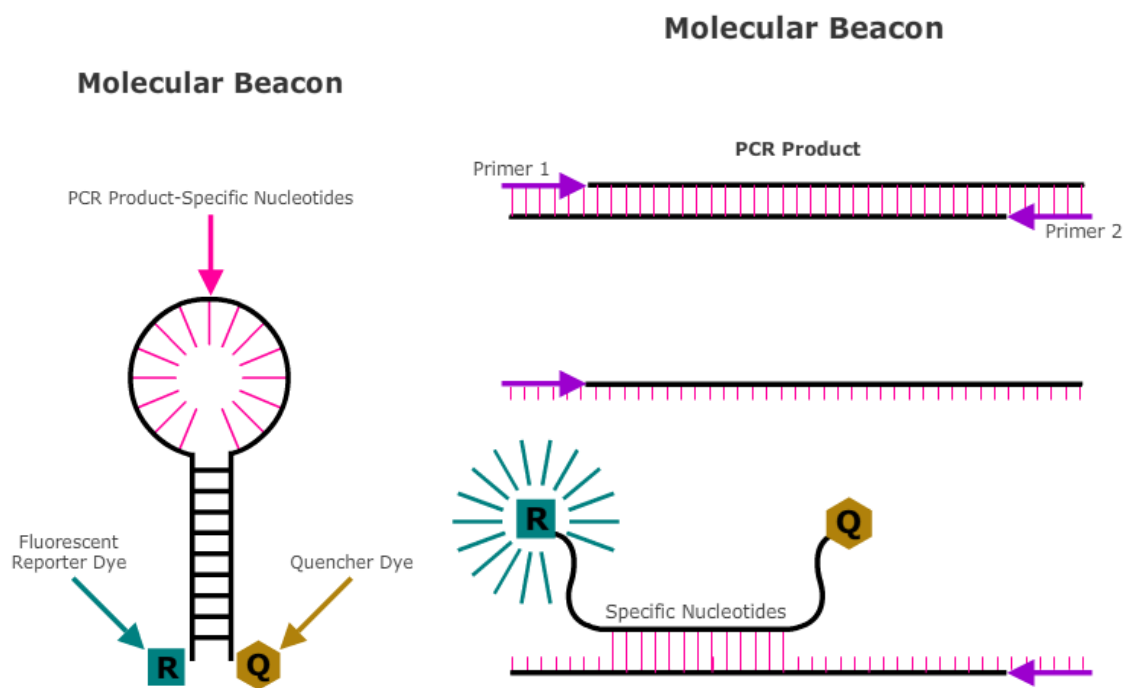


Fig. 14.9 Molecular Beacon (Hairpin loop structure of a molecular beacon)

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Module 3. Genetic engineering technology / recombinant DNA technology

**Lesson 15
DNA MICROARRAY**

15.1 Introduction

DNA microarray is a genomic tool used in molecular biology to monitor the expression of thousands of genes simultaneously on a single chip, thus providing the opportunity to analyse the enormous genetic data in one experiment. The developments are occurring daily making this technology the central platform for functional genomics. DNA microarrays can be used to analyse and measure gene expression, detect single nucleotide polymorphism (SNP), genotype or resequence mutant genomes, comparative genome analysis, drug discovery and biodiversity etc. Their high throughput capabilities also hold tremendous potential for pathogen detection, identification, and genotyping in molecular diagnostic laboratories.

Functional genomics is the study which deals with understanding the function of a genome or its genes with regard to its phenotype

15.2 Definition

Microarray is defined as an array of orderly arranged probes consisting of specific single stranded DNA sequences either short oligonucleotides or cDNAs arrayed on chips (also known as biochips, DNA array, gene chip etc.) which can be used to hybridize with a cDNA sample under high stringency conditions of hybridization.

15.3 Principle of Microarray

The basic principle of DNA microarray is based on complementary base pairing. The DNA double helix's complementary base pairing specificity is exploited in DNA microarray. They are composed of 'DNA probes' that are spotted onto a solid substrate i.e glass, silicon etc. Each spot on the support consists of probes that are complementary to the target gene or gene of interest. The basis behind this is hybridization. In hybridization, two single stranded DNA join together to form double stranded DNA due to their complementarity. Hybridized targets can then be detected using one of the several available reporter systems.

DNA probe is a short single stranded oligonucleotide sequence highly specific for a particular target and explored for detection and identification of the target gene.

15.4 DNA Microarray Technique

DNA microarray technique involves the following basic steps.

- i) Preparation of probes
- ii) Construction of DNA array
- iii) Preparation of samples
- iv) Hybridization
- v) Detection / Scanner / Reader

15.4.1 Preparation of probes

The probes to be spotted on to the array include single stranded short oligonucleotides ranging in length from 9-50 mer. For genotypic microarrays, probes could be the PCR products from the cloned genes.

15.4.2 Construction of DNA array

The probes are printed onto microscopic slides robotically in a specific grid pattern. The delivery of samples on chip can also be performed manually. The delivery technologies used extensively for construction of arrays are:

15.4.2.1 Mechanical microspotting

The use of prefabricated oligonucleotides is the simplest mode to construct microarrays. The oligonucleotide probes can be deposited straight from a reagent tray onto the chemically modified glass surface by a printhead containing microspotting pins, tweezers, capillaries or micropipettes. After the first spotting cycle, the pin is washed and reloaded to deposit on an adjacent spot. This process is repeated. The steps are speeded up by using robotic control.

cDNA is the complementary copy of the mRNA which represents the functional part of the genome

15.4.2.2 'Ink jet' or 'Piezoelectric printing'

Minute volume of reagents are delivered to defined locations on the slide. The printhead moves across the array and at each spot electrical stimulation causes the DNA bases, cDNAs or other molecules to be delivered onto the surface *via* tiny jets.

15.4.3 Preparation of samples

Samples constitute messenger RNA (mRNA) of the cells or tissues of interest. The RNA thus obtained is converted to cDNA (complementary DNA) using reverse transcriptase. During this reaction, cDNA is labeled with fluorescent dyes like Cy3 (red), Cy5 (green), or radioactive nucleotides. The two different samples are labeled with two different colored dyes. The labeled cDNAs are then hybridized to DNA arrays. These steps have also been shown in Fig. 15.1.

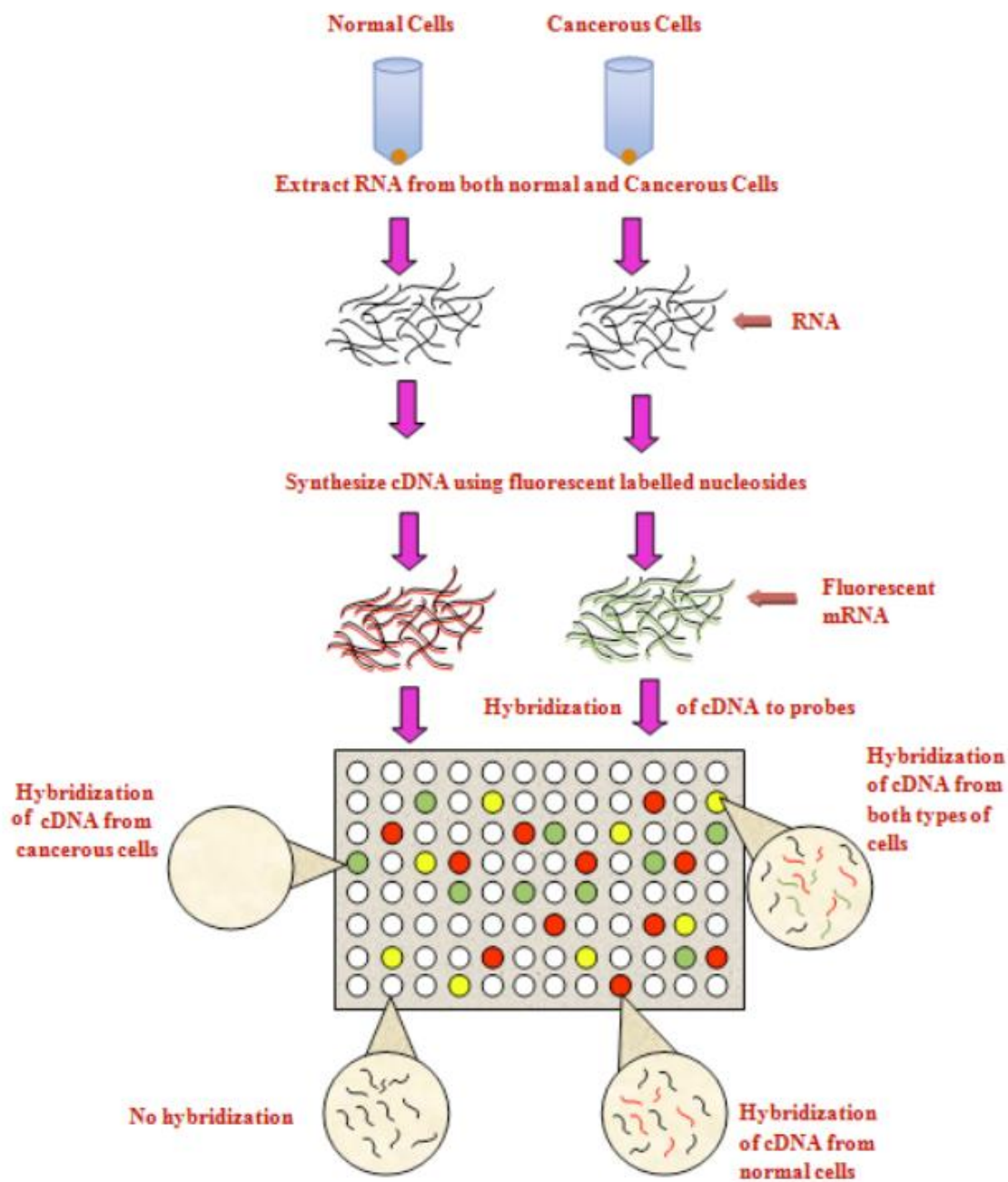


Fig. 15.1 Steps in a Microarray experiment

15.4.4 Hybridization

The labeled cDNA is then hybridized to the microarray slide. Hybridization is the reaction that occurs between the labeled fluorescent cDNA sample with probes on the glass slide. If the target cDNA has complementarity to the probe, it will bind to these probes. The hybridization conditions vary with the type of application of array as some require low stringency (low temperature and high salt) while others require high stringency (high temperature and low salt).

15.4.5 Detection / Scanner / Reader

After hybridization, the microarray is scanned using scanners either based on epifluorescent or laser confocal scanning microscope, which illuminates each spot of DNA and separately measures the fluorescence for each dye or CCD based detection systems. CCD detection systems can even detect very small quantities of array bound molecules. The data thus obtained can be used to find the relative abundance of the sequences of each specific gene in the messenger RNA or DNA samples. The hybridization pattern can then be used to identify the genes that are expressed differentially in the tissues or cells.

15.5 Microarray Equipment

15.5.1 Arrayers

Arrayers are available from several companies e.g. Affymetrix, Agilent, Molecular dynamics, Synteni, Genome systems etc. but are highly expensive. With improvement of technology, these may become cheaper in near future. An arrayer produced by Biorobotics (Micro GridII) is shown in Fig. 15.2.



Fig. 15.2. DNA Arrayers – MicroGrid II (Biorobotics)

15.5.2 Scanners

The principle of scanners is to detect the different levels of fluorescence between the spots on the microarray. The basic principle is that the light source inside the scanner excites the fluorescently labeled samples which is then detected, measured and recorded. The scanners from Affymetrix and Molecular Dynamics is also shown Fig. 15.3.



Fig. 15.3. DNA Scanners from Affymetrix and Molecular Dynamics

15.6 Applications

Microarrays have a number of applications in different areas that may further expand in future.

1. DNA sequencing by hybridization
2. Single nucleotide polymorphism and point mutations
3. Functional Genomics
4. Diagnostics and Genetic Mapping
5. Reverse genetics and DNA chips
6. Genomic mismatch scanning
7. Disease diagnostics
8. Proteomics
9. Transcriptomics



Lesson 16 ANIMAL CELL CULTURE

16.1 Definition

Animal cell culture is the process of culturing animal cells extracted from tissues or organs under *in vitro* aseptically controlled laboratory environment (temperature, gases and pressure) simulating that of *in vivo* system. Under the controlled environment, the animal cells are able to survive and proliferate as under *in vivo* conditions.

16.2 History

The development of animal cell culture can be traced back to 1880 when Arnold showed that leucocytes can divide outside the body. Later, in 1903, Jolly studied the behavior of animal tissue explants immersed in serum and lymph, or ascites fluid. Ross Harison was able to culture frog tadpole spinal chord in a lymph drop hanging from a cover slip of a cavity slide in 1907. In 1912, Carrel initiated a culture of chick embryo heart cells which were passaged for a period of 34 years. The use of trypsin (a proteolytic enzyme) by Rous and Jones in 1916 was another significant break through required for the subculture of adherent cells and in the 1950s, the technique of trypsinization was exploited to produce continuously growing cell lines (HeLa cells). The practice of incorporating antibiotics such as penicillin and streptomycin, to the cell culture medium from 1940s onwards alleviated the problem of microbial contamination. Later, development of chemically defined media (Eagle and Eagle, 1950) led to the advantages of consistencies in various batches, easy sterilization and reduced the chances of contamination. The first product produced for mass vaccination was the polio vaccine which became the first major commercial product of cultured animal cells in 1950s. Animal cell culture has now become an alternative for animal experiments for drug discovery, evaluation of efficacy of several nutrients, herbals, probiotics, absorption and bioavailability studies etc.

16.3 Types of Animal Cell Culture

Animal cells may grow either as adherent monolayers or suspension cells.

16.3.1 Adherent cells

Adherent cells are said to be anchorage-dependent and the attachment to a substrate is a prerequisite for their proliferation. They stop dividing when they reach confluency i.e they cover the whole surface and reach at such a density that they come in contact with each other. However, if they are left in confluent state for long, they lose their viability and die. Most of the cell lines grow in this manner e.g. HeLa cells, CaCO₂, HT-29, INS etc. Adherent cells need to be separated from the culture dish by breaking the bond between cells and the surface using trypsin. The process is called trypsinization. The other proteolytic enzymes can also be used such as collagenase, pronase and papain etc.

16.3.2 Suspension cells

Suspension cells do not adhere to the surface. They are generally in suspension or only loosely adherent. Cells from blood, spleen or bone marrow as well as some transformed cell lines and cells derived from

malignant tumors can be grown in suspension. However, the methods used to propagate these cells are very different from those for adherent cells. These methods are easy to perform since they do not need any trypsinization.

16.3.3 Primary cultures / cell lines

Primary culture involves culturing of cells removed surgically from an animal tissue. The whole process of primary cell culture has been presented in Fig. 16.1. There are two major steps involved in preparation of primary cultures *viz.* explant culture and enzymatic dissociation. Explant culture involves cutting tissues into small pieces and growing them into culture medium. Cells then move from explant and proliferate. The process however can be speeded up by using trypsin or collagenase. Once the cells in primary culture grow, they are subcultured for continuous growth. They are generally harvested by scrapping or trypsinization treatment. They are capable of only a limited number of cell divisions i.e. upto confluency state after which they enter a non-proliferative state called senescence and finally die out. At lower cell densities, however, the normal phenotype can be maintained.

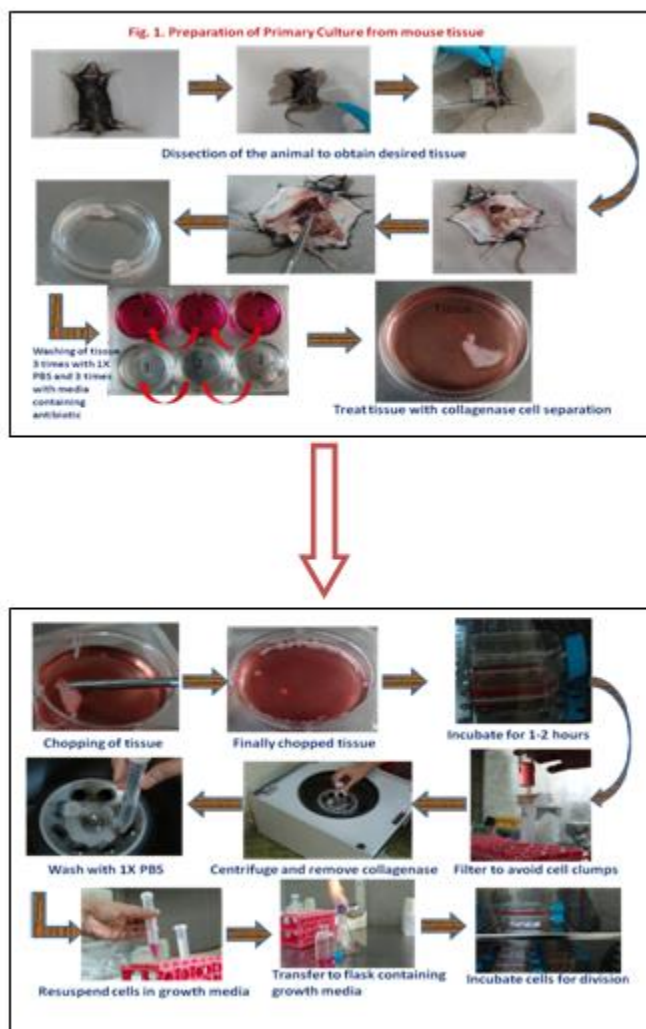


Fig. 16.1 Flow diagram of Primary cell culture development

The advantages of primary cultures are that they are morphologically similar to the parent tissue and hence express tissue specific functions. Primary cells are extensively used by many researchers since they are physiologically more similar to *in vivo* cells. Moreover, cell lines when cultured for longer / extended periods can undergo phenotypic and genotypic changes that can lead to discrepancies when results from

different laboratories are compared using the same cell line. Furthermore, many of the cell lines are not available as continuous cell lines. However, the disadvantage is that every time cells are required to be isolated afresh for each experiment. Secondly, proteolytic enzymes required for disruption can result into damage of membrane receptors, disrupt the integrity of the membrane, and loss of cellular products etc.

16.3.4 Continuous cultures / cell line

Continuous cell lines are developed from the cells that can be passaged indefinitely and express a reasonably stable phenotype. These cell lines have arisen spontaneously in normal cells being passaged in culture, but majority of them have been obtained by culturing tumor cells. In addition to being immortal (infinite life span), they share several additional properties that distinguish them from 'normal' cells in culture. Once a continuous cell line has been established, it is customary to clone the cells in order to obtain a genetically homogeneous population.

16.4 Cell Culture Conditions

16.4.1 Media

Basal Medium

Since cell culture medium affects the growth and proliferation of cell lines, it is extremely important to select a suitable medium. Moreover, different cell lines have different requirements for their growth. The most common basal media include Eagle Minimal Essential Medium (MEM), Dulbecco's Modified Eagle medium (DMEM), RPMI 1640, and Ham F10. All of them contain a mixture of amino acids, glucose, salts, vitamins, and other nutrients, and are available either in powder or in liquid form various commercial suppliers like Sigma, Invitrogen etc.

16.4.2 Supplements

A number of supplements are added to the basal media to enable them to be used for culturing the cells. The optimum pH for most of the cell cultures lies between 7.4 – 7.7. Hence, the type of buffering that is used for the media depends on the growth conditions. Bicarbonate plus CO_2 and N-2-hydroxyethylpiperazine -N'-ethane sulphonic acid (HEPES) are most common buffers. Each type of media has a recommended bicarbonate concentration and CO_2 tension to achieve the correct pH and osmolarity. In addition to buffering the medium, essential amino acids such as cysteine and tyrosine as well as glutamine may be needed to meet certain growth requirements. L-Glutamine is also required by most cell lines since cultured cells use glutamine as an energy and carbon source in preference to glucose, although glucose is present in most defined media. L-glutamine is an unstable amino acid that converts to a form that cannot be used by cells, hence should be added to medium just before use.

16.4.3 Serum

Serum is partially undefined material that contains growth and attachment factors, and may show considerable variation in the ability to support growth of particular cell lines. Most cell lines require calf serum for adequate growth but often fetal calf serum provides the best growth conditions. Fetal calf serum (FCS) is often most commonly used, but for some applications less expensive sera such as horse or calf may also be used. Different serum batches should be tested to find the best one for each cell types since the quality varies a lot.

16.4.4 Antibiotics and fungicides

Antibiotics and fungicides are used to prevent microbial contamination including bacteria, yeasts and molds. These include penicillin, streptomycin, kanamycin, nystatin and amphotericin B etc.

16.4.5 Additional supplements

Primary cell culture requires some additional supplements such as collagen and fibronectin, hormones such as estrogen, and growth factors such as epidermal growth factor and nerve growth factor to attach to the cell culture vessel and proliferate.

Media, serum and supplements should always be tested for sterility prior to their use by incubating a small aliquot at 37°C for 24-48 hours. If microbial growth occurs, it should be discarded.

16.4.6 Incubation

Cell lines should be incubated in a CO₂ incubator with a tightly regulated temperature and CO₂ concentration. Most cell lines grow at 37°C in presence of 5% CO₂ with saturating humidity.

16.4.7 Preservation of cell lines

The cell cultures are required to be stored for long term usage. The general procedure of preservation of all cell cultures is freezing. The cells should be frozen in exponential phase of growth with a suitable preservative like dimethylsulfoxide (DMSO). The cells are frozen slowly at 1 °C/min to -50°C and then kept either at -196°C immersed in liquid N₂ or -70°C. Deterioration of frozen cells has been observed at -70°C, therefore, -196°C is better for storage and preservation.

16.5 Equipment and Facilities Required in Animal Cell Culture

Animal cell culture laboratory requires some specific equipments and techniques which include the following.

16.5.1 Biosafety cabinet class II

Biosafety cabinet class II is a pre requisite for safe handling of human carcinoma cell lines. A class II Biosafety cabinet from Labconco has been illustrated in Fig. 16.2.



Fig. 16.2 Class II, type A2 Biological Safety Cabinets from Labconco

16.5.2 Carbon dioxide incubators

Many cell lines can be maintained in an atmosphere of 5% CO₂:95% air at 99% relative humidity at around 30-40°C using carbon dioxide incubator (Fig. 16.3). The concentration of CO₂ has to be kept in equilibrium with sodium bicarbonate in the growth medium. The incubators are designed to allow CO₂ to be supplied from a gas cylinder which regulates supply of gas (2-5% as required by different cell lines).



Fig 16.3 Carbon dioxide incubator from Sanyo

16.5.3 Microscope

An inverted microscope is essential to examine cell culture in dishes and flasks for their morphology and differentiation. An inverted microscope from Leica has been shown in Fig. 16.4. Additional features of microscope include fluorescence, luminescence, CCD camera and monitor etc. to keep a check on the purity and viability of the cells in good healthy status.



Fig. 16.4 Inverted Microscope from Leica

16.5.4 Cell culture ware/vessel

A variety of cell culture polystyrene plastic ware on which adherent cells can proliferate well, have been shown in Fig. 16.5. Cells can generally be maintained in petri dishes or flasks (25 cm² or 75 cm²) and multi well dishes etc.



Fig. 16.5 Cell culture plastic ware

16.6 Applications

Cell culture techniques are widely used in cellular and molecular biology research. Some of the areas where cell culture finds applications are listed below:

16.6.1 Model systems

Cell cultures provide a good model system for studying basic eukaryotic cell biology, biochemistry, effect of drugs/nutrients on cells etc.

16.6.2 Drug testing and efficacy

Cell culture plays an important role in pharmaceutical industry since these can be used to test the toxicity, efficacy and efficiency of a new drug. The cell lines can be used for high throughput screening of several compounds that may hold promise as drugs.

16.6.3 Production of genetically engineered therapeutic proteins

Animal cell cultures are being extensively used in the production of genetically engineered therapeutic proteins like insulin, hormones, monoclonal antibodies etc. from eukaryotes since they have the ability to introduce post transcriptional and post translational modifications in the expressed proteins to make them biologically active.

16.6.4 Gene therapy

Cells can be removed from a patient lacking a functional gene and then replacing the damaged gene and grown in culture before placing into the patient again.

16.6.5 Cancer research

Cell culture can be used to study normal versus cancer cells and also to look for drugs which can destroy cancer cells selectively.

16.6.6 Vaccine production

Several vaccines have been produced using cell lines like polio, rabies, chickenpox and measles etc.



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Lesson 17

PROTOPLAST / SOMATIC FUSION AND HYBRIDOMA TECHNOLOGY

17.1 Definition

Fusion of one cell with another cell to form a hybrid cell is called protoplast or cell fusion. Protoplasts are prepared by removing the cell wall and are commonly used in bacteria and plants. In animal cell culture, it is known as cell fusion.

17.2 Methods

Several methods/protocols which include chemical, mechanical and electrical are used to fuse dissimilar cells. The methods involve presence or absence of a known fusogen and the most common one is the use of polyethylene glycol (PEG) besides electrofusion, virus mediated fusion (e.g., Epstein–Barr Virus or Sendai virus), liposome-mediated fusion, micro-orifice etc. which are described below:

17.2.1 Polyethylene glycol

Polyethylene glycol (PEG) induced cell fusion has become a standard technique particularly in the hybridoma technology. PEG induced fusions are easy to perform and a high number of cells can be fused in a shorter time. However, the technique suffers from low fusion efficiency and the many unwanted fusion products are created.

17.2.2 Liposome mediated fusion

Liposomes are small lipid molecules in which large number of plasmids are enclosed. They can be induced to fuse with cell cultures using PEG, and therefore have been used for gene transfer. They offer protection to DNA/RNA from nuclease digestion, low cell toxicity, stability and storage of nucleic acids due to encapsulation in liposomes besides having high degree of reproducibility and applicability to a wide range of cell types.

17.2.3 Electrofusion or E fusion

Electrofusion was first given by Zimmerman in early 1980's which led to higher fusion efficiencies under controlled conditions. Electrofusion is the process of combining two cell types (similar or dissimilar) resulting in fusion of cytoplasmic contents of both cells with the help of an electric pulse. These protocols ensure higher viability and cell fusion efficiency. The two cell types are initially kept in a chamber with low conductivity medium. It is important that cell fusion medium has a low conductivity i.e. high resistivity. The cells are then aligned by Dielectrophoresis (application of non-uniform alternating electric fields) and electric pulse is applied. Cell electrofusion begins at the time of application of the high electric field pulses and proceeds for some time after the pulses are applied. After cell membrane fusion maturation, the cells are placed in tissue culture medium to promote cell viability and growth.

17.2.4 Dielectrophoretic cell trapping

Recently, micro-orifice based cell fusion has also been used which assures high-yield fusion without compromising the cell viability. Micro-orifice-based fusion makes use of electric field constriction to assure high-yield one-to-one fusion of selected cell pairs. Dielectrophoresis (DEP) assisted cell trapping method was used for parallel fusion with a micro-orifice array. The method involves construction of a microfluidic chip that contains a chamber and partition. The partition divides the chamber into two compartments and it had a number of embedded micro-orifices. The voltage applied to the electrodes located at each compartment generates an electric field distribution concentrating in micro-orifices. Cells introduced into each compartment move towards the micro-orifice array by hydrostatic pressure. The cells are kept in micro-orifice using DEP assisted trapping to establish cell to cell contact through orifice. Cell fusion occurs by application of a pulse and the fused pair immobilizes at micro-orifice. The unfused cells are removed and the chip is monitored for time lapse imaging of the selected fusants.

17.2.5 Laser induced cell fusion

Initially, laser-induced cell fusion was used to fuse two embryonic cells. Later on, this technique was also applied to fuse B lymphocytes with myeloma cells in suspension as well as to fuse plant protoplasts. The mammalian cells, however, could only be fused by a UV laser microbeam after binding them together via an avidin- biotin bridge. Although, the avidin-biotin bridge created a very specific bond between selected cells, it limited the fusion yield and was time consuming too. This technique was improved by combination of an optical trap with a pulsed UV laser (laser cell fusion trap) which does not depend on any natural cell contact or specific cell receptors. The two cells are selected by simply dragging one cell towards a second cell with the aid of the optical trap. Fusion efficiency can still be enhanced by addition of PEG.

17.2.6 Virus mediated fusion

Cells growing in culture are induced by some of the viruses such as 'Sendai virus' to fuse the cells to form hybrids. The virus induces two different cells first to form heterokaryon and finally the two nuclei fuse together to form fusants. The cells including fusants are plated on a selective medium e.g. HAT which allows the multiplication of hybrid cells only.

17.3 Applications

1. For production of therapeutic hybrids by combining a tumor cell with a dendritic cell to be used in immunotherapy.
2. Cell fusion is extensively used for production of hybridoma for manufacture of monoclonal antibodies.
3. Used for Nuclear Transfer – for fertility treatment and animal propagation.
4. Hybrid cells are used to study gene expression.

17.4 Definition of Hybridoma Technology

The formation of hybrid cell line produced by fusing a specific antibody producing B cell with a myeloma (B cell cancer) cell that is selected for its ability to grow in tissue culture is called hybridoma technology.

17.4.1 History

Hybridoma technology was discovered in 1975 by Georges J. F. Kohler (West Germany) and Cesar Milstein of Argentina (now working in U.K.). They shared the Noble prize for physiology and medicine in 1984 with Niels Kaj Jerne of Denmark (now working in Germany) who made several other contributions in the area of immunology.

17.4.2 Monoclonal antibody production using Hybridoma technology

Monoclonal antibodies (Mab) are nowadays widely used as a therapeutic means to treat cancer, chronic and autoimmune diseases as well as for diagnostic purposes like blood group typing and disease testing/diagnostics. Hybridoma technology is widely used for the production of hybridoma cells. Hybridomas are the cells that have been engineered to produce monoclonal antibodies of consistent quality, high specificity and in large amounts. The various steps for production of monoclonal antibodies using hybridoma technology have been shown in Fig 17.1

Antibodies are proteins synthesized in blood against specific antigens to combat disease causing antigens / organisms and give immunity.

1. The first step to make a hybridoma is to generate antibody producing B cells. The mice are injected by intraperitoneal (IP) route with the antigen against which monoclonal antibodies are to be raised over a course of several weeks until an appropriate antibody titre is achieved.
2. Blood is taken and examined for the presence of antibodies. Some B cells will produce antibodies that bind specifically to epitopes on the antigen of interest while there will be some non-specific antibodies also that will not bind.
3. Splenocytes are then isolated from the mouse spleen and fused with immortalized myeloma cell (that can grow indefinitely).
4. The myeloma cells should be selected which do not produce any antibody and also lack hypoxanthine guanine phosphoribosyltransferase (HGPRT) gene that makes them sensitive to the HAT medium (hypoxanthine aminopterin thymidine medium).
5. Both the B cells and myeloma cells are fused using polyethylene glycol or Sendai virus by making the cell membranes more permeable.
6. Next step is to separate fused hybridoma cells from unfused B cells and myeloma cells.

A highly specific antibody produced by a single clone of cells (specifically, a single clone of hybridoma cells) and specific to one target only is called monoclonal antibody.

7. Fused cells are then incubated in the HAT (Hypoxanthine Aminopetrin Thymidine) medium. Aminopterin in the medium blocks the pathway that allows for nucleotide synthesis. Hence, unfused myeloma cells die, as they cannot produce nucleotides by the de novo or salvage pathways.
8. Unfused B cells die as they have a short life span. Only the hybridoma (hybrid of B cells and myeloma cells) cells survive since HGPRT gene coming from B cells is functional.
9. These cells produce antibodies (a property of B cells) and are immortal (a property of myeloma cells). The medium is then diluted in multiwell plates to such an extent that each well contains only one cell. The supernatant from each well is checked for the desired antibody. Since the antibodies in the well are produced by the same B cell, they will be directed towards the same epitope, and hence are known as monoclonal antibodies.
10. Once a hybridoma colony is established, it will continually grow in culture medium like RPMI-1640 (with antibiotics and foetal bovine serum) and produce antibody. The hybridomas are then screened for the antibody specificity. The desired clones are then transferred to large tissue culture flasks and are cryopreserved. The monoclonal antibodies thus produced are then checked for any cross reactivity.

17.4.3 Large scale production and purification of monoclonal antibodies

Hybridomas are cultured to high densities in culture flasks or roller tubes/bottles. The antibody containing medium is then filter, sterilized and frozen. Alternatively, hybridoma cells can also be grown in dialysis based mini-fermentors which leads to high density cultures. The antibodies are purified from cell homogenate or cell debris obtained from the medium using ion-exchange chromatography or antigen affinity chromatography.

17.4.4 Further advancements in monoclonal antibody production

Monoclonal antibodies can be produced using mouse cell lines. However, these antibodies can cause adverse immune reaction with repeated use. With the advent of recombinant DNA technology, the xenogenic portions of mouse MAb (mouse monoclonal antibody) is replaced with human immunoglobulin to construct humanized monoclonal antibody which still requires refinement or by the use of transgenic mice which contains segments of human gene coding for antibodies. Several new products with MAb for pharmaceutical use are in the market after its approval in 1986 and many more products are under Phase I and II clinical trials. The production of MAbs has been at a fast pace as a result of advent of recombinant DNA technology, new analytical techniques and screening methods.

17.4.5 Applications

1. Monoclonal antibodies are widely used for the treatment of a number of diseases like cancers, allergies etc.
2. Monoclonal antibody therapy can be used to destroy malignant tumor cells and prevent tumor growth by blocking specific cell receptors (immuno-therapy).
3. Find application in diagnostics including ELISA (Enzyme Linked Immuno-Sorbant Assays) based assays.
4. Pregnancy can be detected by assaying of hormones using monoclonal antibodies.
5. Used for detection of pathogens by immunological assays including ELISA.
6. Monoclonal antibodies have been in vogue to immunize against certain diseases in humans and cattle. The most promising outcome is the prospect of developing anti-malarial vaccine.



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Lesson 18 STEM CELLS AND NUCLEAR CLONING

18.1 Introduction

Stem cell research is an emerging field with lot of potential for therapeutic applications in the management of untreatable diseases through cellular or tissue engineering.. Stems cells are useful both in basic as well as translational research. Looking into the tremendous potential of stem cell research, "CMC-DBT Centre for Stem Cell Research" has been established at CMC, Vellore to carry out basic and translational research. Facilities for carrying out research in the area of stem cells have been created at number of centres like PGIMER, Chandigarh; AIIMS, New Delhi; SGPGIMS, Lucknow, KEM hospital, Mumbai and LVPEI, Hyderabad. In the field of animal sciences, facilities have been created at Animal Biotechnology Centre, National Dairy Research Institute, Karnal-132001.

18.2 Definition of Stem Cells

Stem cells are the cells that regenerate and turn into cells that form tissues, organs and systems. The term 'Stem' comes from the word "Root" which means 'source' i.e. stem cells are the source of all cells. The function of the stem cell is not known, however every single cell stems from this cell. Stem cells are undifferentiated cells having a high capacity for self renewal. They have the potential to become different types of cells. When stem cells divide, each new cell has the option either to remain as a stem cell or become a specialized cell with a particular function like muscle cell, blood cell nerve cell etc. Stem cells divide on receiving a signal. On receiving the signal, the genes are activated and stem cells start differentiating into a particular cell type (Fig. 18.1)

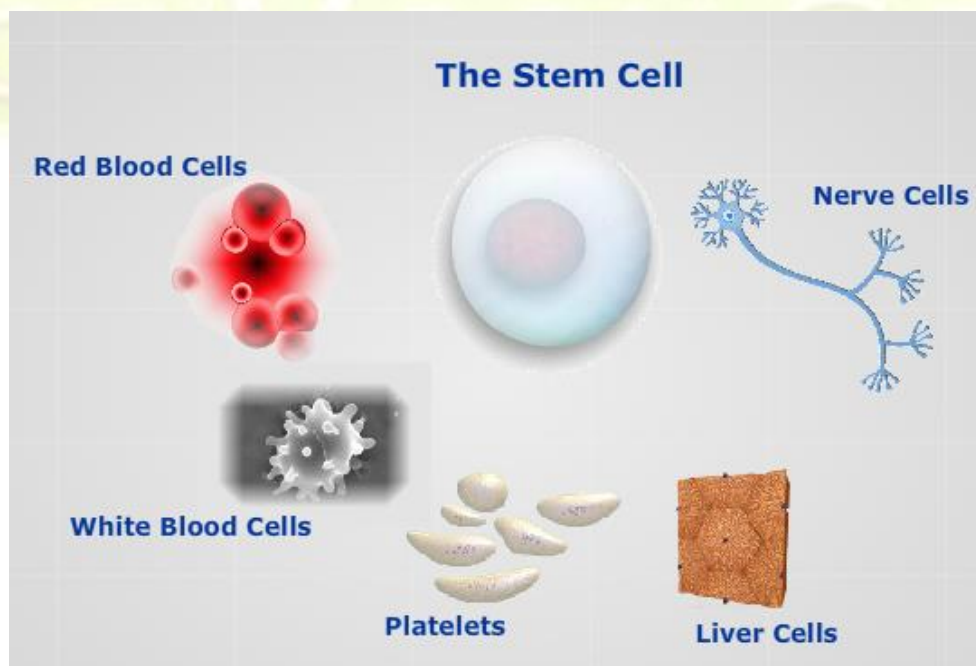


Fig. 18.1 The Stem Cell and its differentiation into different types of cells in the target organ/tissue

Stem cells are categorized into two types:

- i) Embryonic stem cells
- ii) Adult stem cells

18.2.1 Embryonic stem cells

Embryonic stem cells are isolated from the inner cell mass of blastocysts. In a developing embryo, stem cell can differentiate into all of the specialized embryonic tissues as they are totipotent (Fig. 18.2).

18.2.2 Adult stem cells

Adult cells act as a repair system and replenishes the worn out specialized cells and maintain turnover of regenerative organs like blood, skin and intestinal tissues.

18.3 Some Definitions Related to Stem Cells

18.3.1 Totipotent

Totipotent stem cells are those that can become any kind of cell in the body. 'Toti' originates from a Latin word which means whole or complete or total. After the fertilization of an egg, it undergoes a series of divisions to become an embryo before turning into a fetus. The cells that are formed during the first few divisions are 'totipotent'. After 3 - 4 divisions of 'totipotent' cells, these cells will not be able to differentiate into any cell type.

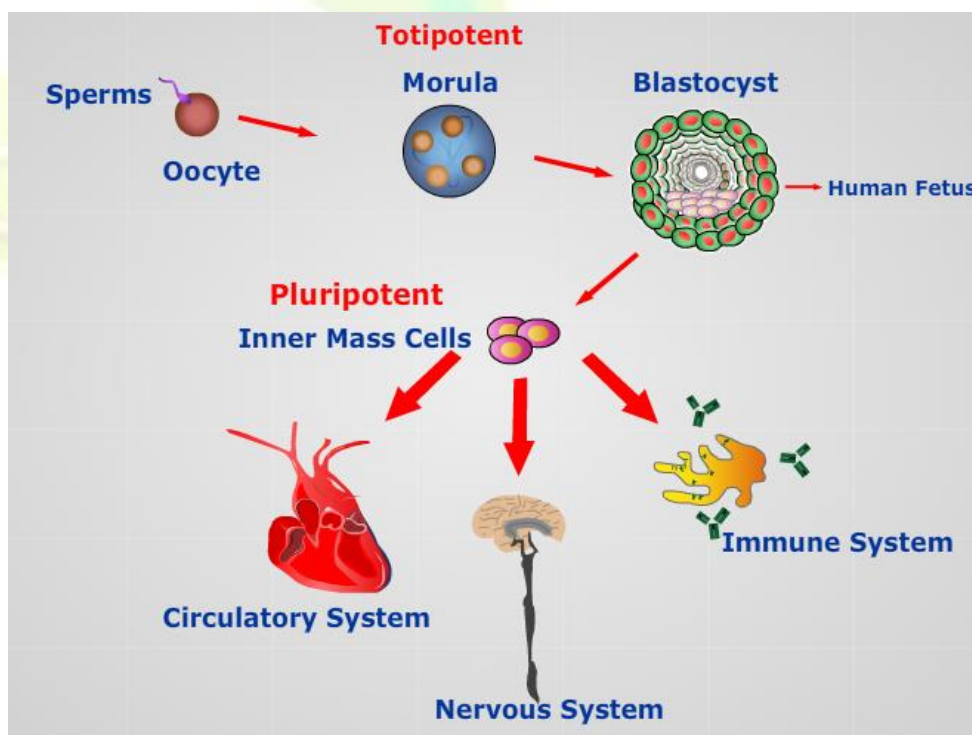


Fig. 18.2 Development of differentiated cells

18.3.2 Pluripotent

Pluripotent stem cells are those that have the ability to become almost any kind of cell in the body. Pluripotent stem cells result from totipotent stem cells after they have undergone first few divisions. Embryonic stem cells at the blastocyst stage and fetal stem cells are pluripotent. Both toti and pluripotent cells are essential for the development of new organism, hence they are found in the early stages of development in the embryo.

18.3.3 Multipotent

These stem cells can differentiate into a number of cells, but only those of a closely related family of cells. They are limited to mostly cells of the blood, heart, muscle and nerves. These cells function as a repair system for damaged tissues. The example in this category are Adult stem cells.

18.3.4 Unipotent

These stem cells can produce only one cell type and have the property of self renewal.

18.4 Applications

1. Stem cell therapy can be used for treatment of several diseases like Parkinson, cancer, myocardial infarction, heart muscle cells or insulin-producing pancreatic cells, missing teeth, crohn's disease, leukaemia etc. (Fig. 18.3). Till date, bone marrow transplantation has been successfully achieved.

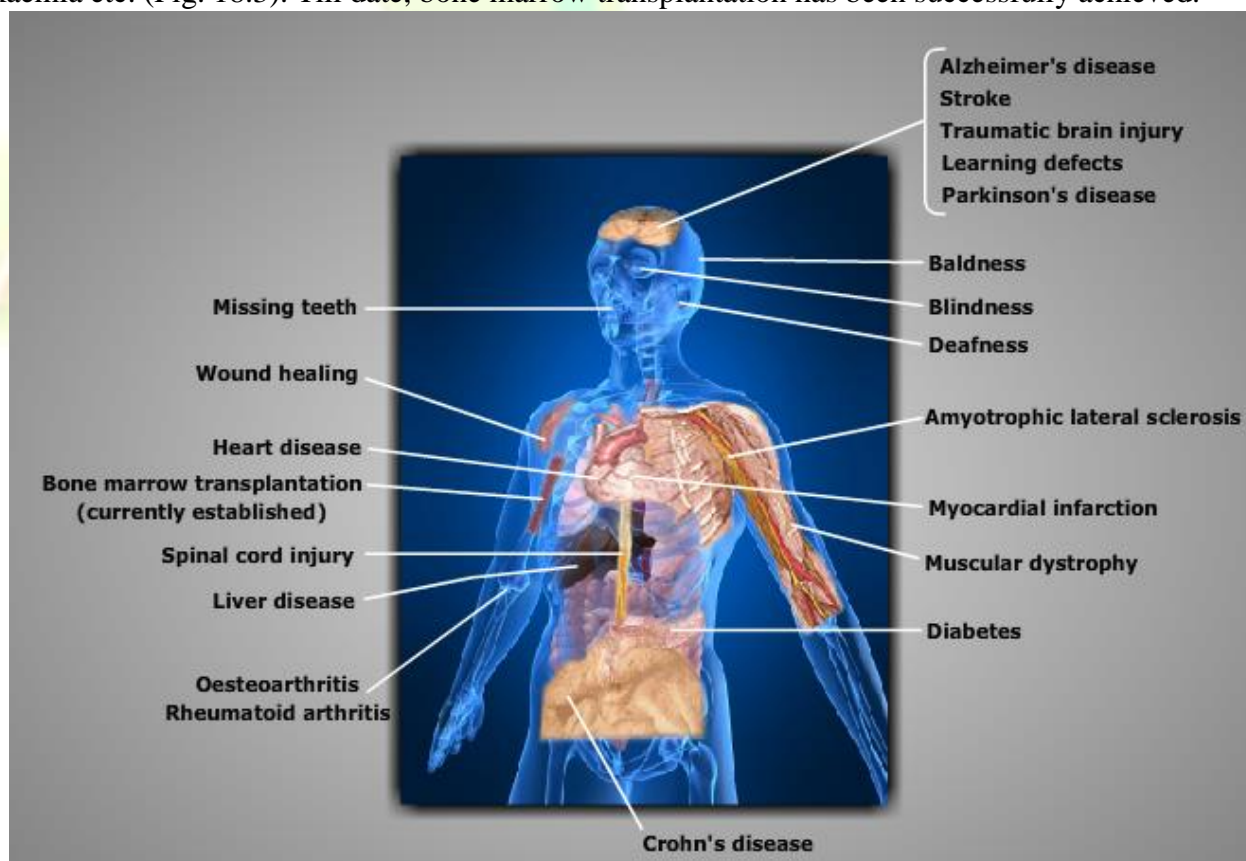


Fig. 18.3 Stem cell therapy of human diseases

2. Stem cells are used in regenerative medicine for replacement of several cells and tissues as these can be directed to develop into heart muscle cells or insulin producing cells.

3. Stem cells can be genetically engineered to accomplish activities that they would not normally be programmed to do. This approach can be used to deliver the chemotherapeutic agents for treatment of cancers and tumors.

18.5 Stem Cell Research in India

India is one of the few countries in the world actually pursuing stem cell research as at least 20 research organizations and 15 companies such as Reliance Life Science and Lifecell are working on stem cells. Reliance Life, the pioneer in stem cell-based research in India on a commercial scale, has commercialized two products i.e. ReliNethra, a first-of-its-kind treatment in India for corneal blindness and ReliHeal-G, which quickly heals wounds. The company has completed clinical trials for treatment for heart attack using stem cells from the bone marrow of the patient. It has also been involved in carrying out clinical trials for application of stem cell-based therapies for skin disorders like stable vitiliago, non-healing diabetic ulcers, Parkinson's disease and spinal cord injury.

LifeCell is involved in developing stem cell-based cardio vascular therapies in India. LifeCell is India's first private stem cell bank, where one can store the umbilical cord of the child for a fee, for future use. LifeCell has the largest network in India. The company has a network of over 50 centers in India and abroad. LifeCell started its operations in 2004 with cryogenic preservation of umbilical cord blood stem cells at its advanced facility in Chennai.

Stempeutics, funded by the Manipal Education and Medical Group, is planning to commercialize two drugs i.e. one for heart complications and the other for limb complications by 2011.

18.6 Stem Cell Therapy Centres in India

1. Tata Memorial - Mumbai
2. Adyar Cancer Center, Apollo Specialty Center- Chennai
3. Apollo Hospital, Global Hospital, NIMS - Hyderabad
4. Narayana Hruduyalaya, Manipal Hospital, Trinity Hospital -Bangalore
5. Christian Medical College - Vellore
6. R & R Army Hospital, AIIMS - New Delhi
7. Inlaks Hospital, Armed Forces Medical College - Pune
8. Sanjay Gandhi PGIMS - Lucknow
9. TRICELL - Chennai (A unit of Life Cell International Pvt. Ltd.)

18.7 Nuclear Cloning

Before learning nuclear cloning, let us be familiar with some terms related to the topic which will be used in this chapter.

18.7.1 What is a clone?

A clone is an exact genetic copy of a plant or animal or human being. It is defined as a group of genetically identical individuals descended from the same parent by asexual reproduction or group of genetically identical cells produced by mitotic division from an original cell.

The term '*clone*' is derived from the Greek word 'κλώνος', which means "trunk, branch", referring to the process whereby a new plant can be created from a twig.

18.7.2 What is a differentiated cell?

Multi-cellular organisms possess million of cells which function differently and become specialized. All cells originate from one unspecialized cell (undifferentiated) which then divides and become specialized cell by "Cell Differentiation" (Fig. 18.4). Differentiated animal cells can be induced to "start over" again and produce generic undifferentiated cells to develop a new organism. Dr. Wilmut succeeded by "reprogramming" a differentiated cell obtained from udder of an adult sheep.

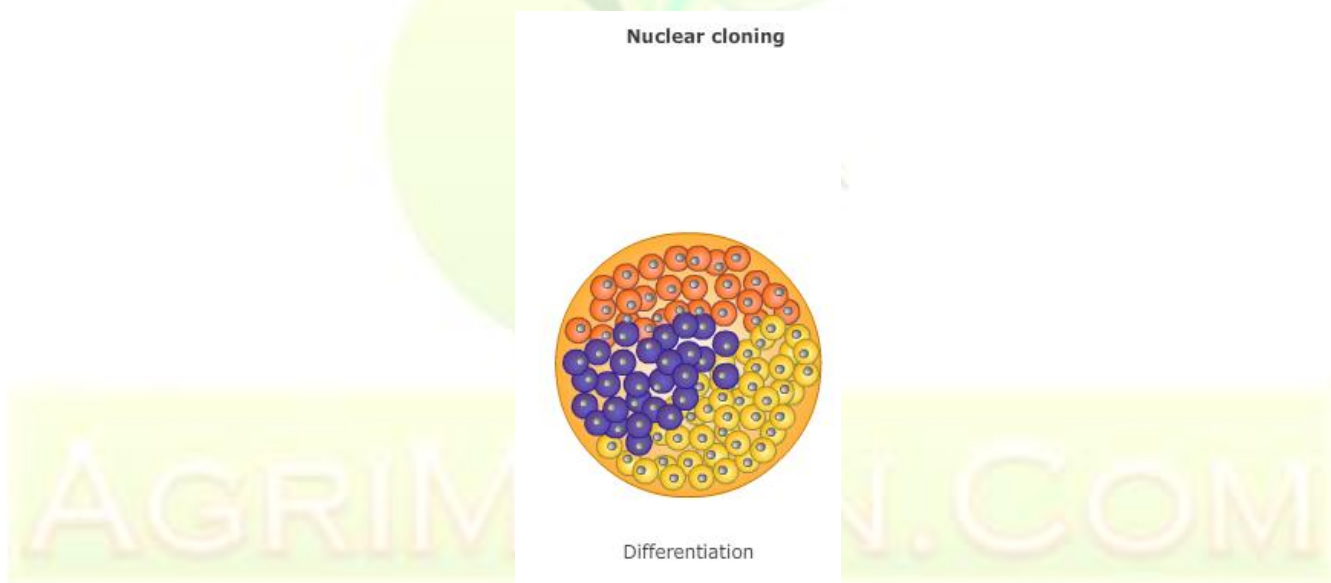


Fig. 18.4 Cell differentiation stages

18.7.3 Quiescence

Quiescence is the state of a cell when it is not dividing i.e. basic function of the cell is stopped. Quiescent cells cycling process is 'arrested' i.e. they stop dividing. Cell is not dormant or inactive e.g. Brain cells (neurons) – quiescent, yet busy in sending signals (Fig. 18.5).

Steps in Nuclear cloning

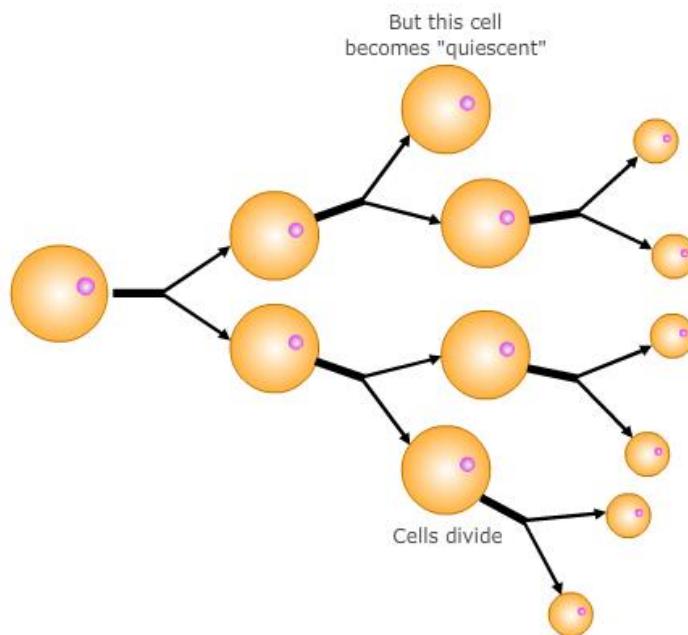


Fig. 18.5 Steps in nuclear cloning

18.7.4 Nuclear cloning or somatic cell cloning

Nuclear cloning also called somatic cell cloning is a technique in which the nucleus of a somatic cell is transferred into an enucleated oocyte for the generation of a new individual, genetically identical to the somatic cell donor. Various steps involved in nuclear cloning are shown in Fig. 18.6.

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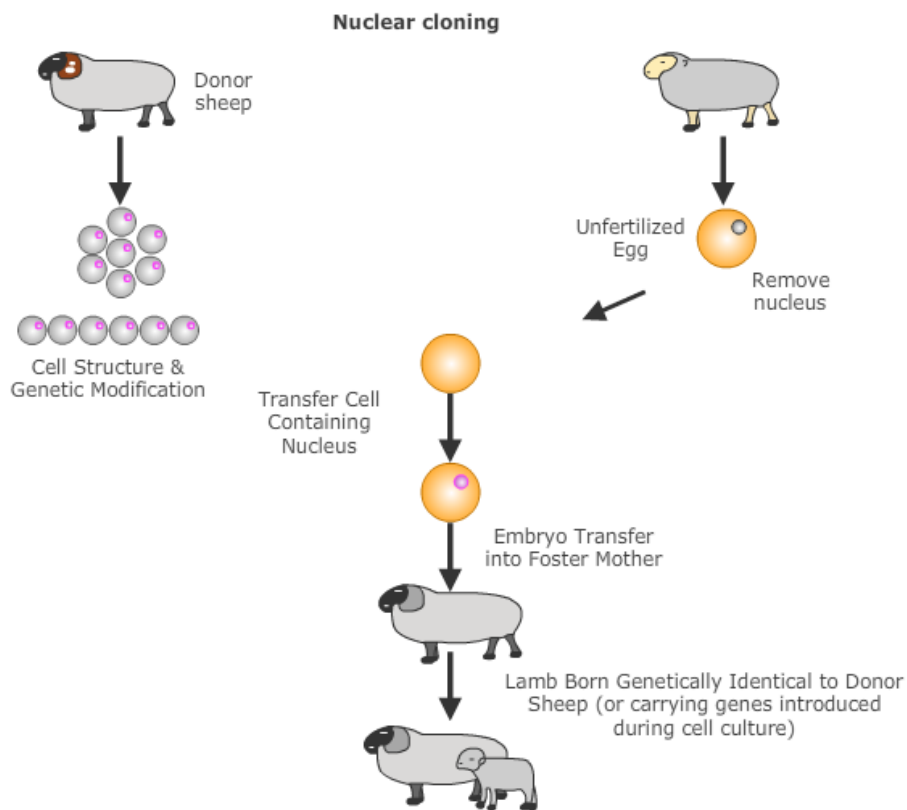


Fig. 18.6 Nuclear cloning

The steps involved in nuclear cloning are given below in a sequential order:

1. Cells are collected from a donor animal and cultured *in vitro*
2. A mature oocyte is then enucleated and a donor cell is transferred into the enucleated oocyte
3. The somatic cell and the oocyte is then fused
4. Embryo is allowed to develop into a blastocyst *in vitro*
5. The blastocyst can then be transferred to the recipient animal
6. Cloned animals are born after completion of gestation

18.7.4.1 Dolly, the first cloned Sheep

Dolly, a Finn-Dorset Ewe, was the first mammal to be cloned by Ian Wilmut, Keith Campbell and colleagues at the Roslin Institute near Edinburgh in Scotland by nuclear cloning technique.. Dolly was born on 5th July, 1996 and died at the age of six due to arthritis and lung disease. The cells used as donor were taken from the mammary gland. The success of cloning an entire animal, Dolly, from differentiated adult mammary epithelial cell created a revolution in science. This demonstrated that the genetic material from a specific adult cell, programmed to express only a distinct subset of its genes, can be reprogrammed to grow an entirely new organism. Later, this technique was successfully performed on several species including cat, rat, dog, monkey etc. and also for transgenic animals. Polly and Molly (Born 1997 - 2000), two ewes, were the first transgenic mammals to have been successfully cloned from an adult somatic cell. However, the difference between Dolly was that the nucleus that was transferred was from mammary gland whereas in Polly and Molly, it was from Fibroblast. Polly sheep clone produced Factor IX blood clotting factor.

18.7.4.2 Cloned Buffalo

Samrupa was the first cloned buffalo born on February 6, 2009, at National Dairy Research Institute, India. However, it died five days later due to lung infection. Later, National Dairy Research Institute (NDRI) succeeded once again in producing a cloned buffalo calf Garima I (Fig. 18.7a and 18.7b) on 6th June, 2010 and Garima-II weighing 32 kg, born through caesarian operation through the new and advanced 'Hand-guided Cloning Technique', at NDRI, Karnal, on August 22, 2010. In the hand-guided cloning technique, the nucleus from the egg is taken by a hand-held fine knife so that the genetic material such as chromosomes, etc. come from single donor's cell, that is, from one parent. Garima II is different from Garima I because, in this case the used donor cell was embryonic stem cell. In the previous cloning, the donor cell was from somatic cells. The donor embryonic stem cell was isolated from the eight-day old blastocyst. These cells were cultured up to 29 passages (117 days) till it expressed pluripotent marker and confirmed to be stem cell. The embryonic stem cells have better cloning ability as compared to somatic cells, as such the epigenetic reprogramming of these cells is much more efficient than the somatic cells, which are already differentiated and lineage committed.



Fig. 18.7 (a) Cloned buffalo calf Garima I at NDRI



Fig. 18.7 (b) One year old Garima I

National Dairy Research Institute, NDRI, Karnal also successfully cloned a male buffalo calf named 'Shresth' (Fig. 18.8) on 26th August, 2010 through the new and advanced 'Hand-guided Cloning Technique'.



Fig. 18.8 Shresth born at NDRI

Shresth weighing 41 kg was born through normal delivery with slight assistance carried out by doctors. This cloned buffalo calf is different from the earlier cloned calves as, in this case, the foster mother was provided opportunity for normal delivery. Shresth was produced from somatic cell from ear of 2 week old buffalo calf, and the embryo which led to successful pregnancy and normal delivery had remained frozen at -196°C for one week in liquid nitrogen and brought back to active life upon thawing at room temperature. The earlier two calves were born through caesarean operation and were produced by using cells from fetus and embryonic stem cell, respectively.

The hand-guided cloning technique developed at NDRI, is an advanced modification of the “Conventional Cloning Technique”. The new technique is less demanding in terms of equipment, time and skill. Immature oocytes were isolated from ovaries and were matured *in vitro*. These were then denuded and treated with an enzyme to digest the outer layer of oocytes called ‘zona pellucida’. The oocytes were then treated with chemicals to push their genetic material to one side of the oocyte. This protruded side was then cut off with the help of “hand held fine blade” for removing the original genetic material of the oocyte. The enucleated oocyte was then electrofused with single cell taken from colony of embryonic stem cells. The resulting embryos were cultured and grown in the laboratory for seven days to develop them to the stage of blastocyst. The blastocysts were then transferred to recipient buffaloes.

This technology could go a long way in increasing the number of superior milch buffaloes in India. Moreover, this technology can also help by breeding bulls in the shortest possible time.

18.7.5 Applications of Nuclear Cloning

1. With the help of nuclear cloning, thousands of genetically identical copies of mammals can be produced.
2. High quality offsprings with specifically selected traits like increased milk production in cows etc. can be produced.
3. It can also be used to produce transgenic animals for pharmaceutical protein production or xeno-transplantation or to preserve endangered species.
4. Somatic cloning is an essential tool for studying gene function, genomic imprinting, genomic re-programming, regulation of development, genetic diseases, and gene therapy.

18.8 Disadvantages of nuclear cloning

1. Low survival rate or low efficiency - 62% fetal loss rate in cloned animals compared to 6% in naturally fertilized animals.
2. High incidence of developmental abnormalities - More than 60% of cloned animals die shortly after birth due to weaknesses and dysfunctional organs.
3. Costly and inefficient.
4. Can lead to a monocultural animal population i.e narrowing of gene pool and thereby, can cut a population's genetic diversity to zero.



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Lesson 19

APPLICATION OF GENETIC ENGINEERING IN DAIRY STARTER CULTURES

19.1 Introduction

Currently genetic manipulation techniques are in extensive use and are playing an important role in enhancing the performance of microorganisms in dairy products processing. The two important variables that can be manipulated through genetic engineering for use in dairy field industry include

- i) Bacteria, used as starter cultures for the production of lactic acid, flavors, proteolysis and also in the by-product utilization and
- ii) Enzymes, used for various metabolic changes in dairy foods for bringing desirable changes.

Genetic manipulation of microorganisms with significant applications to foods was initiated by taking *Escherichia coli* as a microbial model because of its ease of growing and manageability. A number of genetic modifications can be made in industrial as well as laboratory strains of lactic acid bacteria. These include deletion of a gene from a strain; replacing a gene with a similar gene from another strain; replacing a gene in a strain with the same gene that has been modified *in vitro*; increasing the number of copies of a gene; introducing a new gene and using recombinant DNA as selection principle for the isolation of mutants with target DNA.

19.2 Target Areas for Biotechnological Applications in Dairying

Modification of genetic components of bacteria and manipulation of enzymatic action offer dramatic prospects in dairy industry. A potential application of genetic engineering for dairy industry is in the generation of enhanced starter cultures for yoghurt and cheese manufacture. Some of the target areas of genetic manipulation for dairy industry are given in Table 19.1

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Table 19.1 Target areas for genetic manipulation in dairying

Target area	Major organisms	Utility
Lactose utilization	<i>Lactococcus</i> spp., and <i>Lactobacillus</i> spp.,	Stable and rapid production of lactic acid
Proteolytic activity	<i>Lactococcus</i> spp., and <i>Lactobacillus</i> spp.,	Enhanced proteolysis
Citrate utilization	<i>Lactococcus lactis</i> ssp <i>lactis biovar diacetylactis</i> <i>Leuconostoc</i> spp.,	Enhancing flavor production
Bacteriophage resistance	<i>Lactococcus</i> spp., and <i>Lactobacillus</i> spp.,	Preventing phage infections
Bacteriocins	<i>Lactococcus</i> spp., and <i>Lactobacillus</i> spp.,	Bio-preservation of dairy products
Microbial Rennet	<i>Aspergillus niger</i> o <i>Kluyveromyces lactis</i> <i>Mucor miehei</i> ,	Alternative to calf rennet
Proteolytic and lipolytic Enzymes	<i>Bacillus subtilis</i>	Accelerated ripening of cheeses
β -galactosidase enzyme	<i>Kluyveromyces lactis</i> <i>Asperigillus niger</i> <i>E. coli</i> , <i>Bacillus stearothermophilus</i> , Lactic acid bacteria	Accelerating the cheese ripening Tackling lactose intolerance
Epoxy Polysaccharides	<i>L. lactis</i> and <i>Lactobacillus casei</i> Thermophilic LAB	Improving the rheological properties of fermented milk products
Oral vaccines	<i>Lactococcus lactis</i>	Efficient vaccine delivery system

19.2.1 Metabolic properties of dairy starter cultures

Lactose utilization, proteolysis and citrate fermentation are the key industrially important metabolic functions of dairy starter cultures. The pioneering work on genetic engineering in dairy starter cultures was initiated by Larry McKay and co-workers in early 70's. Because of industrial relevance, *L. lactis* has been regarded as a model organism.

19.2.1.1 Lactose utilization

The primary function of starter cultures is the production of lactic acid from lactose which is mediated mostly by plasmid DNA in several lactic acid bacteria. There are ample chances for the loss of lac+ plasmid during the multiplication of starter cultures and hence lactic acid production has been reported to be unstable. The main biotechnological emphasis would be the stabilization lac+ in chromosome of LAB for sustained production of lactic acid from lactose. The first notable success was reported by McKay and Baldwin in 1978 by improving a strain by the stabilization of the lac/prt plasmid in chromosome. In the

last two decades, significant effort was put into the genetic investigations of LAB and the complete genome sequences have been published for four strains i.e. *Lactococcus lactis* IL1403 and MG1363, SK11 and KF147. Recently a nisin-producing starter culture that produced acid at rates suitable for Cheddar cheese manufacture was developed by combining the naturally occurring lactose-fermenting, nisin-producing, and proteinase-positive strain *Lactococcus lactis* ssp. *lactis* NCDO 1404 and the lactose-positive, nisin-positive, proteinase-positive transconjugant *Lactococcus lactis* ssp. *cremoris* JS102.

19.2.1.2 Proteolysis

Caseins are the most significant substrates for proteolysis in the preparation of fermented milks and in cheese ripening. Increasing proteolytic activity and improving starter culture's amino acid converting activities are the major aspects taken into consideration for cheese flavor improvement.

The enzymes involved are *proteinases*, either *endopeptidases* (with active sites in the interior of the polypeptide chain, yielding peptides as the primary product) or *exopeptidases* (with active sites at the ends of the polypeptide chain, yielding amino acids as the primary product). To stabilize these enzyme genes, food-grade approaches to genetic engineering of lactic acid bacteria rely either on a "chromosomal integration of a target gene" approach, or on the construction of food-grade vectors fulfilling the self-cloning definition. Overexpression of genes encoding components of proteolytic system has been achieved in many species of lactic acid bacteria under industrial conditions of cheese making.

19.2.1.3 Flavor production

The two important organisms used in dairy industry for citrate utilization for flavor development are *Leuconostoc* spp. and *L. lactis* subsp. *lactis* biovar *diacetylactis*. Citrate permease the key enzyme in citrate fermentation is linked with an 8-kb plasmid in *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*, whereas in *Leuconostoc* the citrate genes are associated with plasmids as large as 22 kb. This metabolic property is also not stable since they are coded by plasmids, the unstable DNA entities. Two promising approaches are visualized for stabilization of this metabolic property i.e. stabilization of plasmid DNA in LAB or inactivation of the gene encoding α -acetolactate decarboxylase (*aldB*), the enzyme that converts α -acetolactate to acetoin. The flavor and flavor stability of buttermilk was improved by inactivation of the *aldB* gene encoding α -acetolactate decarboxylase (Swindell et al. 1996). This later approach results in accumulation of α -acetolactate, the immediate precursor to diacetyl, which in turn leads to an increased concentration of diacetyl in the growth medium.

19.2.2 Bacteriophage resistance

Bacteriophage infection during the manufacture of fermented milk products is an ever-present danger leading to significant economic losses to fermented milk industry. To address this problem, development of bacteriophage resistance in starter cultures has attracted extensive scientific interest. Many phage-resistance plasmids are conjugative, and this factor has been exploited to improve the phage resistance of phage-sensitive commercial cultures. The technique is relatively simple and involves the isolation of the strain harboring the phage-resistance plasmid and mixed with lac⁺ phage^s recipients. Lac⁺ phage^r transconjugants are selected in the presence of excess virulent phage on lactose agar, which contains a dye to indicate acid production and checked for their ability to produce acid and for the presence of the phage-resistance plasmid. Bacteriophage resistant strains by conjugal transfer of plasmid encoding phage resistant determinants have been developed and successfully used at industrial level.

19.2.3 Natural biological inhibitors

Another starter enhancement that has been extensively studied using genetic manipulation in LAB is the production of small proteins called bacteriocins that inhibit related bacteria.

Some of the well characterized bacteriocins produced by starter bacteria have been shown in Table 19.2

Table 19.2 Examples of well characterized bacteriocins

Bacteriocin	Producer organism	Antimicrobial efficiency
Nisin	<i>Lactococcus lactis</i> subsp. <i>lactis</i> ATCC 11454	Broad spectrum,
Lactococcin B	<i>Lactococcus lactis</i> subsp. <i>cremoris</i> 9B4 <i>Lactococcus lactis</i> subsp. <i>cremoris</i> LMG 2130 <i>Lactococcus lactis</i> subsp. <i>lactis</i> biovar <i>diacetylactis</i> WM4	Narrow
Lactacin F	<i>Lactobacillus acidophilus</i> 11088	Narrow
Pediocin A	<i>Pediococcus pentosaceus</i> FBB61 and L-7230	Broad spectrum,
Curvacin A	<i>Lactobacillus curvatus</i> LTH 1174	Medium
Pediocin A _{CH}	<i>Pediococcus acidilactici</i> H	Broad spectrum,
Leuococin	<i>Leuconostoc gelidum</i> UAL 187	Broad spectrum,
Helveticin J	<i>L. helveticus</i> 481	Narrow spectrum

One such important antimicrobial substance, nisin, is produced by the "food grade" starter strain, *Lactococcus lactis* ssp. *lactis* and is used as a natural preservative in food systems. It has activity against a wide variety of Gram-positive bacteria, including food-borne pathogens such as *Listeria*, *Staphylococcus* and *Clostridium* spp. Genetically engineered *Lactococcus* spp. showed enhanced production of Nisin due to the introduction of multiple copy genes like *nisZ*, *nisRK*, or *nisFEG* that are responsible for Nisin production. María *et al.* (2010) developed a *Lactococcus lactis* UQ2 strain that performed well in milk and synthesized 200 IU/mL nisin, 40 times more than the original strain by conjugal mating.

19.2.4 Rennet Substitutes

Rennet is the key enzyme used as a milk-clotting agent during the production of cheese. Due to acute shortage of calf rennet and or socio-cultural reasons, there is a great emphasis for the use of rennet substitutes due to reduction or elimination of the usage of conventional rennet obtained from calf stomach.

One of the most success stories of commercial genetic engineering is the manufacture of recombinant chymosin. The gene coding for the chymosin enzyme has been cloned into several microorganisms and commercially exploited by successful expression in bacteria (*Escherichia coli*, and *Bacillus* spp.), yeast (*Kluyveromyces lactis*) and moulds (*Mucor miehei*, *Mucor pumilus*, *Aspergillus niger*). Manufacturers claim that these enzymes cause more consistent milk clotting and have better proteolytic activities, offering a more standardized product. This rennet substitute has more predictable properties than animal-derived chymosin and has fewer impurities. In future, genetic manipulation of enzyme together with scientific advances in understanding the protein structure, allow the possibility of improving the properties and making specific changes in the structure of the enzyme.

19.2.5 Accelerated cheese ripening

Traditional ripening of cheese takes 6 to 12 months depending on the type of cheese under rigorously controlled conditions. This enormously increases the cost of cheese production due to maintenance of refrigeration of ripening rooms and blockage of capital cost. Now the enzymes synthesized from genetically modified bacteria like proteinases, lipase and β -D-galactosidase are used for enhancing the ripening of cheese. So far, the most effective example of accelerating the cheese ripening process by metabolic engineering has been the nisin-induced expression of bacteriophage lysin and holin in *L. lactis*, resulting in complete lysis of the cells, complete release of peptidases and of other enzymes and a sharp increase in production of free amino acids and flavor compounds in cheese. Yet another example for commercially successful enzyme is Neutralse produced from *Bacillus subtilis* which is reported to be helpful in decreasing the ripening period by 50%.

19.2.6 Tackling lactose intolerance

Lactose intolerance problem has gained worldwide dimensions as a vast majority of population is not able to digest the lactose due to inability of synthesizing lactase enzyme in their intestine. Biotechnology has offered a solution by utilizing β -D-galactosidase to hydrolyse lactose into glucose and galactose. This enzyme is commercially obtained from yeast (*Kluyveromyces fragilis*), fungi (*Aspergillus niger*) Bacteria (*E. coli*, *Bacillus stearothermophilus*, lactic acid bacteria used as dairy starters)

Ex: In USA Lactaid, a brand of modified milk is available in which 70% of lactose is hydrolysed.

19.2.7 Microbial epoxy polysaccharides

The conventional hydrocolloids of polysaccharides are derived from gums, mucilages and extracts of plant origin. These are having different functional properties owing to their molecular structural diversity and are useful in providing stability and preventing wheying off of fermented products. Due to high cost and increasing demand for hydrocolloids, attention has been shifted for the development of bacterial cultures capable of producing polysaccharides and having synergistic growth capability with other dairy cultures. FAO has already permitted many microbial polysaccharides and some are under toxicological safety testing.

EPS synthesis might be plasmid mediated as seen in *L. lactis* and *Lactobacillus casei*, or located on the chromosome, as the case may be in all the thermophilic LAB. Enhanced EPS synthesis can be achieved by

- a. Complete gene clusters, encoding exopolysaccharide producing enzymes can be transformed from one LAB strain to another one and the newly generated strains could influence viscosity and texture of the fermented product (Germond *et al.* 2001)
- b. In *S. thermophilus*, the phosphoglucomutase gene was inactivated resulting in improved exopolysaccharide production enhancing the viscosity of the fermented food product (Levander *et al.* 2002).
- c. Using a self-cloning strategy also, exopolysaccharide production in *L. lactis* can be enhanced

19.2.8 Vaccine delivery

Recently *Lactococcus lactis* has been shown to deliver antigens that stimulate mucosal immunity to non

respiratory pathogens as well as Human papilloma virus and the malarial parasite. Lactic starter cultures also possess strong adjuvant properties that can also be explored during oral vaccine development for boosting antibody production in the host. *L. lactis* being the non-pathogenic food grade bacteria shows much efficacy as live antigen and enzyme carriers, thus it proves beneficial for the oral administration than the attenuated pathogenic microorganisms like *Salmonella typhi* and *Vibrio cholerae*. The bacterium can be genetically engineered to produce proteins from pathogenic species on their cell surfaces. Intra nasal inoculation with the modified strain will elicit an immune response to the cloned protein and provide immunity to the pathogen.

As per the reports available, *Lactococcus* spp., could be engineered to present the conserved portion of the streptococcal M protein required for streptococcal adherence and colonization to the nasopharyngeal mucosa, in order to provide immunity to *Streptococcus pyogenes*. Consequently, the resulting local immune response could protect the individual from strep throat caused by the streptococcus. Similar approach can be used for other human pathogens such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycobacterium tuberculosis* and *Neisseria meningitidis*. Moreover, *L. lactis* is non-invasive and does not colonize the gut and thus has less potential to trigger immune-tolerance or side effects upon prolonged use

19.2.9 Other areas of importance

a. Single cell protein (SCP) represents microbial cells (primary) grown in mass culture and harvested for use as protein sources in foods or animal feeds. Synthesis of SCP by employing genetically engineered yeast cultures of *Saccharomyces* spp. and *Kluyveromyces* sp. has been successful and is in wide use.

b. Many health benefits have been identified with probiotic use and it is possible to identify several desirable enhancements that might be made to existing probiotic bacteria. The combination of a probiotic and a prebiotic is known as a 'synbiotic' and the prebiotic is thought to enhance the survival of the probiotic. The desirable property could be the generation of probiotics that are genetically modified to digest speciality 'prebiotic' carbohydrates. There is considerable interest at the present time in identifying novel prebiotic oligosaccharides and synbiotic combinations and many new possibilities would be offered if this activity were to proceed in parallel with the development of probiotic strains designed to metabolize them.



Lesson 20
ACCELERATED CHEESE RIPENING

20.1 Introduction

Ripening of cheese refers to physical and biochemical changes that take place in cheese when it is being held under specific and controlled conditions. Ripening process is considered as the most crucial step in cheese making as it has greater influence on the development of characteristic cheese flavor. Most varieties of cheeses require 6-12 months of ripening period depending on the type of cheese and/or conditions of ripening. Two important parameters that influence cheese ripening are temperature and relative humidity.

20.2 Ripening Changes

A series of microbiological and biochemical changes that occur during ripening process are responsible for converting fresh, bland cheese curd into a final mellow, waxy product having a characteristic taste, aroma and texture. Ample information with regard to contribution of milk components to maturation is available in most varieties of the cheeses due to break down of components by enzymes derived from rennet, milk and added cheese starters to control the overall intensity of flavor.

20.2.1 Physical changes

Gradual change occurs from rubbery curd of green cheese to the mellow, waxy ripened product, due to proteolysis and velvetiness of cheese due to lipolysis.

20.2.2 Chemical changes

The major chemical changes occurring during ripening are

- (i) glycolysis i.e. conversion of residual lactose into lactic acid and metabolism of lactate and citrate yielding end products such as diacetyl, acetyl methyl carbinol etc;
- (ii) proteolysis, wherein proteins are broken down to amino acids and further to simpler compounds like amines (Histamine etc.); and
- (iii) lipolysis which involves hydrolysis of milk fats to fatty acids, further leading to the formation of keto-acids etc.,

Among these, proteolysis is considered to be the most important change occurring in cheese and often used as ripening index of cheese.

20.3 Approaches for Accelerating Cheese Ripening

The ripening of cheese is a slow and consequently an expensive process as it involves heavy expenditure on refrigeration and labour apart from resulting in loss of product due to microbial damage. Attempts are being made to reduce ripening period by accelerating the bio-chemical changes. Two important approaches employed or tried by the cheese industry for accelerating the cheese ripening process include (1) Technological and (2) Bio-technological.

20.3.1 Technological approaches

Technological approaches involve the manipulation of curing conditions such as elevated temperatures or by increasing the moisture content of cheeses and making use of these as cheese slurries.

Cheese is ripened at elevated temperature i.e. at 15°-20°C for about 2-3 weeks depending upon the required flavor intensity and on the type of cheese before transferring to traditional normal temperature of ripening. As a result of elevated temperature, bacterial growth is stimulated resulting in increased release of enzymes for bringing about enhanced proteolytic and lipolytic changes. However, controlling the temperature is very critical as even 1°C rise in normal ripening temperature results in improper balance between proteolysis and lipolysis.

Cheese slurries are prepared by raising the moisture content in the product by adding at various stages as per the convenience and incubating at higher temperatures (30°C) for few days (7-10 days). The slurries containing 40% total solids are being reported to have developed strong flavor in days rather than in months. However, these methods suffer due to lack of control and often the overall flavor scores of such cheeses were depressed by a higher incidence of off flavors. Moreover, with cheese-slurry method the contamination with undesirable organisms is quite possible because of exposure to air, or due to aeration during incubation. In view of such drawbacks, development of flavor through natural and rapid methods by biotechnological approaches for accelerating the ripening process is most promising.

20.3.2 Biotechnological approaches

Biotechnological approaches involve addition of exogenous enzymes (proteinases, peptidases, and lipases), use of attenuated starter cultures, adjunct cultures, or genetically modified lactic cultures.

20.3.2.1 Exogenous microbial enzymes

Addition of exogenous enzymes enhances the enzyme pool and thus can help in accelerating the ripening changes. These include addition of β -galactosidases, proteolytic enzymes, lipolytic enzymes or mixture of these.

20.3.2.1.1 β -galactosidase

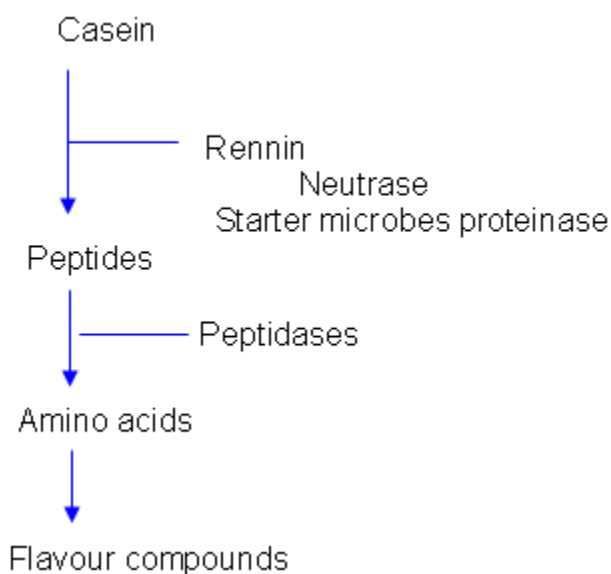
β -galactosidase hydrolyses the lactose in milk yielding glucose and galactose. Glucose is better utilized by the microorganisms than other carbohydrates and thus helps in rapid proliferation of microbes. β -galactosidase helps in accelerating the cheese ripening by reducing the cheddaring time due to faster development of acidity due to enhanced growth and activity of microbes and reduces ripening period by providing proteinases indirectly due to increased population of starters. The basis of the increased flavor may have been due to more rapid proteolysis in cheeses caused by either higher proteinase content contributed by increased populations of starter bacteria or by contaminating proteinases of β -galactosidase preparation. However, clear distinction is still under investigation.

20.3.2.1.2 Proteinases

Proteinases include chymosin, natural proteinases present in milk, and proteinases produced from starter and non-starter microorganisms. Proteinases and peptidases break down proteins to peptides and further to free amino acids. Proteinases reduce the ripening period due to availability of increased substrate resulting from casein hydrolysis for microbial peptidases, thus accelerating production of flavor precursors and flavor compounds.

Three different types of proteinases namely acid proteinases, alkaline proteinases or neutral proteinases can be added. Acid and alkaline proteinases produce weak, soft body with crumbly texture, while neutral proteinases produce good quality cheese. The proteinases derived from *Aspergillus* spp. *Pseudomonas fluorescens*, *Micrococcus caseolyticus*, *Bacillus subtilis* etc have been tested. Although the ripening period can be reduced by the use of proteinases by 35 to 50%, it results in development of bitterness which is undesirable and requires more attention.

Peptidase extract alone results only a minor effect on flavor intensity because no additional proteolysis can be achieved to provide more substrate peptides. If only proteinases are added, then more peptides are accumulated resulting bitterness. Instead of using proteinases or peptidases alone, a combination of proteinases and peptidase is being increasingly used. When they are added in combination, proteinases form peptides and peptidases utilize these peptides forming free amino acids.



20.3.2.2 Lipases

Lipolysis is desirable in strongly flavored cheeses such as Italian and Blue veined cheeses but not in delicately flavored varieties like cheddar.

20.3.2.3 Mixtures of enzymes

For proper and balanced flavor development in cheese it is preferred to use a mixture of enzymes as they enhance the rate of multiple reactions aimed at increasing or modifying particular aspects of cheese ripening instead of individual enzymes which can control only one particular type of reaction during ripening. Few examples for commercially available cheese enzyme mixtures are Accelase™, Neutrased™, DCA 50 etc.

20.3.2.4 Methods of enzyme additions

Enzymes are added to milk or to cheese curd at different stages. Addition of enzymes to milk in solution form helps in uniform mixing and final homogenous distribution of enzymes in curd. It also ensures

maximum interaction between coagulated particles and enzymes. The limitation of such additions is the loss of about 60-90% of enzymes in whey as they are water soluble contaminating the whey and thus making it unsuitable for other purposes. In addition, this protocol often results in poor gel strength and cause difficulties during working of the cheese curd for expulsion of moisture due to early breakdown of casein. Alternatively enzymes are mixed with salt and sprinkled on the surface of cheese curd after milling, wherein salt acts as vehicle. However, the major limitations of salt enzyme mixtures that the salt dissolves and penetrates the curd more rapidly than the enzymes and leaving the enzymes to remain on surface and developing 'Hot spots'. The other defects are mottled wavy colour defects. Moreover, this method is not suitable for surface-salted cheeses.

20.3.2.5 Microencapsulation

Another promising method of exploiting enzyme technology for accelerated cheese ripening includes encapsulation of enzymes. In this method, enzyme of interest is encapsulated in some type of artificial sacs usually with the object of protecting it from particular environment. The sac dissolves at the required target site and stage, and releases the enzyme for its designated action. In cheese manufacture, microencapsulation helps in entrapment of more enzymes in cheese curd. Various substances have been used for the encapsulation of enzymes but most widely used ones are liposomes. These are artificial sacs/capsules in micro vesicle form consisting of a central aqueous core surrounded by one or more eccentric layers of fat. Liposomes are formed from phosphatidyl choline. These are stable in milk for long time enough to be included in curd and being pressed along with curd. Size of liposomes is ~1 micron. When added to milk, because of its particulate nature, majority of them are retained and entrapped during coagulation in curd matrix and thus enzyme loss in whey is minimized. The encapsulated enzymes are released subsequently due to the dissolution of capsule by acid environment or other causes and the liberated enzymes cause biochemical changes. This method has several advantages such as simplicity in addition, reduction in loss of enzyme in whey (only less than 10% of enzyme is lost), maintaining flavor balance as there will be regulated seepage of enzyme from capsule into cheese, absence of body and textural defects as enzymes are uniformly distributed in addition to providing better and good mouthfeel as it contains high fat due to presence of capsules.

20.3.2.6 Manipulation of starters

Many of starter organisms produce proteinases and peptidases. For accelerated cheese ripening, addition of increased volumes of starter culture or enhancing their growth is necessary for producing more proteinases or peptidases. But increasing the starter culture would affect the manufacturing of cheese and produce "atypical" cheese by increased production of acid and hence modification of cheese starters is necessary. The concept is based on the augmentation of normal starter inoculum with starter preparations which have been treated in such a way so as to prevent them from metabolizing and producing acid during cheese making but yet have their important degradative enzymes left intact.

20.3.2.7 Attenuated starters

Attenuated starters are the modified lactic acid bacteria that lack the ability of producing sufficient levels of acid during the cheese making but provide active enzymes which can play a significant role in maturation and flavor development in cheese. However, attenuated starters are always added to cheese milk along with regular starter cultures. Various approaches for the production of attenuated starters are

heat or freeze-shocked cells, solvent treated cells, natural mutant starters, lysozyme treatment, spray drying etc.

In one of these approaches i.e. heat-shocking of starter cells by sublethal heat treatment at 60°C or 70°C for few seconds substantially delays lactic acid production of mixed strain mesophilic starter cultures or thermophilic streptococci and lactobacilli, while only marginally reducing their proteinase or peptidase activity. Several studies have shown that addition of heat shocked cells significantly reduces the period of cheese ripening and intensifies the flavor production. But heating procedure was found to be critical since slightly higher temperatures caused dramatic inactivation of proteinases and some of peptidases. The cost is prohibitively high, as higher volumes of heat shocked cells ($>10^9$ cfu/g) is necessary for bringing about desirable effect.

Freezing and thawing of lactic acid bacteria kill them without inactivating their enzymes. For this kind of attenuation, concentrated bacterial cells are subjected to freezing at -20°C overnight and rapidly thawing the cells to 40°C . The other methods of attenuation are freeze drying or spray drying of concentrated starter cultures. However, these methods have not gained much commercial value.

In lysozyme treated cells, the lysozyme hydrolyses β 1-4 linkage of peptidoglycan of bacterial cell wall i.e. linkage between N-acetyl Muramic acid and N- acetyl Glucosamine, resulting in weakening of cell wall followed by lysis of cell. Lysozyme treatment has been used to prevent acid production by starters. However, the enzyme is too expensive to apply to large scale cheese making and the results with cheddar cheese suggested that typical flavor was only marginally increased even by increased starter proteinase and peptidase activity equivalent to 10^{10} cells/g cheese.

In case of solvent treated cells, treatment of starter cell suspension with organic solvents results in activation of some membrane bound proteolytic enzymes. Now it is possible to generate cells which do not produce acid but have 10 times greater peptidase activity than normal cells. But this approach suffers due to prohibition by regulatory authorities across the globe.

20.3.2.8 Use of mutant starter strains

It consists of lactase negative (Lac^-) or proteinases negative (Prt^-) or lactase negative proteinases positive ($\text{Lac}^- \text{Prt}^+$) derivatives which have also been found to significantly influences ripening changes in cheeses. These mutant strains can be obtained either by selection from natural pool of mutants or through induction of mutation using x-rays or chemicals such as ethidium bromide, nitrosoguanidine (NTG) etc., The Lac^- strains of LAB with higher proteinases and or peptidase activity are the most promising ones. Extensive research on the use of these mutant strains by and large has been highly promising till date, but their commercial use is yet to be explored.

20.3.2.9 Adjunct starters

This approach relies heavily on the use of lactobacilli which intensify the cheese flavor production when used in combination with lactococci. It is believed that addition of small volumes of these adjunct lactobacilli modify the proteolysis resulting in higher concentration of amino acids. The lactobacilli can be mesophilic or thermophilic strains. In comparison with mesophilic strains, the thermophilic lactobacilli die out rapidly during cheese making, lyse and release intracellular proteolytic enzymes to produce higher

amounts of free amino acids. Thermophilic lactobacilli do not grow in cheeses like cheddar, but when added with other cheese starters intensify the flavor production.

20.3.2.10 Genetic engineering

The decoding of complete genome sequence of *Lactococcus lactis* ssp. *lactis* IL 1403 strain has raised the hopes of exploiting the genetic engineering of starter cultures as the best options available for finding a viable, sustainable and promising tool for accelerating the cheese ripening. Most of the strains of starter cultures used in cheese industries can be modified by DNA recombination mechanisms. It is possible to produce starter cultures expressing proteinases and many intracellular enzymes which play significant role in secondary proteolysis during cheese ripening. The plasmid controlled characters like lactose utilization; protein metabolism and citrate metabolism can be transferred to suitable recipient strains. For example, more proteolysis producing strain can be allowed to take up lactose utilizing plasmid (if it is lac⁻) and thus two characters can be combined.

Genetic engineering research is also capable of delivering the technology to make cultures tailored to specific cheese flavor and texture profiles. However, identifying the key enzymes and their role in ripening the cheese is yet to be established and in the absence of such knowledge, the exploitation of genetic engineering for acceleration of cheese ripening will be a limiting factor, in spite enormous potential of this technology. In future, if this technique is specially developed by the dairy researchers to be safe and food compatible, it will play a vital role in proving the efficacy of this metabolic, rather than enzymatic approach.



Lesson 21

DAIRY ENZYMES AND WHOLE CELL IMMOBILIZATION

21.1 Introduction

Enzymes are complex proteins which act as catalysts to accelerate the chemical reactions of living cells and bring specific chemical changes for converting specific set of reactants or substrates into specific products, without being changed themselves. These are not permanently modified by their participation in reactions and have a great specificity. In dairy industry, the use of enzymes, particularly exogenous enzymes are not fully exploited and limited to a few major and some minor applications. Enzymes play important role in the preparation of certain dairy products like cheeses, yoghurt etc., by improving texture, flavor and bringing about desirable changes in the product. Lipolytic and proteolytic enzymes can accelerate the production of flavor compounds. Successful use of preparations containing these enzymes is complicated by the need to attain a satisfactory balance among the various enzymes involved in the cheese ripening process.

21.2 Important Enzymes Used in Dairy Industry

The most important enzymes used in dairy industry are rennet, protease, lipases, lactase etc. as the principal constituents of milk are proteins, lipids and lactose.

21.2.1 Rennet

Rennet, an exogenous enzyme, is used as a milk-clotting agent in cheese industry for the manufacture of quality cheeses with good flavor and texture. The action of milk clotting enzyme i.e. rennet in cheese making is splitting of κ -casein which causes destabilization of casein micelles and subsequently leads to the formation of a coagulum.

21.2.2 Proteases

These are enzymes that are added to milk during cheese production for hydrolyzing caseins, specifically κ -casein, which stabilizes micelle formation preventing coagulation. Certain proteinases are used in enhancing the cheese flavor and also in acceleration of ripening process. An extract of *B. subtilis*, which contains a neutral proteinase, active at higher temperature seems to be quite suitable in this regard. Moreover, it has the advantage of being barely active below 8°C, and even sometimes inactivated at lower temperatures. Hence, over ripening can be prevented. Flavor defects can be kept to a minimum by combining the endopeptidase activity (catalyze the hydrolysis of peptide bonds in the interior of peptide chain or protein molecule) of enzyme preparations with the exopeptidase activity (catalyze the cleavage of the terminal/last or next-to-last peptide bond from a polypeptide or protein, releasing a single amino acid or dipeptide) of extracts of lactic acid bacteria.

21.2.3 Lipases

These are used in the preparation of stronger flavored cheeses, such as Italian, Romano cheese etc., by breaking down milk fats and giving rise to characteristic flavors to cheeses. Lipase acts on Triglycerides

and on hydrolysis yields fatty acids, partial glycerides plus glycerol. This process is very critical in natural cheese making and should be controlled as milk fat contains high levels of short chain fatty acids, which when in free form are very volatile and have low flavor threshold.

21.2.4 Beta-galactosidase (lactase)

It helps in the hydrolysis of lactose to glucose and galactose, and hence play a significant role in dairy processing. Lactose intolerance in humans is characterised by the typical symptoms like severe tissue dehydration, diarrhoea, and at times, even death and such condition results due to the lack of ability of individuals to synthesize lactase enzyme which hydrolyses milk sugar i.e. lactose. Hence exogenous lactase is used in preparation of lactase-treated milk and in the manufacture of lactose-free products, particularly milk, for such individuals. Other applications of lactase enzyme include modification of functional properties and preparation of dietetic products, in accelerating the process of cheese ripening.

The other enzymes that have limited applications in dairy industry are catalase, lactoperoxidase, and lysozyme.

21.3 Immobilization of Enzymes

It is always cost-effective to use the enzymes more than once. Most of the enzymes are in solution with the reactants and/or products and hence it is difficult to separate them after completion of chemical reaction. In order to reuse the enzymes again after separation from the products during chemical reaction, it is necessary to employ techniques that are helpful in attaching the enzyme to the reactors. This idea has led to the employment of immobilization techniques for enzymes. The concept of enzyme immobilization was first evolved, when difficulties were experienced during the use of crude enzyme preparations in the production of wine, cheese or in tanning. The phenomenon of immobilization of enzyme on a support, was first reported by J.M. Nelson and E.G. Griffin in 1916. They reported the adsorption (immobilization) of invertase on charcoal/alumina without loss of activity. However, the technique of enzyme immobilization could be established only after a lapse of about 40 years, in 1954- 1961 when many researchers developed relevant procedures and the equipments.

In simple terms "immobilized" means unable to move or stationary. An **immobilized enzyme** is an enzyme which is attached to an inert, insoluble material such as calcium alginate over which a substrate is passed and converted to product. This technique has revolutionized the prospects of enzyme application in industry.

Immobilization is defined as the imprisonment of a biocatalyst in a distinct phase that allows exchange with, but is separated from, bulk phase in which substrate, effector, inhibitor molecules are dispersed and monitored.

Immobilized enzyme is physically entrapped or covalently bonded by chemical means to an inert and usually insoluble matrix, where it can act upon its natural substrate. The matrix is usually a high molecular weight polymer such as polyacrylamide, carrageenan, chitan, cellulose, starch, glass beads, etc.

The use of insoluble form of an enzyme in a process offers a number of advantages as given below:

- Immobilization allows separation of enzymes from the products after completion of chemical reaction and thus can be reused or recycled

- Immobilized enzymes have ability to bind to a matrix, by which it typically possess greater resistance to change in pH and temperature and have operational stability than the soluble form of the enzyme.
- Reaction mixture or products specifically contain only solvent and reaction products and so more or less do not require complex purification as the processed product is not contaminated with the enzyme.
- Immobilization improves the efficacy and efficiency of an enzyme
- Certain manipulations of chemical reactions are better possible with immobilized enzymes

21.3.1 Methods of immobilization

It is very important to choose a method of attachment that will prevent loss of enzyme activity by not changing the chemical nature or reactive groups in the binding site or active site of the enzyme during its immobilization to a surface. The selection of immobilization method depends upon the application as well as the resources available.

The Traditional Methods of Enzyme Immobilization can be classified as Follows

21.3.1.1 Carrier binding

In this method, the enzymes are bound to water in-soluble carrier molecules. Based on the technique of binding, this is further divided into

a. Physical adsorption

This is the immobilization of an enzyme on the surface of water-insoluble carriers.

b. Ionic binding

This process involves ionic binding of the enzyme to water-insoluble carriers containing ion-exchange residues as indicated in Fig 21.1. e.g. *Polysaccharides* and *synthetic polymers* having ion-exchange centers

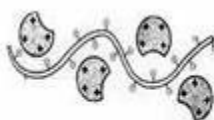


Fig.21.1 Ionic Binding

c. Covalent binding

This is based on the binding of enzymes and water-insoluble carriers by the formation of covalent bonds between the enzyme and the support matrix (Fig 21.2) e.g. Glutaraldehyde

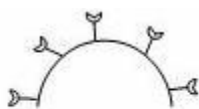


Fig.21.2 Covalent Binding

21.3.1.2 Cross linking

This is the process of intermolecular cross-linking of enzymes by bi-functional or multi-functional reagents (Fig 21.3).



Fig.21.3 Cross linking

21.3.1.3 Entrapment

Here enzyme is trapped in insoluble beads or microspheres i.e. incorporating enzymes into the lattices of a semi-permeable gel or enclosing the enzymes in a semi-permeable polymer membrane (Fig 21.4).



Fig.21.4 Entrapment

21.3.2 Application of immobilized enzymes

Application of immobilized enzymes is increasing day by day and the Table 21.2 given below elucidates the use this technology in various food applications.

All About Agriculture...

Table 21.1. Immobilized enzymes and their uses

Industry	Enzymes	Application
Cheese	Renin, Lipase	Curdling and lipolysis for flavor production
Beverage industry	Enzymes from yeast	Convert sugars to alcohol and CO ₂ .
Juice and wine processing	Pectinases	Hydrolyse the pectin making the juice or wine clear.
Chocolate and candies	Invertase	Manufacture of chocolate covered berries and other such candies.
Soft drinks industry	Glucose isomerase	Production of fructose and high fructose syrups from hydrolysed corn starch.
Ice-cream industry	Lactase	Prevention of lactose crystals in ice-cream.

21.4 Whole Cell Immobilization

The term “immobilized cells” refers to keeping the cell in one place. Generally, in a reaction environment, cells are floating around in nutrient liquid whereas in cell immobilization, the cells are trapped or stuck to a sticky surface while nutrient flows over them. This method is more appropriate and useful when there is a need for using or when a series of enzymes are required in the process.

Whole cell immobilization was defined as "the physical confinement or localization of intact cells to a certain region of a space with preservation of some desired catalytic activity", and is a process that often mimics what occurs naturally when cells grow on surfaces or within natural structures.

Full-scale systems using immobilized higher cells came into use in the eighties. A well-known example is the plant of Bayer, USA, to produce factor VIII that plays a key role in blood clotting. Here, the cells are not immobilized on a carrier but retained by a membrane in a continuously operated system.

21.4.1 Advantages of whole-cell immobilization

- Enzyme isolation and purification steps are not required
- Show the higher stability in the catalytic power and enzyme activity,
- Have multivariate enzyme applications, eliminating the need for immobilization of multiple enzymes
- Intracellular enzymes need not be extracted prior to the reaction; they may be used directly
- The end products can be recovered in a simple manner.
- The technique is cost effective

21.4.2 Disadvantages

- Low productivity
- Lower resistance
- Limitation of mass transfer
- Problems with degradation of product
- Byproducts are formed due to lysis of cells or toxic metabolites

21.4.3 Methods of whole cell immobilization

Most of the cell immobilization techniques are modifications of the techniques developed for enzymes. The techniques used for whole cell immobilization are briefly described below:

21.4.3.1 Binding

These methods are further classified based on the chemical forces used in the binding process of microbial cells with carrier molecules.

a. Weak bonds

Flocculation: The microbial cells are encouraged to aggregate to form clumps and thus resulting in floc formation under the influence of the weak chemical forces such as Van Der Waal forces, hydrogen bonds etc. However, such weak chemical forces are strongly influenced by the pH, ionic strength and reagents that influence flocculation.

Adsorption: Living microbial cells are weakly adsorbed onto the non-charged surfaces with Van Der Waal forces, hydrogen bonds and hydrophobic interactions, so that the cells can be easily shed from the surface under the influence of shear forces of flow. However, the microbial cells will be replenished due to continuous multiplication with the passage of time.

Ionic binding: This is mainly due to the ionic charge interactions between positively charged anionic ionic exchangers made of cellulose or sephadex and negatively charged microbial cells.

b. Strong bonds

Covalent binding: Covalent binding process involves the immobilization of whole cells on inert, stable carrier by covalent binding e.g. silanized or derivatized porous glass. The method is not very successful for immobilization of whole cells because of general toxicity of reagents used and low loading capacity of the carrier molecule, even though it is very effective and efficient in immobilization of enzymes.

Cross linking: Microorganisms are crosslinked by chemical substances, e.g., by glutardialdehyde. The surfaces (especially the proteins) of microorganisms are linked with the surfaces of other microorganisms by aldehyde groups of glutardialdehyde

21.4.3.2 Physical retention

The physical methods of whole cell immobilizations are broadly categorized as entrapment and membrane retention.

a. Entrapment

The method depends on the entrapment of microbial cells in the pores of polymer material. Microbial cells are entrapped by gels that permit the diffusion of small molecules, both substrate and product, at rates that are adequate for the cells' viability and functioning. Entrapment can be carried out using by introducing microbial cultures into polymerization medium i.e. cells are included in polymerizing monomer or into polymer solution which is then used to form flat, biologically active membrane. The variations in the entrapment procedures are as follows:

Ionotropic gelation: Ionotropic gelation is based on the ability of polyelectrolytes to crosslink in the presence of counter ions to form hydrogels. Entrapment by ionic gelation, especially in the form of alginate beads, is the most widely used method. Alginate is a polysaccharide that forms a stable gel in the presence of cations, such as calcium. Beads of alginate-containing cells, are formed by dripping a cell suspension-sodium alginate solution mixture into a stirred calcium chloride solution.

Polymerization: Gel entrapment by polymerization is most commonly carried out by using polyacrylamide. However, toxicity of cross-linking agents used in polymerization may cause a loss of cell viability. Among the polymerization agents available, k-carrageenan is reported to be more advantageous.

Microencapsulation: Microencapsulation is a process by which very tiny droplets or particles of liquids or solid materials or microbial cell suspensions are surrounded or coated with a continuous film of polymeric material. A prevalent objective is to protect the cells from degradation by reducing their reactivity to environmental conditions. Biopolymers, such as alginate, gellan-gum, xanthan, κ -carrageenan and more recently proteins are used as matrix materials are being widely used for microencapsulation

b. Membrane Retention

Semipermeable membranes with porous structures are used for the retention of microbial cells. The porous membranes offer the advantage of allowing the passage of substrates and products during reaction processing. The methods include dialysis tubing (a type of semi- or partially permeable membrane tubing made from cellulose or cellophane which selectively allows some molecules to pass through the membrane through passive transportation or active transportation), ultrafiltration units (hollow-fiber anisotropic membranes most commonly used for downstream processing of conventional fermentation).

21.4.4 Biomass production

Methods are used to support cell release from gel beads that occurs suddenly as microcolonies form near the surface of the biocatalysts. The release of cells growing in the peripheral layer of highly colonized gel beads can be used to efficiently produce biomass in the bulk liquid medium. This cell release activity can be used for producing single or mixed strain cultures and to continuously inoculate food liquids to process fermented foods such as fermented milk products.

21.4.4.1 Production of bulk lactic starter cultures

The LAB are largely used in single and mixed cultures for the production of fermented milks like yoghurts and cheeses. The Immobilized Cell (IC) technology can be explored to produce mixed lactic starters in continuous fermentation. The high IC concentration results in higher productivity and decreases contamination risks, due to the high dilution and inoculation rates provided by cell release from beads. Immobilisation also improves plasmid stability in the starters to express desirable functions optimally during pilot production of fermented dairy foods.

21.4.4.2 Production of mass Probiotic cultures

IC technology can be used to continuously and stably produce mixed-strain starters containing fastidious and non-competitive micro-organisms, such as bifidobacteria, with a high volumetric productivity and high biomass concentrations in the outflow of the continuous fermentation, even at high dilution rates.

21.4.4.3 Pre-fermentation of milk

Starter culture preparation is of paramount importance in the manufacture of fermented dairy products. The continuous inoculation-prefermentation of milk for yoghurt production in a stirred tank reactor by separately entrapped cells of *Lactobacillus delbruekii* ssp. *bulgaricus* and *Streptococcus salivarius* ssp. *thermophilus* in Ca-alginate gel beads was the first studied dairy application of IC. This technology allowed a reduction in fermentation time by approximately 50 and 20% compared with freeze-dried strains and a liquid yoghurt culture respectively. The inoculation-prefermentation of milk for fresh cheese production with a mixed culture entrapped in κ -carrageenan/LBG gel beads has also been extensively studied. Fermentation time to produce the fresh cheese curd was considerably reduced by more than 50% compared with the traditional industrial process.

21.4.4.4 Metabolite production

The ultrafiltration of cheese whey yields large volumes of low-value whey permeate, which has limited uses. The immobilized cell technology can be used for production of different metabolites and functional ingredients from LAB using this low value whey permeate containing high lactose and mineral contents, used as a culture medium for the production of lactic starter cultures or metabolites.

21.4.4.5 Lactic acid production

Lactic acid is widely used as an acidulant and preservative agent in foods and as a precursor for production of emulsifiers, such as stearyl-2-lactylates, in baking industry for the baking. High lactic acid productivities and long-term stability have been obtained during continuous IC fermentation of yeast extract-supplemented whey permeate by *Lactobacillus helveticus* immobilized in κ -carrageenan/locust bean gums gel beads, but with limited conversion of lactose.

21.4.4.6 Exopolysaccharide production

Very little research has been done on cell immobilization for Extracellular polysaccharide (EPS) production. Mucoïd properties of *L. Rhamnosus* RW 9595M were explored for cell immobilization by adsorption on solid porous supports (ImmobaSil®). The production of EPS was investigated during pH-controlled IC repeated-batch cultures in SWP. IC technology results in changes in cell morphology and physiology, and the formation of very large aggregates containing very high cell and insoluble EPS concentrations. The high potential of the strain, *L. rhamnosus* RW9595M, and of IC technology has been judiciously exploited for the production of EPS as a functional food ingredient. In addition, the production of insoluble EPS allows an easy recovery of the product and the aggregates containing high EPS and viable cell concentrations could have interesting applications as symbiotic product, combining both probiotic and prebiotic activities.

21.4.4.7 Bacteriocin production

Cell immobilization has been used to increase cell density for bacteriocin production in supplemented whey permeate medium. A very high nisin Z production was demonstrated in the broth after repeated-cycle pH-controlled batch (RCB) cultures of *Lactococcus lactis* ssp. *lactis* biovar *diacetylactis* UL719 immobilized in κ -carrageenan/LBG gel beads in Supplemented Whey permeate (SWP).

21.4.4.8 Other applications in food industry

Novel technologies were developed based on immobilized cells in gel beads, which are principally

involved in fermentation of liquid substrates, and the immobilized cells are not found in the product. Thus the most important industrial exploitation of immobilized cell technology is found mainly in beer and wine making.

Some potential applications of IC technology in the food industry have been given in Table 21.2

Table 21.2 ICT Applications in the food industry

SECTOR	PRODUCTS
Sugar conversion	High fructose syrup, lactose-free milk
Sauces	Soy sauce
Enzymes	Amylases, proteases, lactase, invertase
Amino acids	Aspartic, phenylalanine, glutamic, lysine
Organic acids	Acetic, lactic, citric, propionic, malic
Alcohols	Ethanol, glycerol, xylitol, sorbitol, mannitol
Flavors / aromas	Fruit juice debittering, acetaldehyde, diacetyl, caffeine



Lesson 22

CLONING AND EXPRESSION OF GENES IN BACTERIA AND YEAST

22.1 Introduction

Bacteria can serve as factories for production of industrially important native proteins /recombinant proteins. Recombinant DNA technology has been employed to construct/ design strains that over-produce native proteins or foreign proteins. The standard protocols of strain construction involve use of strong promoters, insertion of multiple copies of expression cassettes into the genome and, in some cases, secretion of a foreign protein as a fusion with a native, secreted protein. However, for economical large industrial scale production, further manipulations are required.

22.2 What is a Recombinant Protein?

A protein obtained by introducing recombinant DNA into a host cell causing it to produce the gene product or a protein whose amino-acid sequence is encoded by the cloned gene is called as recombinant protein.

22.3 Basic steps in Recombinant Protein Production

PCR amplification of gene of interest if from prokaryotic source or isolation of RNA/ mRNA and cDNA synthesis if from eukaryotic source



Cloning of amplified PCR product in a PCR cloning vector like pGEM-T or pUA (pDrive)



Transformation into *E. coli*



Sequencing of the cloned insert



Sub cloning into expression vector



Transformation into expression host e. g. *E coli* or yeast



Confirmation of identity of recombinant protein using SDS-PAGE, western blot or any other enzyme assay available for a particular gene



Large scale production in fermenters



Downstream processing and purification of recombinant protein

All the above steps are described in the following section:

22.3.1 PCR amplification of gene of interest

22.3.1.1 Prokaryotic source

The gene sequence encoding the protein to be expressed is retrieved from the NCBI database available as the public domain. Primers are designed from the flanking region of the gene sequence and used for amplification of the gene of interest from the genomic DNA of the prokaryotic donor.

22.3.1.2 Eucaryotic source

In case of eukaryotic system, RNA is isolated from the donor and cDNA is synthesized using random primers or oligo dT or specific primers as shown in Fig. 22.1. The cDNA is then amplified using the specific primers.

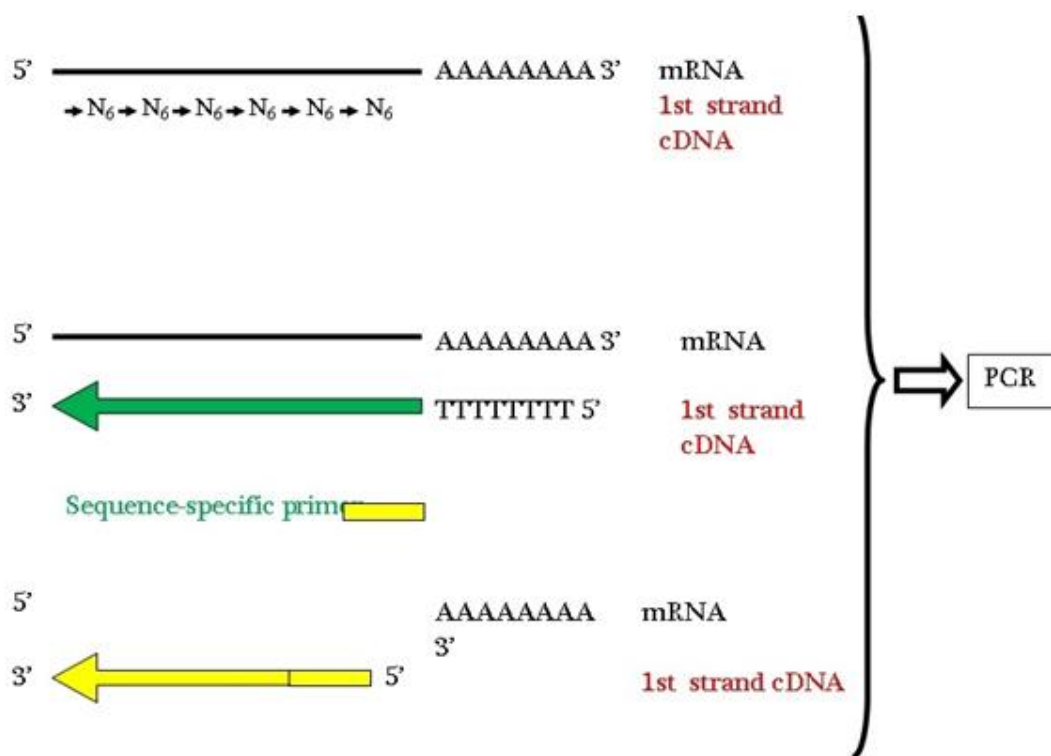


Fig. 22.1 Synthesis of first strand of cDNA from RNA / mRNA

All the above mentioned products are then purified using PCR product purification kits available from several manufacturers e.g. Promega, Qiagen, Invitrogen, Sigma, Banaglore Genei etc.

22.3.2 Cloning of PCR product in a PCR cloning vector like pGEM-T or pDrive

The purified PCR products obtained as above are cloned using PCR cloning vectors e.g. pGEM-T or pUA (TA or UA based cloning).

22.3.3 Transformation into *E. coli*

The recombinant vector is then transformed into competent cells of *E. coli*.

22.3.4 Sequencing of the cloned insert

The transformants are picked up and the recombinant plasmid vector carrying the cloned gene as insert is isolated and purified using plasmid purification kits. The nucleotide sequence of the inserted gene is determined and analysed.

22.3.5 Sub cloning into expression vector

Once the sequence of the target or insert DNA matches with the original gene sequence, it can be sub cloned further into an expression vector (for production of protein form the gene of interest).

22.4 What are Expression Vectors?

Expression vectors are the vectors that allow the exogenous / insert / target DNA to be inserted, stored and expressed.

Expression vectors are the basic tools used for the production of proteins of food and pharmaceutical applications such as chymosin, lactoferrin, insulin and streptokinase etc. that are important for food/industrial applications and medical treatments of specific diseases.

22.4.1 Components of an expression vector

An expression vector consists of the following elements important for the production of proteins as shown in Fig. 22.2.

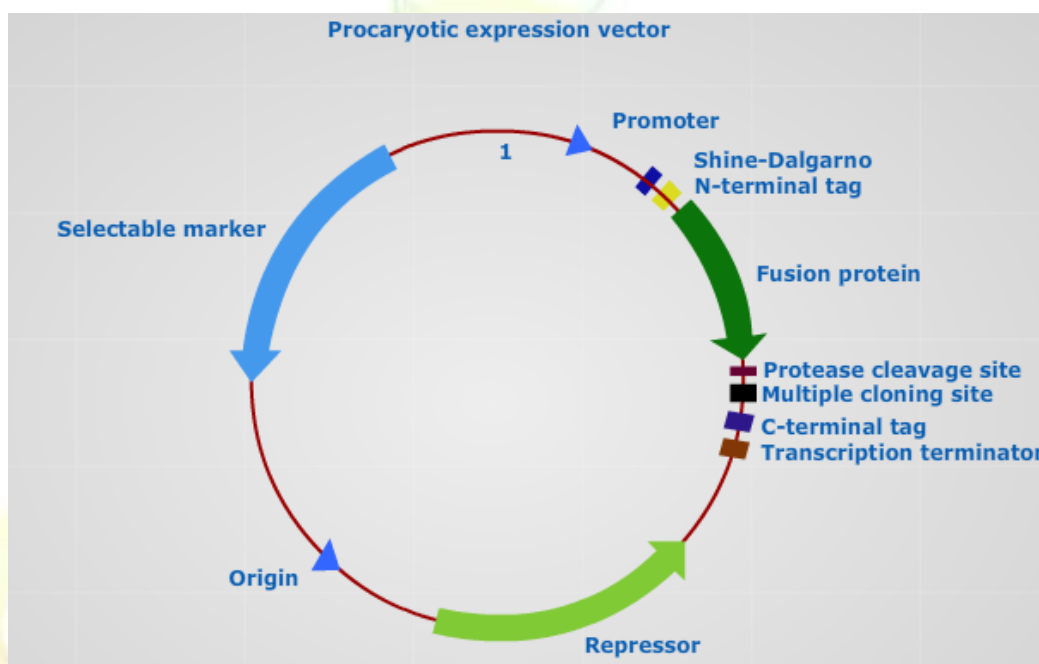


Fig. 22.2 Procaryotic expression vector

- Origin of Replication (ColE1) – responsible for replication of plasmid DNA
- Selection marker (ampicillin, kanamycin or chloramphenicol resistance) – antibiotics used as selection markers in order to select recombinant clones / transformants
- Multiple cloning site (MCS) – comprises of several restriction enzyme sites for cloning the gene of interest
- Transcriptional promoter (T7, T5, T3 or tac) - DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. Strong promoter causes mRNAs to be over expressed at high frequency.
- Intact ORF and ribosomal binding sites (RBS) near start codon are required for translation
- Fusion tags at N terminal or C terminal ends
- Terminator sequence to terminate transcription.
- Regulatory genes for controlled expression of the target protein

22.4.2 Strategies for the construction of expression vectors

- Vectors that can synthesize pure proteins exclusively encoded by inserted gene (Transcriptional Fusion)
- Vectors which can allow the synthesis of fusion proteins encoded by the sequence in the vector and those encoded by the inserted gene (Translational fusion)

22.4.2.1 Prokaryotic expression vector

Plasmid derived vectors – A typical plasmid vector has been shown in Fig.22.3.

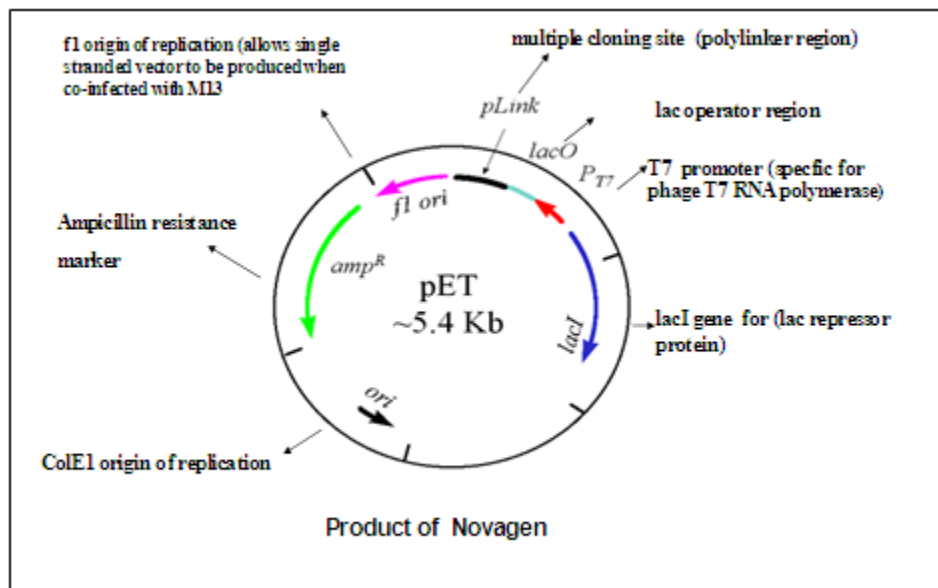


Fig. 22.3 Genetic Map of pET vector from Novagen

22.4.2.2 Eukaryotic expression vector

Yeast Expression vectors – A vector for expression in methylotrophic yeast *Pichia pastoris* has been shown in Fig. 22.4.

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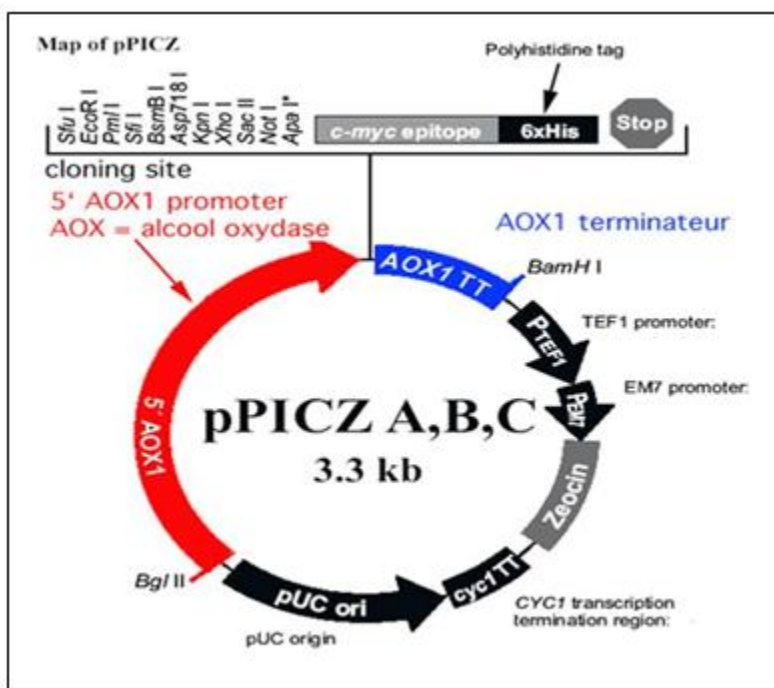


Fig. 22.4 Genetic Map of pPICZ A, B, C vector from Invitrogen

Other common eukaryotic expression vectors include
 Baculo-virus expression vectors
 Mammalian expression vectors (SV40, Retrovirus and Adenovirus vectors)

22.5 Transformation into Expression Host e. g. *E coli* or Yeast

After the construct has been designed, it is transformed into expression host compatible with the vector.

22.5.1 Expression systems

Expression systems are based on the insertion of a gene of interest into a host system (prokaryotic or eukaryotic) for its efficient translation and expression into a protein.

Host expression systems are of the following types:

Prokaryotic system – Bacteria like *E. coli*, *B. subtilis*, *Lactobacillus*, *Lactococcus* etc.

Yeast expression system

Cultured insect cells

Cultured mammalian cells

Transgenic Animals (mammary gland as bioreactors)

22.5.1.1 Prokaryotic / Bacterial expression systems

The great demand for production of high amounts of pure protein for pharmaceutical applications and for research made *Escherichia coli* as one of the most important cell factories for recombinant protein production. Although, it is a well studied model organism showing high productivity, the recombinant protein frequently aggregates and forms the so called inclusion bodies. In spite that several strategies have

been designed to solubilize these inclusion bodies, the recovery of biologically active protein is very low. However, *E. coli*, the model organism, is the most commonly used expression host for the production of recombinant proteins because of the several advantages.

- Non –pathogenic
- Fast growth (less generation time) approx. 20 min.
- The genome has been well characterized
- Choice of a large number of commercially available vectors
- Can be transformed easily using calcium chloride induced transformation
- The recombinant proteins can be purified using simple techniques
- Can produce recombinant protein at a very high level – gm/L

1. Advantages of prokaryotic expression systems

- Gene expression can be easily controlled by using inducible vectors.
- It is easy to grow the bacteria with high yields.
- The protein can be secreted into the medium.

2. Disadvantages

- Bacteria may recognize the proteins as foreign and destroy them.
- Absence of post translational modifications required for eukaryotic genes expression
- Bacterial environment sometimes may not permit correct protein folding and leads to production of inactive protein
- Eucaryotic proteins when expressed at very high levels form inclusion bodies
- Biological activity and immunogenicity may differ from natural protein.

The most common *E. coli* host systems are *E. coli* BL21, *E. coli* BL2(DE3) and origami using pET series of vectors from Novagen.

22.5.1.2 Eukaryotic expression vectors

- Proper Post translational machinery is present e. g. glycosylation, adenylation
- Disulphide bond formation.
- Proper protein folding.

- Endotoxin free recombinant protein.

22.5.2 Expression in yeasts

Yeasts offer a number of advantages as expression systems for complex mammalian proteins. They combine the ease of manipulation and growth of unicellular organisms to an eukaryotic subcellular organization enabling post translational processing and modification. The two most common hosts used for gene expression in yeasts include *Saccharomyces cerevisiae* and *Pichia pastoris*. Some of the examples of commonly used vectors for *Saccharomyces cerevisiae* are pYES2, pYES2.1/V5-His-TOPO, pYC, pFLAG etc. Recently, *Pichia pastoris* has been developed into a highly successful system for the production of a variety of proteins. The increasing popularity of this particular expression system can be attributed to several factors which include 1) the simplicity of techniques required for the molecular genetic manipulation of *Pichia pastoris* and their similarity to those of *Saccharomyces cerevisiae*, one of the most thoroughly characterised experimental systems in modern biology 2) The ability of *Pichia pastoris* to produce foreign proteins at high levels either intracellularly or extracellularly 3) The capability of performing many eukaryotic post translational modifications such as glycosylation, disulphide bond formation for correct folding and proteolytic processing and 4) The availability of the expression system as a commercially available kit. Currently, *Pichia pastoris* expression system has been developed for commercial application by SIBIA, CA with selling rights given to Invitrogen Inc. The most common *Pichia* expression vectors are pPIC9, pPICZ α A, B, C and pGAPZ α A, B, C etc. and the hosts are *P. pastoris* X-33 etc. Many proteins have been expressed in the *Pichia* Expression System, including enzymes, proteases, protease inhibitors, receptors, single-chain antibodies, and regulatory proteins.

22.6 Confirmation of Recombinant Protein using SDS-Page, Western Blot or any other Enzyme Assay Available for a Particular Gene

Analysis of crude as well as purified product for the presence of target protein is an essential step. Recombinant protein recovered after large scale production and downstream processing also needs to be confirmed once again using these methods.

22.6.1 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a widely used technique in biochemistry, genetics and molecular biology to separate proteins according to their electrophoretic mobility. The migration of proteins depends on both charge and size. It is basically used for checking purity of the proteins and also determine their molecular weight. Electrophoretic mobility is dependent on molecular weight, higher order protein folding and post translational modifications. A wide range of proteins can be separated by preparing varying concentrations of polyacrylamide gels. Generally, 10% polyacrylamide concentration of gel is sufficient enough to resolve the proteins ranging from 10-150 kDa. The protein mixture to be analyzed is mixed with an anionic detergent such as SDS along with a reducing agent like mercaptoethanol or dithiothreitol (DTT). SDS denatures secondary, tertiary and quaternary structures of proteins and applies a net negative charge to each protein in proportion to its mass. Mercaptoethanol and DTT denature all the disulfide bonds. The protein samples are boiled before loading onto the polyacrylamide gel. Boiling of the protein samples further denatures the protein molecules. SDS then binds strongly along the polypeptide chain and provides a net negative charge to it. Generally, one molecule of SDS binds two amino acid residues. When the electric current is applied, the negatively charged protein migrates from cathode to anode relative to their size. A tracking dye (BPB,

Bromo phenol blue) is added to the protein solution to allow the tracking of the progress of the protein solution through the gel during the electrophoretic run. Two different types of apparatuses are shown in Fig. 22.5. Finally, the gels are stained with Coomassie Brilliant Blue R-250 (CBB) or with silver stain.

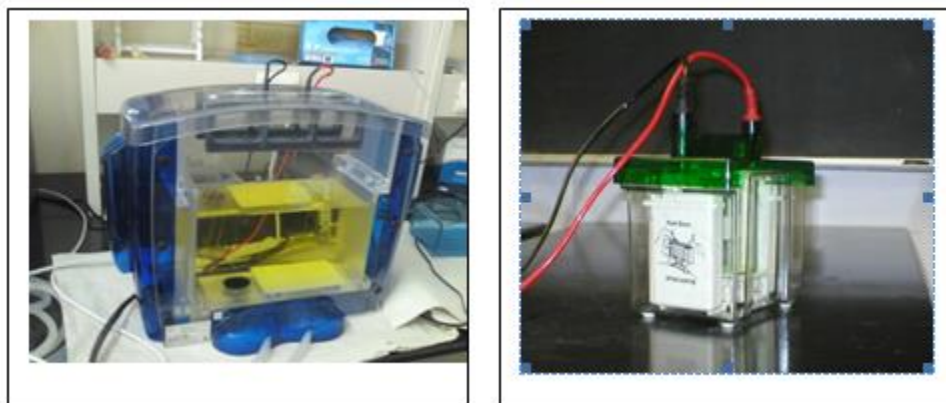


Fig. 22.5. SDS-PAGE apparatus

22.6.2 Western blot

Western blotting or protein immunoblotting is a technique which is used to identify and locate proteins based on their ability to bind to specific antibodies. It is a widely used technique for confirmation of expression of recombinant protein. Proteins separated onto SDS-PAGE are transferred to Nitrocellulose or PVDF (polyvinylidene difluoride) membrane either by capillary action by stacking or electroblotting. The transferred recombinant or target protein is detected using specific primary antibody and secondary enzyme labeled antibody and substrate using colorimetric, fluorescent, chemiluminescent or radioactive labeling etc.

Some of the other techniques which include application of antibodies to detect proteins are immunostaining and enzyme-linked immune-sorbent assay (ELISA).

22.6.3 Enzyme-linked immunosorbant assay (ELISA)

ELISA also called enzyme immunoassay is a very common and powerful method extensively used for estimating protein, antigen or antibody in quantities upto even ng/ml to pg/ml in a solution. ELISA is also extensively used in diagnostics. The protein (antigen) detection method is basically the same as in western blot except that it is carried out in 96 well microplate. The sample containing recombinant protein or any other sample is immobilized onto microtitre plate and then detected by the specific antibody.

22.6.4 Other enzyme assays available for a particular gene

The expression of the recombinant protein can also be detected by using enzyme assays if available for that particular protein like β -galactosidase, protease, cellulase, milk clotting activity etc.

Module 5. Application of biotechnology in dairying

Lesson 23

LARGE SCALE PRODUCTION AND DOWNSTREAM PROCESSING OF RECOMBINANT PROTEINS

Once a transformant or recombinant clone is selected based on detection of expressed protein by any of the above methods, the conditions for maximal expression of recombinant proteins are standardized. Further scale up is then carried out in fermenters (Fig. 23.1).



Fig. 23.1 Fermenter from Applicon

Even the industrial micro-organisms are grown under controlled conditions with an aim of optimizing the growth of the organism or production of the target microbial product at industrial level. Fermentation is carried out in vessels called fermenters which have complex integrated system of automatic controls of pH, temperature, oxygen demand etc. Fermentation in liquid media is generally of three types depending upon the mode of operation *viz.* Batch Fermentation, Fed-batch Fermentation and Continuous Fermentation.

23.1 Batch Fermentation

It is the simplest mode of operation. The reactor is filled with the growth medium and fermentation is allowed to proceed. After inoculation, no medium is then added. After completion of fermentation, reactor is emptied and refilled again for new batch of fermentation.

23.2 Fed-Batch fermentation

This is most widely used process in industries and is a modification of Batch type fermentation only. The reactor is filled with the growth medium and fermentation is then allowed to proceed. However, medium is periodically added after inoculation as the feed added at the start of fermentation gets exhausted. The product is obtained periodically and as well after completion of the fermentation process.

23.3 Continuous Fermentation

In continuous fermentation, fresh medium is supplied continuously. Products and cells are continuously removed for processing so that cells receive fresh medium continuously. It can be operated for longer

periods. Growth rate of the cells can also be optimized by controlling the flow rate of the feed entering the reactor thus resulting in high productivity

23.4 Downstream Processing and Purification of Recombinant Proteins

Downstream processing refers to the recovery and purification of proteins/products either from

- Natural sources such as animal or plant tissue.
- Fermentation by wild micro-organisms or genetically modified

The products range from antibiotics, enzymes, hormones, therapeutic antibodies used in diagnostics, vaccines and several other recombinant proteins.

If the protein is intracellular, recovery involves the following steps.

i) Cell harvesting by centrifugation:

Fermentation process results in Biomass containing cells which must be separated for further processing. The separation of microbial cell normally involves either Filtration or Centrifugation.

23.4.1 Filtration

Filtration is generally used in case of very small sized microbial cells. It is useful for industries being cheapest option since slurry/microbial biomass can be fed directly to filtration unit attached with the output of the bioreactor.

23.4.2 Centrifugation

It is used in laboratory scale applications wherein cell biomass is separated by applying centrifugal force using a centrifuge (Fig. 23.2).



Fig. 23.2 Centrifuge from Sanyo

- i) Cell disruption generally involves ultrasonication, bead beater, microfluidizer and cell disruptor etc. for recovery of intracellular proteins
- ii) Purification of the required product from cell extracts or the culture supernatant
- iii) Confirmation of the target protein using SDS/PAGE, western blot or enzymatic assay

If the protein is extracellular, the recovery involves:

- i) Concentration of the fermented medium either by dialysis, concentrator or ultra filtration.
- ii) Purification of required product from cell extracts or the culture supernatant
- iii) Confirmation of target protein using western blot or enzymatic assay

23.4.3 Ultrafiltration

Concentration involves passing of supernatant either from intracellular or extracellular extract through membranes of different cutoff values i.e. pore sizes (3, 5, 10, 30 or 100kD etc.). Molecules larger than the membrane pore size rating will be retained at the surface of the membrane. Hydrostatic pressure is applied that forces the liquid against a semipermeable membrane and this process is widely used in industry. For smaller volumes, centricons and ultrafiltration unit (Fig. 23.3) are used while for larger volumes tangential flow cartridge is preferred.



Fig. 23.3 Ultrafiltration unit

23.4.4 Chromatography

23.4.4.1 Gel filtration

Gel-filtration chromatography separates molecules on the basis of size (Fig. 23.4). It involves passing a protein solution through a column which is packed with beads of a hydrated insoluble material (matrix) such as dextran, agarose or polyacrylamide. Larger molecules cannot enter the beads, hence come out first in void volume. On the other hand, smaller molecules enter the beads and take more time for elution.

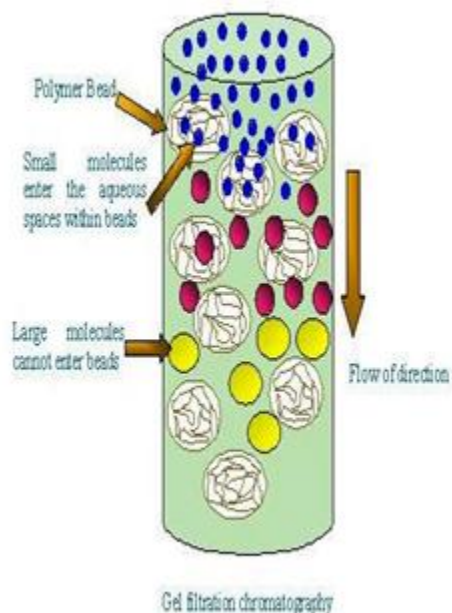


Fig. 23.4 Gel Filtration chromatography

23.4.4.2 Ion exchange chromatography

Ion-exchange chromatography separates molecules based on differences in net charge (Fig. 23.5, 23.6 and 23.7). Proteins with a net positive charge will be retained on negatively-charged columns such as carboxymethyl-cellulose (CMC) and those with a net negative charge will be retained on positively-charged columns such as diethylaminoethyl-cellulose (DEAE-cellulose).

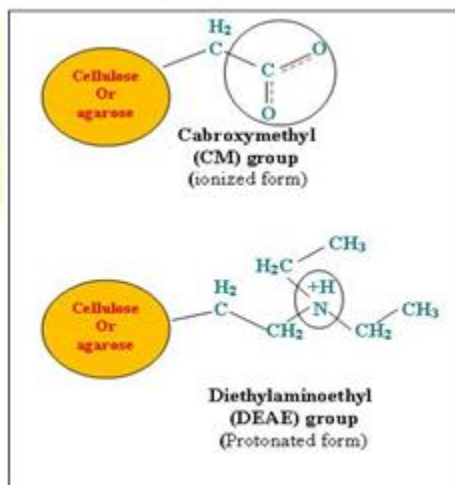


Fig. 23.5. CMC and DEAE groups chromatography

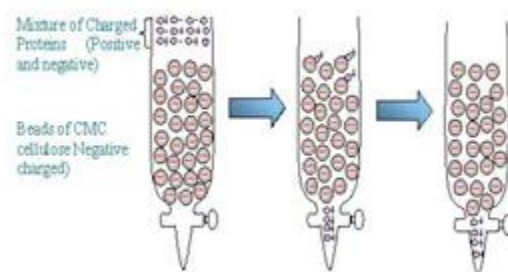


Fig. 23.6 Ion exchange

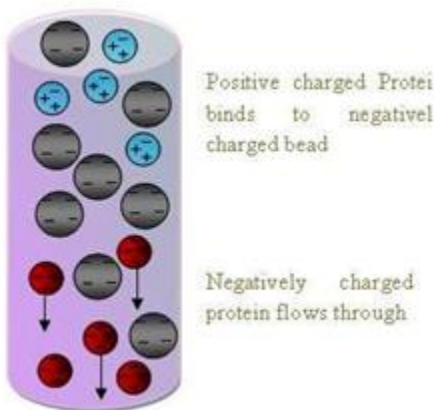


Fig. 23.7. Ion exchange chromatography

23.4.4.3 Hydrophobic interaction chromatography

Hydrophobic word is assigned to molecules that distracts water. Hydrophobic Interaction Chromatography (HIC) is a liquid chromatography used to separate proteins on the basis of relative hydrophobicity wherein hydrophobic ligands bind to hydrophilic ligands on the separation matrix. Hydrophobic amino-acids can interact with HIC gel under high salt concentrations and then to bring about desorption, the salt concentration is lowered gradually and the proteins elute based on their hydrophobicity.

23.4.4.4 Reversed phase

Reversed Phase Chromatography (RPC) separates molecules according to differences in their hydrophobicity. RPC has become increasingly important for high-resolution separation and analysis of proteins, peptides and nucleic acids.

23.4.4.5 Affinity chromatography

Affinity chromatography is a purification technique that offers purities >95% in just one step. It makes use of a specific native or added property of the target molecule to isolate it from all other contaminants in the sample and is most popularly used in separation of recombinant protein in one step. Recombinant proteins can be purified using affinity columns since most of them are expressed as fusion proteins which helps in quick separation of proteins from several other proteins. Most common fusion partners include Glutathione – S- transferase (GST), Thioredoxin, Maltose binding protein and six histidine tags (His6) which use glutathione, phenylarsine oxide, amylose and nickel respectively (Fig. 23.8). One of the most

common affinity chromatography technique is Immobilized metal ion adsorption chromatography (IMAC) also known as metal chelate affinity chromatography. IMAC is an excellent chromatography technique for optimization and purification of histidine tagged proteins since most of the vector systems used for expression of recombinant proteins have six his tag. The bound proteins can then be eluted by competitive elution with, for example, imidazole, or by lowering pH. Strong chelating agents, such as EDTA can also be used.

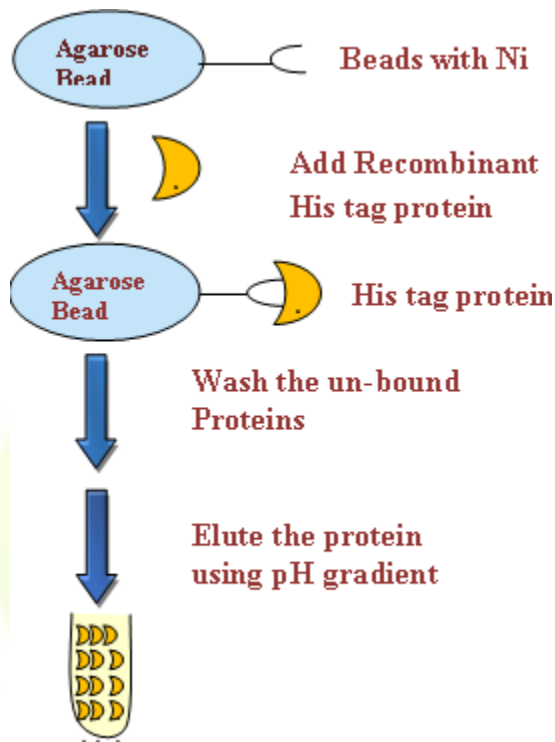


Fig. 23.8 Separation of protein using His tag affinity chromatography

23.4.5 Isoelectric Focussing

Every protein has a unique isoelectric point, a pH at which the net charge on the molecule becomes Zero. In presence of ampholytes and a charged field, protein molecules migrate till the net charge on them becomes nil, thus resulting in efficient separation. It does not require denaturation of proteins thus maintains their biological activity. Rotofor (Bio-rad, Fig. 23.9) is commonly used for separation of proteins based on their isoelectric point.



Fig. 23.9 Rotofor from Bio-Rad

23.5 Confirmation of Identity of Recombinant Protein Using SDS-PAGE, Western Blot or any Other Enzyme Assay Available for a Particular Gene

After purification of the recombinant protein already described above from fermentation broth, SDS-PAGE is run to confirm the purity as well as molecular weight of the protein with the help of western blotting, ELISA or protein/enzyme assay etc.

23.6 Production of Specific Recombinant Enzymes/Proteins of Commercial interest for application in dairy/food industry

The detailed protocols for the production of two industrially important proteins having prospects for application in dairy industry are given below.

23.6.1 Buffalo chymosin

Chymosin is an aspartyl protease (Fig. 23.10) secreted in the fourth stomach (abomasum) of the suckling ruminants. Bovine (cattle) calf chymosin popularly known as **calf rennet** is the milk clotting enzyme present predominantly in the cheese rennet which has been traditionally used as the key ingredient for cheese making in the dairy industry and the typical cheese flavor is principally attributed to the activity of this enzyme. Chymosin specifically recognizes the k-casein sequence from amino acid position His98 to Lys111 and cleaves peptide bond between Phe105 - Met106 in casein.

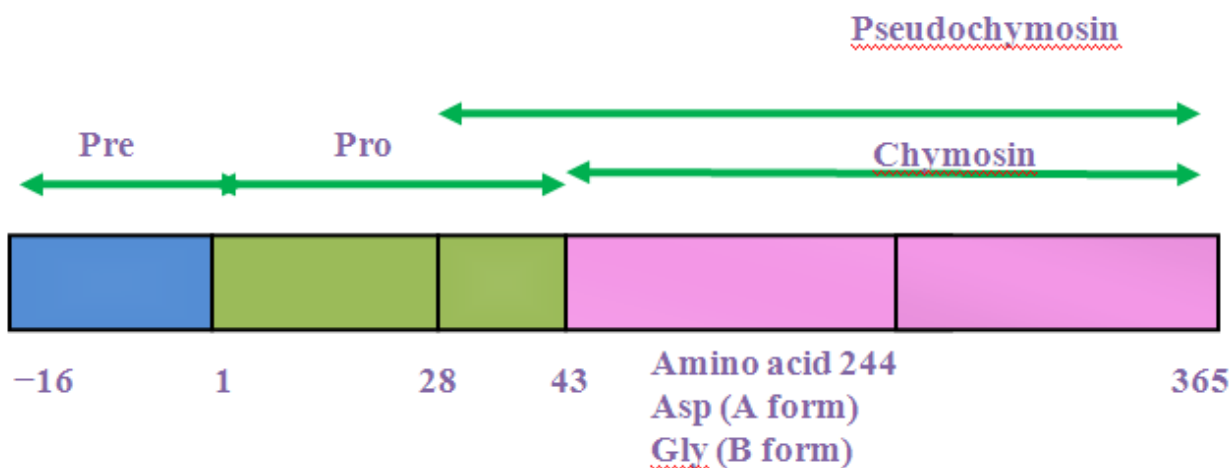


Fig. 23.10 Primary structure of bovine *chymosin*

Traditionally, calf rennet is derived from the abomasal tissue of the suckling bovine calves after slaughter. However, the production of bovine chymosin is now greatly limited due to decreasing bovine calf population all over the world. This situation coupled with tremendous growth of cheese industry has considerably accelerated the demand for calf rennet in cheese industry. Hence, to meet this growing demand for cheese manufacture, concerted efforts are now being directed to find appropriate substitutes for bovine calf rennet. Although milk clotting enzymes from microbial sources have found wide applications in dairy industry, the quality of processed cheese using these enzymes has been inferior to that made from cattle calf rennet. Such products have limited consumer acceptability due to bitter flavor produced in the cheese. However, chymosin from abomasum of young buffalo calves could serve as a very attractive substitute for calf rennet as it has been reported to possess higher milk clotting activity compared to cow, goat and porcine chymosin. In addition, the chymosin from buffalo is expected to have an inherent compatibility with buffalo milk and is better in milk clotting activity than chymosin from

heterologous sources. Therefore, the use of buffalo chymosin could be a preferred option for processing buffalo milk into common cheese varieties like cheddar and Mozzarella especially in countries like India with large population of buffaloes. India also harbors a major population with vegetarian food habit. As a result of growing public awareness, cheese produced using calf rennet is not acceptable by the vegetarian consumers due to religious sentiments. Hence, concerted efforts have been made in the last decade to explore biotechnological interventions to produce recombinant chymosin from sources other than cattle through heterologous expression of chymosin gene. The buffalo recombinant chymosin will not only provide a viable and cheap substitute for calf rennet but could also cater to the needs of vegetarian population by producing vegetarian cheese varieties specifically Mozzarella cheese extensively used in the preparation of Pizza. NDRI scientists at Molecular Biology Unit, Dairy Microbiology Division have expressed goat chymosin in *E. coli* and buffalo chymosin in *Pichia pastoris* - a methylotropic yeast.

23.6.1.1 Expression of Goat Chymosin in *E. coli*

Goat prochymosin cDNA was cloned and characterized by sequence analysis. The prochymosin cDNA spanned 1101 nucleotides and was predicted to code for 365 amino acids with a proregion of 42 amino acids. The cDNA fragment containing goat prochymosin was then subcloned in to pET43.1a (+) and expressed as a NusA fusion protein in *E. coli* which showed low level of milk clotting activity after activation at acid pH.

23.6.1.2. Expression of Buffalo Chymosin in *E. coli*

Buffalo chymosin was also cloned, sequenced and expressed in *E. coli*. The expressed protein has been shown on SDS-PAGE in Fig. 23.11. However, the expression level was not adequate and cost effective for its large scale production. Hence, further attempts were made to express buffalo chymosin in *Pichia pastoris*.

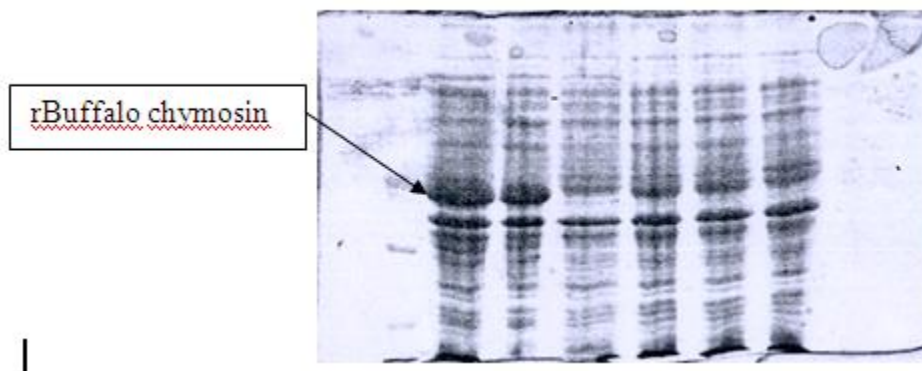


Fig. 23.11 SDS-PAGE showing expression of buffalo chymosin

23.6.1.3 Expression of buffalo chymosin in *pichia pastoris*

The strategy used to clone buffalo prochymosin insert from *E. coli* to *Pichia pastoris* vector pPICZαA has been outlined below in Fig. 23.12. After ensuring the in frame insertion of buffalo prochymosin insert, the construct was digested with SacI for linearization and electroporated into *Pichia pastoris* host X-33. Further to this, expression studies were carried out to study the level of expression in shake flask and various parameters were attempted to optimize the expression level. The expressed protein chymosin has been shown on SDS-PAGE (Fig. 23.13).

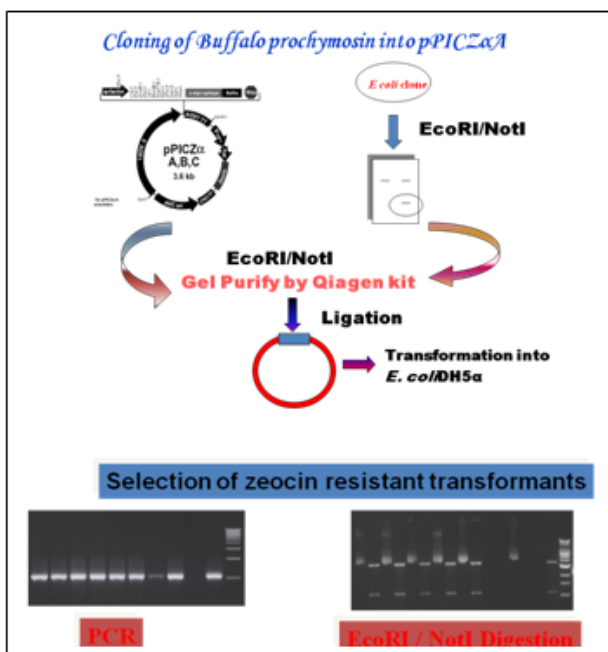


Fig. 23.12 Cloning strategy of buffalo chymosin in pPICZα

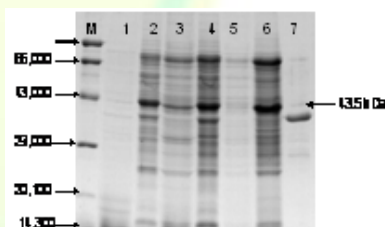


Fig. 23.13 SDS-PAGE showing recombinant buffalo chymosin

Buffalo chymosin has been expressed in the culture supernatant of *P. pastoris* in a 10 liter fermenter (Fig. 23.14) and production conditions and down stream processing have been optimized for the recovery of the recombinant product. The expression of the protein has been achieved to the level of 200-300 mg recombinant protein/L. Partially purified recombinant buffalo chymosin was successfully used as a milk clotting enzyme in the production of Mozzarella cheese (Fig. 23.15) from buffalo milk and the quality of the cheese made with recombinant enzyme has been comparable with that of cheese made with Meito rennet.



Fig. 23.14 Production of Recombinant Buffalo Chymosin in 10L fermenter



Fig. 23.15 Mozzarella cheese prepared using recombinant buffalo chymosin

23.6.1.4 Applications

- Production of all varieties of cheeses
- Production of casein hydrolysates
- Pizza market through production for Mozzarella cheese

23.6.2 Human lactoferrin

Lactoferrin – a naturally occurring unique glycoprotein found in milk of mammalian species has been receiving increasing attention and interest due to its multiple bioactive functions beneficial for human health. It is a unique multifunctional protein expressed in the milk of mammalian species such as the cow, buffalo, pig, equine, goat and mouse and particularly human. Its major functionality is related to its role in iron absorption and strong iron-binding properties. Lactoferrin is also used in the treatment of bacterial, viral and fungal infections, sepsis, cancer, tumors and immunosuppressory illnesses both in human and veterinary medicine. Human lactoferrin (hLf) with several bioactive functions in particular can find potential market in health / functional foods for elderly or immuno-compromised hosts for recovery from gastro-intestinal infections, and prophylactic products for traveler's diarrhoea as well as can address the problem of antibiotic resistant strains especially methicillin resistant staphylococci. Lactoferrin fortified dairy foods will also have extended shelf life and hence can find potential applications in dairy, food and meat industry. Alternatively, the iron-loaded lactoferrin can be added to foods such as baby formula, cereal, and ice cream to enhance the nutritive value of the food. However, it is impractical to purify native lactoferrin particularly from human milk in order to make it a commercially viable product. Nevertheless, worldwide production of bovine lactoferrin (Lf) has increased tremendously during the last decade. Currently, for commercial production of Lf, bovine colostrum is used and Lf is isolated from cheese whey or from skim milk. Due to minute concentration of Lf in bovine milk, the recovery is too low that adds to the cost of production thereby making it commercially nonviable. Amongst the different MNCs, DMV is marketing bovine lactoferrin. However, bovine lactoferrin does not have the same biological effect since it has lower affinity for human lactoferrin receptors. Hence, NDRI scientists at Molecular Biology Unit expressed the same in two of the yeast systems namely *Saccharomyces cerevisiae* and *Pichia pastoris*. The cloning strategy was similar to that used for chymosin as described previously for chymosin. Human lactoferrin (hLf) cDNA was cloned and sequenced both from mammary gland tissue and neutrophils and finally expressed in *Saccharomyces cerevisiae* and in *Pichia pastoris*. Recombinant human lactoferrin is being currently produced by Agennix, a Houston based US Biopharma company and Ventria Bioscience, a California based company. The Agennix has expressed human lactoferrin in *Aspergillus* and Ventria exploits their ExpressTec for expression of Lf in rice.

Human Lactoferrin has been expressed intracellularly in *S. cerevisiae* at 5 mg/l level. The yeast biomass as such can be incorporated into food products which will serve as the source of lactoferrin as well as vitamins. The yeast host has GRAS status. Human lactoferrin has also been expressed in *Pichia pastoris* at a level of approximately 10-20 mg/l in a 10 L fermenter. Total protein was calculated to be 6.3 mg/l in supernatant and about 12 mg in cell pellet. The recombinant lactoferrin has been shown in Fig. 23.16.

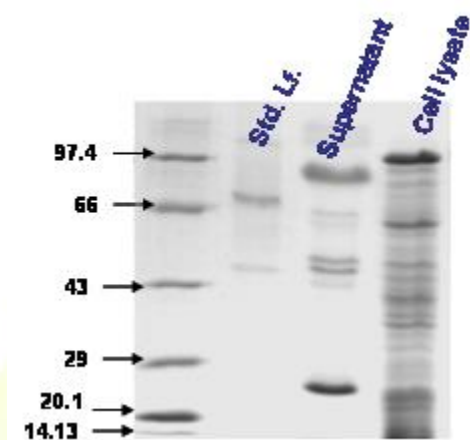


Fig. 23.16 Recombinant human lactoferrin

23.6.2.1 Application of human lactoferrin

a) Pharmaceuticals

- Stimulation of immune system, regulation of iron metabolism, control of cell or tissue damage and as an antioxidant, prevention of osteoporosis, vaginal candidiasis and treatment of peptic ulcers
- Treatment and prevention of opportunistic bacterial, viral, and fungal infections such as pneumonia, acquired immune deficiency syndrome (AIDS), candidiasis, diarrhoea, and neonatal sepsis.
- Anticarcinogenic - treatment of tumors such as brain tumors
- Lactoferrin can aid in reducing heart attacks
- Against Dental caries
- As an Anti-inflammatory drug

b) Functional / Health / Nutraceutical Foods

- Lactoferrin can have potential market in health / functional foods for elderly or immuno-compromised hosts, recovery from gastro-intestinal infections, and prophylactic products for traveler's diarrhoea as well as to address the problem of antibiotic resistant strains especially methicillin resistant staphylococci. Lactoferrin fortified dairy foods will also have extended shelf life.
- As nutritional supplements that include tablets, gelatin capsules, or liquids containing the lactoferrin together with adjuvants or diluents.
- Alternatively, the iron-loaded lactoferrin can be added to foods such as baby formula, cereal, and ice cream to enhance the nutritional value of the food.
- As a food preservative in food and meat industry.

c) Other Applications

- As an antiseptic either alone or in the form of a powder, solution, ointment, aerosol spray, or cream.



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Module 5. Application of biotechnology in dairying

**Lesson 24
DESIGNER MILK**

24.1 Introduction

Although, milk is often considered as nearly the most perfect and ideal food to meet our nutritional requirements, there is still considerable scope to improve its functional properties to suit to the needs of the consumers by introducing appropriate modifications in its composition. The attention is now focused on adding more value to milk and studying its health implications. With the recent developments in the Biotechnological techniques e.g. Genetic Engineering, rDNA technology, Protein Engineering and advances in animal cloning and transgenic techniques, it is now possible to alter milk composition at will for better manufacturing / technological properties to add variety to our traditional dairy products and also to produce variants of milk to cater to the needs of specific consumers from health and nutritional perspectives. With the advent of modern gene transfer and expression methodologies, new opportunities have been created for the modification of animal production traits including milk production with altered composition. Animal udder can virtually be used now as an efficient biological vat or Bioreactor for the production of homologous and heterologous proteins, sugars and fats. Transgenic animals which constitute a useful experimental tool for assessing the ability and effect of transgenic mammary gland specific expression are mainly concerned with either producing biologically important and active proteins such as pharmaceuticals in milk of transgenic animals or to alter the intrinsic properties and composition of milk itself by genetically adding a new or modified protein for better manufacturing properties for dairy industry. Our growing understanding of the lactation process in the ruminants at molecular level and continual innovations in dairy processing have presented exciting opportunities for genetic manipulations that are not possible through traditional, nutritional, and classical genetic approaches.

24.2 Biotechnology based strategies for altering the Properties of Milk

Advances in biotechnology and genetic engineering have led to exploring new initiatives that were hitherto not even thought possible in the field of dairying. It is now firmly established that a new generation of value-added products can be produced and harvested from milk and milk products. While until recently, emphasis has been on breeding large animals to produce more milk, the current interest of animal scientists is now on producing designer milk by expressing homologous/heterologous proteins and introducing appropriate alterations in the major milk constituents in the milch animals through animal cloning and transgenic technology. By a thorough understanding of the biochemistry, genetic traits and changes in the cows diet that affect milk synthesis and composition, ways and means to manipulate milk composition to suit specific needs can now be explored judiciously. By combining the two approaches of nutritional and genetic interventions, researchers are now hoping to develop '**designer milk**' tailored to consumer preferences or rich in specific milk components that have implications in health as well as milk processing. The current interest in the modification of milk composition include the healthful and therapeutic aspects of milk and milk products. To realize the full potential of these advantages, it would be desirable to have the opportunity to alter milk composition in several ways. For diet and human health measures, the actions that would be beneficial include: a) generate a greater proportion of unsaturated fatty acids (USFA) in milk fat b) reduce lactose content in milk in order to cater to persons suffering from lactose intolerance and c) remove β -lactoglobulin (β -lg) from milk. From a technological stand point, there exist vast opportunities in: a) alteration of primary structure of casein to improve technological properties of milk b) production of high-protein milk c) engineering milk meant for cheese manufacturing

that leads to accelerated curd clotting time d) increased yield and/or more protein recovery e) milk containing nutraceuticals and f) replacement for infant formula.

Five basic areas that might be highly useful for introducing desired alterations in milk are listed in Table 24.1. Within these broad areas, a wide variety of modifications to milk can be exploited. These include; adding extra copies of an existing gene (α s1, κ - and β -casein), down regulating the expression of a gene (α -lactalbumin), adding new genes such as those encoding human lysozyme or lactoferrin, removal of a gene (β -casein, β -lactoglobulin or acetyl-CoA carboxylase), and adding a mutated gene (α s1, κ - and β -casein) etc. In this direction, preliminary research has already been carried out using transgenic mice as model systems in the first four of these categories.

Table 24.1 Potential areas for introducing desired manipulations in milk targeting its key components for value addition

Altering the proteins to change the manufacturing properties of milk
Changing the amino acid composition of milk to improve human nutrition
Increasing the overall protein content of milk
Altering the type and amount of fatty acids in milk
Increasing the antimicrobial activity of milk

24.2.1 Alteration/ Manipulation of milk proteins

24.2.1.1 Caseins

The major milk proteins comprise of four variants of caseins namely α s1, α s2-, β - and κ -caseins and two whey proteins *viz.* alpha lactalbumin (α -LA) and beta lactoglobulin (β -LG) along with serum proteins and immuno-globulins which are present in milk of most of the mammals. The relative abundance of the various protein constituents of cow's milk varies between breeds and genotypes with the general proportions being approximately 31.5% α s1-casein, 29.5% β casein, 8.5% α s2-casein, 11% κ -casein, 10% β -lactoglobulin, 4% α -lactalbumin and 5.5% serum proteins and immuno-globulins. Caseins which include alpha, beta and kappa caseins are the major proteins of milk representing > 78% of the proteins. Normal Dairy Cattle have a single copy of alpha-CN, beta-CN and kappa-CN within each cell. Introduction of one extra copy of casein in the bovine genome can have a drastic impact on dairy industry. An increase of 20% in the content of α s1-CN of milk would result in an increase of over US\$ 190 million/year for the dairy industry. Similarly increased β -CN content in milk reduces rennet clotting time and increases the extent of syneresis. Therefore, higher protein content obtained by over expressing additional copies of endogenous bovine casein genes would be a significant advantage to the dairy industry.

Further modifications at the level of milk proteins can be achieved through molecular cloning of milk protein genes using appropriate vector–host systems by introducing alterations in the nucleotide sequences before putting the construct in the milch animal for expression of the altered protein. This step allows for their genetic modification in order to improve the nutritional quality or functional properties of milk. By using this strategy, more economical cheese products could result from a more efficient cleavage of a κ -CN by chymosin. Storage time for cheese maturation may be considerably reduced by altering the chymosin cleavage site of α s1-CN to more efficiently hydrolysable peptide linkages. Other manipulations

include alteration of physical properties particularly thermostability of casein. Thermostability can be achieved by increasing the expression of κ -casein genes and masking the expression of beta-lactoglobulin in the mammary gland. Since caseins are relatively low in sulphur containing amino-acids, their nutritional value could be improved by increasing their methionine content.

Desirable manipulations of the target milk proteins can also be introduced at protein sequence level as listed below with two specific examples

- Amino-acid sequence of α s1 casein can be manipulated by directed mutagenesis to make it more suitable for the reaction catalyzed by enzyme casein kinase which causes phosphorylation of caseins and this makes them highly thermostable.
- Amphiphilicity of the caseins can also be augmented by gene technology. This property is associated with the surface activities of the casein that govern their emulsification and foaming properties. This type of manipulation can be extremely useful in making ice-creams, softies, candies and whipped toppings.

24.2.1.2 Whey proteins

The two major whey proteins namely β -lactoglobulin and α -lactalbumin are separated during milk curdling. These proteins have been completely sequenced, crystallized and subjected to X ray diffraction studies. β -lactoglobulin is thought to function as a retinal binding protein that favors absorption of vitamin A in the gut of milch animals. β -lactoglobulin can specifically be targeted at the genetic level by site directed mutagenesis for extinction because its presence in milk confers some undesirable manufacturing properties. This would also aim towards utilization of the spared amino-acids pool in the cells made available to the protein synthesis apparatus to produce more desirable proteins.

The second most abundant whey protein in human milk, α -LA is a minor component in bovine milk. Because α -LA is a small protein of 123 amino-acids, it is amenable to genetic engineering by site directed mutagenesis to modify its amino-acid composition. This technique has been used to modify α -LA and make it consumable by patients with phenylketonuria who lack the enzyme that metabolizes phenylalanine and thus require low phenylalanine diets. Since the three dimensional structure of α -LA is known, the sensitivity of four phenylalanine residues in α -LA can be engineered in-vitro without disrupting the structure of the molecule. The modified gene is then microinjected into the pronucleus of a fertilized egg and the protein is produced in milk and can be used as a supplement for an improved diet for phenyl-ketonuria patients.

The aforesaid manipulations can be explored by using the following approaches:

(i) Adding extra copies of an existing gene

The addition of more κ -casein to the milk protein system could affect the physical properties of the milk since κ -casein is directly involved with micelle formation, structure and size. An increase in κ -casein could increase the thermal stability of casein aggregates and act to decrease micelle size. A smaller micelle diameter would lead to a larger available surface area, which would result in a more consistent and firmer curd as well as an increase in cheese yield. These modified properties of milk could be of great benefit and interest to the dairy industry.

(ii) Down-regulating the expression of a gene

The expression of a gene can be down regulated *in vivo* in a developmentally and the tissue-specific manner using transgenes expressing antisense of ribozyme messages

(iii) Removal of a gene

In order to determine the consequences of milk protein system of deleting a major milk protein, a β -casein knockout line of mice was produced. It was concluded that β -casein was a non-essential component of the milk protein system, thus illustrating that profound changes can be made in the composition of milk without disrupting the general organization of the micellar systems.

24.2.2 Fat content

It is now possible to manipulate even the fat content in milk by disrupting the fat synthesis in the mammary gland. By using this strategy, milk with 2% fat content (40% reduction) might be achievable. It has been suggested that de novo fat synthesis in mammary glands might be reduced by blocking the expression of acetyl CoA-Carboxylase gene through stem cell (knock out), antisense or ribozyme technology. Natural milk with reduced fat content could be extremely beneficial for patients with heart ailments.

24.2.3 Removing components of milk by genetic manipulation

The essential availability of bovine ES cells should enable undesirable components of the milk to be removed by disrupting the target gene in the bovine genome using homologous recombination.

24.2.3.1 Lactose

Lactose intolerance is a serious problem in 70% of the world population due to deficiency in the intestinal lactase (β -galactosidase) needed to hydrolyze milk lactose into its constituent monosaccharides leading to gastrointestinal upset. Genetic manipulation can now be applied to reduce lactose content in milk by either removal of α -LA by ES cells and gene 'knock out' methodologies or by introducing a lactase enzyme (β -galactosidase) into milk via mammary gland specific expression. The important role of α -lactalbumin in lactose formation has been shown in experiments involving transgenic animals. Lactation is disrupted in α -lactalbumin knockout mice, but it can be restored by human α -lactalbumin gene replacement thereby making it an attractive target for genetic manipulation to produce low lactose milk. An alternative approach for suppressing the α -LA gene expression is the use of antisense and ribozyme sequences. It is, however, imperative that such studies are conducted in ruminant mammary tissues in order to bring about the actual reduction in lactose and the economic benefits.

24.2.3.2 β -Lactoglobulin (β -LG)

Whey proteins have nutritional benefit in fluid milk market but represent a less valuable milk component for cheese industry. β -LG is the most abundant (upto 50%) whey protein. Human milk is devoid of β -LG which is considered to be the main allergen in bovine milk. However, it is not required in the lactation

process as β -LG has no known function in the process of milk secretion. Further upon heat treatment, β -LG forms gel aggregates with other β -LG molecules because of the exposure of a thiol group. This thiol group interacts with a disulphide group of κ -CN and interferes with chymosin mediated hydrolysis which is important for curd formation and cheese production. It is possible now to manipulate the level of β -LG at genetic level. However, because β -LG is also the major source of cystine in milk, removal of β -LG may result in a milk of inferior nutritional value. Hence, it would be desirable to delete β -LG coding sequences and neutralize the endogenous regulatory elements to drive expression of other proteins i.e. casein and lactoferrin.

24.2.4 Humanization of bovine milk

Breast milk is nature's perfect food for human infants providing them with all aspects of nutrition and protection against infections. However, a considerable number of infants are fed formulae based on bovine/buffalo milk. The composition of these infant formulations can be improved if the proteins contained therein resemble more closely to those of human milk. It is now possible to add human milk proteins including lactoferrin to bovine milk by genetic engineering to produce humanized milk and new functional foods that mimic human breast milk for providing better digestion and improved health and nutrition to the children. The shelf life of such products is also expected to be very high due to antimicrobial activity associated with some of these proteins.

24.2.4.1 Gene manipulation for enhanced shelf life

Milk and milk products have got a limited shelf life as they provide an ideal medium for the growth of a wide range of spoilage and pathogenic micro-organisms. The contamination of these perishable dairy foods with microbial contaminants would render them unfit for human consumption and also can cause huge economic losses to the dairy industry due to excessive food spoilage. However, the shelf life of these foods can be extended considerably by incorporating proteinaceous antimicrobial factors namely bacteriocins produced by food grade lactic acid bacteria. These bacteriocins can be expressed under the control of milk protein gene promoter in the mammary gland. Nisin is one such broad spectrum bacteriocin used extensively in dairy industry as a food-grade biopreservative. Nisin happens to be the first bacteriocin which was granted GRAS (Generally Regarded As Safe) status by FDA for application as food-grade biopreservative in canned processed cheese. It is produced by *Lactococcus lactis* subsp. *lactis* from where it can be isolated and purified. Nisin has been thoroughly characterized at molecular level. Hence, it can be explored as a potential target for its expression in milk through transgenic technology to provide inbuilt protection to milk and milk products against the common microbial contaminants without affecting the natural flora of milk, thereby, extending the shelf life and safety of raw milk considerably.

24.2.4.2 Adding new genes

In order to explore the possibility of introducing novel human genes encoding commercially important proteins such as lysozyme, attempts have been made to produce transgenic mice expressing human lysozyme in milk to determine the consequences of transgene on some basic rheological and antimicrobial properties of their milk. Lysozymes are ubiquitous enzymes found in avian egg whites and mammalian secretions such as tears, saliva and milk, that are positively charged at physiological pH and have an inherent antimicrobial activity. If human lysozyme is present in bovine milk at a significant level, two main effects could be expected. First, because of its antimicrobial activity, lysozyme may reduce the

overall level of bacteria in milk thus decreasing disease in the udder and overall bacterial levels in the milk. As lysozyme is considered to be a part of the passive immunity and the natural defense against bacteria, viruses, parasites and fungi in human milk, it could also exert human health advantages as well. Second, due to the net positive charge, lysozyme may be able to interact with the negatively charged caseins to produce milk with altered functional and physical properties. In studies using transgenic mice expressing human lysozyme in their milk at an average concentration of 0.38 mg per ml, the rennet clotting time of milk was decreased by 35%, gel strength of rennet induced gels was significantly higher in milk from the transgenic mice than in milk from control mice, while the average size of the micelles tended to be smaller. Milk from these transgenic lines was found to be bacteriostatic against two cold spoilage organisms- *Pseudomonas fragi* and *Lactobacillus viscosus* and a mastitis causing isolate of *Staphylococcus aureus*.

24.3 Animal Pharming

Animal pharming is defined as the use of transgenic animals as bioreactors for the production of pharmaceutical proteins and bioactive peptides for therapeutic applications in the treatment of different human diseases. Ever since this highly specialized technique was evolved, it was acclaimed as an highly efficient and cost effective method for the production of pharmaceuticals. The first therapeutic product produced in milk of transgenic live stock that got approval turned out to be recombinant human antithrombin-III which was produced by GTC Biothrapeutics under the brand name “ATryn”. This was indeed a new milestone with lot of impact on human health. Since then, quite a few other important therapeutic products have been developed through this technology by expressing these in the milk of cattle. However, there is some amount of skepticism whether animal pharming in the near future would be able to attract the new generation of investors to take up this technology at commercial scale and stimulate the society and the pharma industry to accept the transgenically derived live stock and products as an alternative to well established drug based production systems. Nevertheless, there is cause for optimism since pharmaceuticals represent a fast growing sector with lot of commercial stakes and has made considerable strides in this upcoming area of considerable health significance. Animal pharming seems to have immense potential and perhaps could be the future technology with bright prospects for managing chronic human diseases in accost effective manner.

24.4 Future Strategy

The ultimate goal of the dairy industry has been to create an efficient, healthy cow or buffalo that can serve all the needs of the industry in totality. Genetic engineering offers tremendous opportunity for a paradigm shift in reshaping of the industry from the producers to the processing plants. Dairy producers now find the prospects of applying advanced biotechnological tools a feasible strategy to add enormous value to milk by producing high protein milk, milk destined for cheese manufacture that has accelerated curd clotting time, milk containing nutraceuticals, orally administered biologicals that provide health benefits or a replacement for infant formulae. Such a scenario would be a radical change for the dairy industry. There is now little doubt that the products of genetic engineering will become a part of the dairy industry in the new millennium.

Hence, it can be concluded that it is now feasible to produce milk of altered chemical composition particularly in respect of homologous / heterologous proteins of considerable commercial value by appropriately manipulating genes encoding such commercially important proteins. Over production of

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such useful proteins in milk could not only improve the quality of dairy products made out of such milk but also be instrumental in increasing their shelf life and safety from public health point of view. Furthermore, it is possible to humanize bovine/buffalo milk by expressing human lysozyme, lactoferrin and other human milk protein in the target milch animal so that the milk produced from the transgenic animals mimic human milk in nutritional and therapeutic functionality and better digestibility.



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Lesson 25

APPLICATION AND IMPACT OF BIOTECHNOLOGY ON FOOD INDUSTRY

25.1 Introduction

Staple food constitutes the most indispensable and basic need of man for fulfilling minimal nutritional requirements to sustain human life on earth. Man has been traditionally depending upon agriculture and livestock to meet food demands since times immemorial. It is obligatory on the part of all Governments to provide safe, wholesome and nutritious foods to their citizens belonging to all the sections of society. A healthy diet can play a significant role in creating a healthy mind and healthy society in the country. Adequately nourished and healthy citizens can serve as the work force in building a Nation by boosting the growth, prosperity and productivity. However, the overall quality and safety of food commodities can be considerably influenced by the food processing and packaging to provide optimal nutritive value to the consumers. With the advent of new scientific knowledge and technological innovations, food sector is witnessing a phenomenal growth across the world particularly in developed countries. Although, developing countries like India are the potential markets for variety of such processed foods, their indigenous food processing industry is still in the transition stage to adopt modern and advanced food processing tools to compete with the developed countries. One such powerful technique that can be very promising and highly relevant to food processing industry in countries like India is the Biotechnology. By judiciously applying biotechnological tools and processes, the quality, safety and nutritive value of processed foods can be improved considerably with lot of value addition.

25.2 Role of Biotechnology in Food Sector

Biotechnology has already benefitted the food industry in a big way. It has given us high quality foods that are tasty, nutritious, wholesome, convenient, shelf stable and safe. As research and development initiatives continue, it seems inevitable that biotechnology will have an increasing impact on the food we eat. It offers huge potential for increasing the range and quality of food available to us, particularly more nutritious and palatable foods. It also seems likely that it will continue to bring advantages to the processing and safety monitoring of food supply due to emergence of new technologies at a faster pace.

Although, traditional biotechnology that makes use of natural microbial fermentations has been playing a vital role in the development of our food supplies such as cheese and yoghurt-making and the use of yeast to leaven bread and ferment alcohol. for thousands of years, the second-generation food biotechnology is based on initiatives to screen enzymes and micro-organisms in the natural environment and exploit them for useful applications such as food ingredients, microbial fermentation to manufacture several products like lactic acid, citric acid and other flavor enhancers etc. However, the major focus is now on exploring the modern biotechnology which is based on a combination of molecular genetics, applied enzymology and fermentation technology for value addition to foods. It is the modern Biotechnology which is becoming increasingly important part of the over all efforts to improve methods of food production and to increase the variety, quality and safety of foods we eat.

25.3 Potential Areas in Food Processing for Biotechnological Applications

There are several potential areas in the food industry where the traditional and modern biotechnological tools can be applied during processing for the overall improvement of the nutritional quality, safety and

health promoting attributes of the processed foods specifically with regard to the dairy based fermented products. Some of the potential areas of considerable commercial interest in food industry that can be targeted for biotechnological interventions are listed below:

1. Food fermentations
2. Starter cultures technology and genetic manipulation
3. Recombinant Enzymes
4. Biopreservation of foods
5. Functional / Health foods and Nutraceuticals
6. Probiotics, prebiotics and symbiotic foods
7. Genetically modified foods (GM Foods)
8. Milk derived bioactive peptides and other functional ingredients
9. Low calorie foods
10. Food packaging
11. Diagnostic tests for food safety and quality assurance
12. Biosensors

The scope and impact of biotechnological interventions in these areas will be briefly described and highlighted below:

25.3.1 Food fermentations

Biotechnology as applied to food processing makes use of microbial inoculants to enhance properties such as the taste, aroma, shelf-life, texture and nutritional value of foods. The process whereby micro-organisms and their enzymes bring about these desirable changes in food materials is known as fermentation. Fermentation processing is also widely applied in the production of microbial cultures, enzymes, flavours, fragrances, food additives and a range of other high value-added products. These high value products are increasingly produced in more technologically advanced developing countries for use in their food and non-food processing applications. Fermentation is one of the oldest biotechnological processes traditionally used by man since time immemorial for food preservation. Fermented foods such as bread, beer, wine, vinegar, sauerkraut, pickles etc. and traditional products like dahi, lassi and shrikhand account for one third of the human diet across the world. Other fermented dairy products such as cheese, yoghurt, kumis, kefir and others like sausages and soya sauce etc. are now being produced commercially and marketed globally. Food fermentations contribute substantially to food safety and food security particularly during the off season when there is decline in the production of raw material. The fermentation bioprocess is one of the major biotechnological applications in food processing and often constitutes an important step in a sequence of food-processing operations, which may include cleaning, size reduction, soaking and cooking. Fermentation bioprocessing makes use of microbial inoculants for enhancing properties such as the taste, aroma, shelf-life, safety, texture and nutritional value of foods. Microbes associated with the raw food material and the processing environment serve as inoculants in spontaneous fermentations, while inoculants containing high concentrations of live micro-organisms, referred to as starter cultures, are used to initiate and accelerate the rate of fermentation processes in non-spontaneous or controlled fermentation processes.. Current literature documents volumes of research reports on the characterization of microbes associated with the production of traditional fermented foods in developing countries. The development and improvement of microbial cultures has been a driving force for the transformation of traditional food fermentations in developing countries from an “art” to a science. Microbial culture development has also been a driving force for innovation in the design of equipment suited to the hygienic processing of traditional fermented foods under controlled conditions in many developing countries.

Improvements in the commercially important properties of microbial cultures, together with the improvement and development of bioreactor technology for the control of fermentation processes in developed countries, has played a pivotal role in the production of high-value products such as enzymes, novel microbial cultures, and functional food ingredients. These products are produced in more advanced developing economies, and are increasingly imported by less advanced developing countries, as inputs for their food processing. Although, the fermentation technology has been in vogue for many years in different countries, the output resulting from these technologies is not very high and hence needs optimization to minimize losses and maximize product recovery to make the technology efficient, commercially viable and cost effective. Microbial cultures can be genetically manipulated using both traditional and molecular approaches to improve their fermentation characteristics for producing better quality fermented foods through enhanced enzymatic activity and flavor development. However, genetic improvement of bacteria, yeasts and moulds has often been the subject of intensive debate because of safety and health concerns likely to be associated with such genetically modified microbes.

25.3.2 Starter culture technology in food fermentations and their genetic manipulation

One of the most important areas relevant to food processing industry is the use of lactic starter cultures. Starter cultures, comprising of Lactic Acid Bacteria (LAB) such as *lactococci*, *lactobacilli*, *pediococci* and *propioni* bacteria are used in the production of cultured dairy products such as dahi, yogurt and cheese etc. A starter culture is bound to provide particular characteristics in a more controlled and predictable fermentation. The availability of good starter bacteria is an essential pre-requisite for preparing quality fermented foods. The commercial value of the fermented products is, therefore, chiefly dependent upon the performance of the starter cultures. With the days of spontaneous fermentation and back slopping far behind us, we dwell in an era where the burgeoning fermented milk industry today demands “multifunctional” or “tailor made” starters fulfilling technical and metabolic requirements. Utility of starters go beyond imparting preservation and palatability to the final product. They could be selected for accelerated acid, flavor and, bacteriocin production in the fermented food to suppress spoilage and pathogenic bacteria apart from expressing additional health promoting functions. The deliberate use of functional traits within bacteria is supported by knowledge on their phylogenetics, characterization of genome structure and flexibility, gene regulation and gene functionality particularly in relation to their commercially important traits. The strategies used for genetic manipulation of lactic starters to enhance their commercially important metabolic activities in the fermented foods for value addition have been described previously.

Recent advances in the field of metabolic engineering, genomics, and bioinformatics are expected to contribute to the future development of functional starter cultures to be used for food processing industry. Exploration studies of the natural diversity of wild strains occurring in traditional, artisan foods, fermented dairy products etc. along with newer approaches such as comparative genomics, microarray analysis, transcriptomics, proteomics, and metabolomics, will generate useful information leading to the generation of new, industrial starter strains with increased diversity, stability, and industrial performance. These techniques will permit rapid, high-throughput screening of promising wild strains with interesting functional properties and lacking negative characteristics, as well as the construction of genetically modified starter cultures with a tailored functionality. Bioinformatics can be used to search genomes for essential components, for instance with regard to flavor development, such as peptidases, amino-transferases, enzymes for biosynthesis of amino acids, and transport systems for peptides and amino acids.

25.3.3 Recombinant enzymes

Food industry is constantly in search of advanced technologies to meet consumer demand for nutritionally balanced and safe food products. Enzymes are a useful biotechnological processing tool whose action can be controlled in the food matrix to produce high quality products. The emerging area of enzyme engineering addresses the requirement of food processing sector, reducing the investment and the processing cost dramatically. Currently-used food enzymes are extracted from animals and plants (for example, a starch-digesting enzyme, amylase, can be obtained from germinating barley seeds) but most of the enzymes come from beneficial micro-organisms by large scale fermentation through optimization of temperature, nutrients and air supply and later purified. Fermentation-derived enzymes are now the tools of choice for the innovative food processing industry. Moreover, several of the enzymes used in food processing industries are produced using recombinant micro-organisms. The industrial production of enzymes for use in food processing dates back to 1874 when Danish scientist Christian Hansen extracted rennin (chymosin) from calves' stomachs for use in cheese manufacturing. Bovine chymosin was the first enzyme to be produced through biotechnological approaches in *E. coli*. Since then, genetic manipulation has been used to make tailor made enzymes for specific consumer requirement. Now enzymes can be produced through recombinant DNA technology in large quantities for their subsequent application in food industry. Some of the recombinant enzymes produced through genetic engineering approaches are given the following Table 25.1

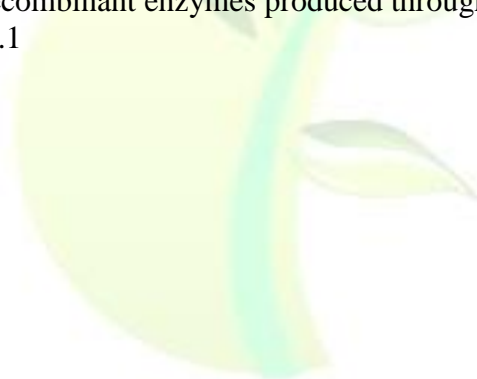


Table 25.1 Recombinant enzymes produced through genetic engineering and their application in foods

Enzyme Name	Host	Applications
Alpha-acetolactate decarboxylase	Bacteria	Removes bitter substances from beer
Alpha-amylase	Bacteria	Converts starch to simple sugars
Aspartic protease (chymosin /rennet)	Bacteria/fungi/Yeast (<i>Pichia pastoris</i>)	Used in production of cheese
Catalase	Fungi	Reduces food deterioration, particularly egg-based products
Cyclodextrin-glycosyl transferase	Bacteria	Starch/Sugar modification
Beta-glucanase	Bacteria	Improves beer filtration
Glucose isomerase	Bacteria	Converts glucose sugar to fructose sugar
Glucose oxidase	Fungi	Reduces food deterioration, particularly egg-based products
Lipase	Fungi	Oil and fat modification
Xylanase	Bacteria/fungi	Enhances rising of bread dough
Novamyl	Bacteria	Used in baked goods to preserve freshness
Pectin esterase	Fungi	Improves fruit juice clarity
Protease	Bacteria	Improves bread dough structure
Phytase	Bacteria, Yeast and Fungi	Phytate digestibility – phosphorus release
Pullulanase	Bacteria	Converts starch to simple sugars
Pectinase	Fungi	De-pectinization, mashing.

Most of these enzymes including bovine, goat or buffalo rennet are now being produced through rDNA technology for commercial applications in the food industry. Although, these recombinant enzymes are highly cost effective, their application in foods require approval from the regulatory agencies (DBT) from safety point of view.

25.3.4 Bio preservation of foods

Although, recent developments in innovative modern technologies implemented in food processing and more stringent microbiological food-safety standards have reduced the incidences of food borne illnesses and product spoilage, they can not completely rule out the possibility of health risks associated with such foods. Improved food safety has been achieved through drastic physical treatments like high temperatures, high pressure technology as well as chemical preservatives. Toxicity of many of the commonest chemical

preservatives (e.g. nitrites, sulphites), the alteration of the organoleptic and nutritional properties of foods, and especially consumers demand for safe and minimally processed foods without chemical additives have necessitated the need for alternative food grade safe biopreservatives. Hence, the food industry is constantly looking for new procedures and methods to produce minimally processed, ready to eat food with intact nutritional, taste, and flavor. Biopreservation of ready to eat processed foods is one such safe approach. It is defined as the extension of shelf life and safety of foods using the natural food grade antimicrobial compounds that are of plant, animal and microbial origin and do not pose any adverse effect on human health. The most common form of biopreservation of food products is through fermentation. The fermentation is brought about by food grade GRAS status lactic acid bacteria (LAB) belonging to *lactococci*, *lactobacilli*, *streptococci*, *pediococci*, *leuconostocs* etc. which are being extensively used as starter cultures in the manufacture of dairy, meat and vegetable food products. These bacteria preserve the nutritional qualities along with inhibition of pathogenic and spoilage organisms due to the production of organic acids, hydrogen peroxide as well as proteinaceous metabolites such as bacteriocins.

25.3.5 Functional / Health foods and nutraceuticals

Functional/health foods and /nutraceuticals are currently the focus of attention across the world because of their immense health potentials and commercial value. Although, the term “functional foods” currently lacks a common definition, this category is generally thought to include products that influence specific functions in the body and thereby offer benefits for health, well-being or performance, beyond their regular nutritional value. The concept of functional foods is not new as its origin dates back to prehistoric days. The old practice of using specific foods for some ailments figured prominently in ancient Hindu scriptures like Sushrita. The famous Greek physician Hippocrates also strongly advocated this concept through his tenet “**Let food be thy medicine and medicine be thy food**”. However, the relevance of this concept gained sudden momentum during the last few decades due to unprecedented interest evoked amongst the health conscious consumers. This has been largely attributed to radical change in the modern lifestyle and perception of consumers towards their diet beyond nutrition. As a result of shift in the mindset of consumers towards the linkage of diet with their health, the commercial interest in functional food market has grown enormously and there is a boom in the functional foods and nutraceutical products in the market as can be reflected from the availability of variety of health foods in the food counters. The driving forces behind the reemergence of functional food concept in the present context is driven by a number of factors including the increasing life expectancy of people, quest for safe alternative to drugs, self care movement, rising health care costs, overwhelming scientific evidences to link diet with health, advances in food and ingredients technology for product diversification and the greater media coverage given to these high profile foods with novel health claims. These products result from technological innovations, such as cholesterol lowering spreads, xylitol sweetened chewing gum and dairy products fermented with specific lactic acid bacteria, or are from a naturally functional food such as soy, oats and grains high in fiber. Functional foods have been developed in most food categories and the global market size is conservatively estimated to exceed that for organic foods. In addition to providing new options for improving health and well-being, the functional foods sector offers potential for new economic opportunities.

Biotechnology has a key role to play in this new industry. Traditionally, the application of biotechnology techniques in the food industry focused on the major energy-providing foods, such as bread, alcohol, fermented starch, yogurt, cheese, vinegar, and others. More recently, there has been increased interest in biologically active non-nutritive ingredients from natural products like herbals or foods. The functional food concept has in recent years moved progressively towards the development of dietary supplements that may affect the intestinal microbial composition and activities and hence may influence the gut health. In this context, dairy based food products which form an integral part of our diet can be very attractive candidate for application as functional or health foods after fortification / supplementation with novel

bioactive ingredients which have the ability to trigger general health promoting and specific physiological functions in the host. These bioactive ingredients include Probiotics/ prebiotics, bioactive peptides, biotherapeutic proteins, omega 3, CLA to low calorie sugars, PUFA, isoflavones etc. which can be added to dairy foods to enhance their functionality for protecting health of the consumers against chronic diseases such as gastro-intestinal illnesses, CVD, strokes, hypertension, diabetes, cancers etc.

Major breakthroughs have occurred and enormous progress has been made in this area of considerable health significance during the past few decades due to new advancements in biotechnological tools particularly with regard to genetic engineering and biotechnology. Nutrigenomics is the new era in the development of third generation of health/functional foods and is expected to revolutionize wellness and disease management across the world. Very soon need based customized health foods with specific bioactive functions intended for the target population will appear at the counters in the super markets and food outlets. This effort, however, requires a strong proactive synergy between Food and Pharmaceutical industry as well as Nutritionists, Biotechnologists and Dietetic and Medical professionals

25.3.6 Probiotics as functional foods

The term “probiotics” refers to live microorganisms that confer a health benefit to the host when ingested in adequate amounts (FAO/WHO, 2002). They are usually bacteria selected from species found in the intestinal tract. *Lactobacilli* and *bifidobacteria* are the two key members of this group used extensively in the production of probiotic food formulations for health applications across the world particularly in the developed countries. Milk and milk products specifically fermented dairy foods are considered as excellent carriers of probiotic strains to express their health promoting functions most optimally. Probiotic microorganisms may be concentrated and added directly to a food or to a milk product in small amounts and allowed to grow. Yoghurt is a classical example of a functional food with probiotics. Yoghurt with probiotics, called bio-yoghurt, should contain living bacterial cultures. Probiotics have been used as dietary supplements and oral agents for intestinal disorders. Probiotics have recently emerged as one of the most valuable bugs on account of expressing a multitude of novel health promoting functions which are highly strain specific. The most notable probiotic functions include immuno-modulation, restoring the balance of disturbed gut flora, strengthening the mucosal barrier function, prevention of lactose intolerance etc. However, the current focus of attention is to explore probiotics as possible biotherapeutics against chronic inflammatory metabolic disorders such as diabetes, CVD, obesity, irritable bowel disease (IBD) and syndrome (IBS), Ulcerative Colitis (UC), Crohn’s disease (CD), acute diarrhea, serum cholesterol reduction, shortening of the duration of respiratory infections, blood pressure control, colon cancer, and urinary tract infection (UTI) etc. Because of their immense health potentials, probiotics are now recognized as the vital health care concept of 21st century. Probiotics are one of the fastest growing food category within functional foods. And, as the list of health benefits accredited to them continues to expand, so does their use in new dairy and functional food applications. (Table 25.2).

Table 25.2 List of some popular brands of dairy based probiotic foods and probiotic strains used therein with different health claims

S. No.	Brand name	Company	Probiotic strain	Health claim
1	b Active	Mother Dairy's	<i>Bifidobacterium</i> BB-12	Enhance the body's natural digestive system and nutrient absorption capability
2	Nutrifit	Mother Dairy's	<i>L. acidophilus</i> LA5	Strengthen body's defences
3	ActiPlus Dahi	Nestle	<i>L. acidophilus</i> NCFM	Healthy digestive system
4	Culturelle Digestive Health Capsules	Culturelle	<i>L. rhamnosus</i> GG	Restores the natural balance of good bacteria in digestive tract: help to reduce digestive upset, help with occasional diarrhea and help with gas and bloating
5	Valio	Gefilus	<i>Lactobacillus</i> GG	Health-promoting effect
6	Yakult probiotic drink	Yakult Honshe	<i>L. casei</i> strain Shirota	Improve digestion and build immunity
7	Yo plus	Yoplait	<i>L. acidophilus</i>	Digestive health
8	Proviva	Probi AB	<i>L. plantarum</i> 299v	Digestive health
9	Activia light	Danone	<i>B. lactis</i> DN-173 010	Regulate digestive system
10	Danimal smoothies	Danimal	<i>L. rhamnosus</i> GG	General health benefits
11	Vaalialow fat yogurt	Vaalialow	<i>L. acidophilus</i> , <i>B. bifidus</i> and <i>L. rhamnosus</i> GG	Maintain and regulate digestive system
12	BioLife Yogurt Nectarine	Easiyo	<i>Bifidobacteria</i> and <i>L. acidophilus</i>	Digestive wellbeing

13	Yosa smoothie	Bioferme	<i>Bifidobacteria</i> spp. and <i>Lactobacillus rhamnosus</i>	Increase the amount of beneficial bacteria in the gut, balance the digestion and boost the immune system of gut
14	ProBugs (Goo-Berry Pie, Orange Creamy Crawler, Sublime Slime Lime and Strawnana Split)	Lifeway-kefir	<i>L. lactis</i> , <i>L. rhamnosus</i> , <i>S. diacetylactis</i> , <i>L. plantarum</i> , <i>L. casei</i> , <i>Saccharomyces florentinus</i> , <i>Leuconostoc cremoris</i> , <i>B. longum</i> , <i>B. breve</i> , <i>L. acidophilus</i> , <i>B. lactis</i> and <i>L. reuteri</i>	Promote healthy gastrointestinal functions
15	Activia organic yogurt	Stoneyfield	<i>B. lactis</i> DN-173 010	General health benefit

Recent advances in biotechnological tools such as genetic engineering, recombinant DNA technology, PCR and availability of whole genome sequences of common probiotic strains, have improved the prospects of designing novel probiotics with improved functional efficacy and safety for human health applications and new product development. The most notable novel recombinant probiotic at present is a derivative of *Lb. johnsonii* La1. La1 is a well characterized probiotic strain used extensively in commercial preparation of probiotic foods due to its strong health-related attributes and positive immuno-modulatory effects on the host. Milk fermented with this culture normally produces a racemic mixture of D and L-lactate in the ratio 60:40. Presence of D-lactate in milk fermented with La1 and ability of the strain to produce D-lactate after ingestion does not pose any problem to most of the adult population. But, it can indeed cause D-acidosis and encephalopathy in patients suffering from bowel syndrome and intestinal failures, and in new born infants with immature liver. However, inactivation of the single copy D-lactate dehydrogenase (LdhD) gene of La1 resulted in rerouting of pyruvate mainly to L-lactate with no D-lactate production. This novel strain has the same beneficial properties as the parent probiotic while the absence of D-lactate makes it a safer alternative for specific populations.

Amongst several other possibilities is the design of recombinant strains with novel properties that confer competitive advantage to their survival. One way to accomplish this strategy is by expressing and secreting colicin V, a narrow host range antibacterial bacteriocin produced by *E. coli* in La1. This strategy has allowed the expression and secretion of the Gram negative antimicrobial in probiotic organisms to extend their inhibitory spectrum to Gram negative enteropathogens too. Established probiotic lactobacilli can also serve as attractive candidates for oral vaccination against HIV, tetanus, Rota virus, *E. coli*, *Salomonella* and *H. pylori* etc. in view of their long history of safe use, ease of oral administration, low intrinsic immunogenicity and extensive industrial handling experience. Robust genetically engineered probiotic bacteria have also been developed by applying powerful genetic engineering techniques for

better survival and stability during the harsh technological processing conditions used in the product development and hostile gut environment. In foods, genetically engineered bacteria have been used to improve the flavor and stability, or to block the formation of unwanted flavors. Metabolic engineering and Genetic engineering should make it possible to strengthen the effects of existing bacterial strains and create new ones. Global gene and protein expression techniques as well as metabolomics are now extensively being used to provide evidence of probiotic adaptations in food products, their survival and host-microbe interactions in the mammalian gut. However, acceptance of genetically engineered probiotics in product development is a subject of intensive debate due to long term safety and public health concerns and requires approval from the regulatory bodies constituted for this purpose in the country.

25.3.7 Genetically modified and transgenic foods

Within the last two decades, the application of recombinant DNA techniques in the production of foods and food ingredients has developed from the level of basic research into a commercial business. From the very beginning, development and use of genetic engineering have been accompanied by strict regulations. These regulatory requirements cover the contained use of genetically modified organisms (GMO), their deliberate release into the environment as well as the placing on the market of products containing or consisting of GMO. So far, there is no report on any adverse effects on humans resulting from the consumption of foods produced by application of recombinant DNA techniques. Nevertheless, the sensitive nature of the subject (ethical issues) and the speed of the developments elicited fear among consumers. Potential hazards of the new technology are automatically projected into risks. In most developed countries, the willingness to accept such (perceived) risks is low, because the first generation of GM foods (herbicide-tolerant, insect-resistant crops and products thereof) were of no obvious advantage to regular consumers. The potential of recombinant DNA techniques in food production goes far beyond the applications reported so far. However, the benefits expected from the next generation of GM crops (improved nutritional value, functional foods) will result in new issues (complex metabolic changes, significant impact on overall nutritional status) and thus will pose new challenges in terms of food safety assessment. Nevertheless, it continues to be a grey area with high commercial stakes and as a result of that, the list of GMO and transgenic foods/crops has been expanding steadily (Table 25.3) While consumers accept the functional food concept readily, the acceptance of novel foods as they are defined according to the EU Regulation (EC, 1997), and particularly transgenic food, is controversial.

Table 25.3 Genetically modified foods/crops with regard to specific traits for value addition

All About Agriculture...

S.No.	Food	Traits	Modifications
1	Soybean	Resistant to herbicides (glyphosate or glufosinate)	Substitution of the <i>Agrobacterium sp.</i> (strain CP4) gene EPSP (5-enolpyruvyl shikimic acid-3-phosphate) synthase
2	Golden rice	Rich in vitamin A	Rice transformed with two beta-carotene biosynthesis genes: <i>psy</i> (phytoene synthase) from daffodil (<i>Narcissus pseudonarcissus</i>) and <i>crtl</i> from the soil bacterium <i>Erwinia uredovora</i>
3	Tomatoes	Enriched with vitamins, having extended shelf life (FlavrSavr tomato) and Virus resistance	Suppressed polygalacturonase enzyme production and inhibited production of ethylene by introducing genes for enzymes that degrade ethylene precursors Resistant against "cucumber mosaic virus" by transforming a virus capsid protein in plant
4	Potato	Fungal resistance, herbicide resistance and increased levels of amino acids	Resistant against the pathogen causing potato late blight (<i>Phytophthora infestans</i>) by transferring bacterial genes encoding anti-fungal enzymes, e.g. chitinase or glucanase. Tolerant against the glyphosate. Indian researchers developed a genetically modified potato that contain up to 60 percent more protein and increased levels of amino acids by inserting a gene from amaranth to the potato.
5	Corn	Herbicides, insect resistant and vitamin enriched	Resistant to glyphosate or glufosinate herbicides. Insect resistance via producing Bt proteins. Vitamin-enriched corn derived from South African white corn variety M37W has bright orange kernels, with 169x increase in beta carotene, 6x the vitamin C and 2x folate.
6	Papaya	Virus resistance	Immunisation through transfer of the gene for the envelope protein of the pathogenic virus (particularly papaya ringspot virus – PR virus)
7	Sugar cane	Virus and herbicide resistance	Resistant against mosaic viruses. Tolerant to Roundup Ready (active ingredient: glyphosate) and Liberty Link (active ingredient: glufosinate).
8	Sugar beet	Virus and herbicide resistance	Resistance against the rhizomania virus. Resistance to glyphosate or glufosinate.
9	Canola	Resistance to herbicides	Resistance to glyphosate or glufosinate
10	Brinjal	Pest-resistant	Bt crystal protein gene added/transferred into plant genome

Complementary to conventional breeding techniques, gene technology allows the transfer of genes between unrelated species. Thereby, breeding targets can also be achieved more quickly both in plant and animal breeding. This is one of the key, but hotly debated technologies of our times. Important topics that need to be addressed vary from legislation, such as labeling requirements, to safety and environmental issues. Concerns about application of this agricultural biotechnology are on the ecological impact of growing genetically modified foods, the impact of these crops on biological diversity, and on the safety of food supply, or the development of resistance by insect pests. However, the potential of the agricultural new biotechnologies is enormous also for developing countries. Therefore, questions about agricultural biotechnology must be addressed for people in both developed and developing countries, as we have to address the issue of food security for a world population of some 9000 million people in the year 2050. Furthermore, genetic engineering is not just a new technology for crop improvement, it is a powerful research tool that is helping to provide fresh and better insights of molecular mechanisms involved in biological processes.

25.3.8 Milk derived bioactive components

Milk and milk products are functional foods. Milk contains bioactive components beyond proteins, minerals and vitamins. These minor elements include immunoglobulins, hormones, growth factors, cytokines, nucleotides, polyamines, enzymes and bioactive peptides. Many bioactive peptides are embedded within milk proteins and remain inactive until released and activated by gastrointestinal digestion or during food processing. Bioactive peptides are naturally found in milk, fermented milk and cheese. Successful commercialization of milk bioactives is dependent upon developing new technologies for their production, producing innovative food and health ingredients, studying the mechanisms of actions, and conducting clinical studies to verify health effects. These bioactive peptides have been reported to exhibit anti-hypertensive activity, immune-modulatory properties and antimicrobial activity against high risk pathogens and hence can play a very important role in alleviation of gut related diseases such as peptic ulcers. Whey proteins such as lactoferrin, beta-lactoglobulin and alpha-lactalbumin also enhance immune cell function. The metabolic activity of probiotic lactic acid bacteria generates de novo immunoregulatory peptides from milk via enzymatic degradation of parent milk proteins. Opioids refer to natural opiates (opium) and synthetic narcotics (i.e. morphine, heroin) that induce sleep and soothe pain. The opioid agonists in milk peptides, derived from casein or whey protein, have shown morphine-like activity. For example, caseinomorphins prolong gastrointestinal transit time, prevent diarrhea, stimulate secretion of insulin and somatostatin and could play a role in appetite suppression. Opioid antagonists such as casoxins and casoplatelins block the agonist effect of externally administered opioids and enkephalin (the endogenous neurotransmitter) thus affecting the release of pain-reducing endorphins.

25.3.9 Packaging of processed foods

The rising sales of convenience processed foods / ready to eat foods and developments in their packaging have been a major issue in innovative packaging to attract the consumers. Packaging techniques have developed to an extent to provide very attractively packed foods and consumers are prepared to pay a premium for quality and safe ready to eat foods. Modern consumers being highly health conscious and with the improvement in economy and rising financial status, demand for bottled water, fruit and vegetable juices, milk drinks and milk products, functional foods, sausages etc along with increasing demand for packaged fresh food products is all time high. In emerging markets where super and hypermarkets are expanding very rapidly, the demand for high barrier materials, active packaging, intelligent packaging, modified atmosphere packaging (MAP), active packaging with antibacterial activity

for increasing shelf life of processed foods, nanotechnology and digital print for packaging is rising extraordinarily. This is precisely due to the growing awareness of health conscious consumers towards safety and demand for processed foods with extended shelf life. Packaging industry in India is one of the fast growing industries which has its influence on all the other industries directly or indirectly.

Recently, nanotechnology has been significantly increasing its impact on the food and beverage packaging industry by altering the structure of the materials on the molecular scale, to give the materials desired properties which can significantly enhance the shelf life, efficiently preserve flavour and colour as well as facilitate transportation and usage. Nanotechnology applications for food contact materials / matrices (FCM) and food packaging constitute the largest share of the market for applications in the food sector. Nano-structured film can effectively prevent the food from the invasion of microorganisms and ensure the food safety. Sensors can alarm us before the food goes rotten or can inform us the exact nutrition status contained in the contents. Active FCMs generally incorporate nanoparticles with antimicrobial or oxygen scavenging properties whereas Intelligent food packaging can incorporate nanosensors to monitor and report the condition of the food. Based on the antimicrobial action of nano silver, a number of active FCMs have been developed that are claimed to preserve the foods for longer period by inhibiting the growth of microorganisms. Examples include “Fresher Longer™ Miracle Food Storage Containers” and “Fresher Longer™ Plastic Storage Bags” from Sharper Image® USA, “Nano Silver Food Containers” from A-DO Korea and “Nano Silver Baby Milk Bottle” from Baby Dream® Co. Ltd. (South Korea). The embedded sensors in a packaging film can detect food-spoilage organisms and trigger a colour change to alert the consumer about the end of the shelf life. One of the examples is Nano Bioswitch/“Release-on-Command” system that releases a preservative if food begins to spoil. Nanoscale-sensing devices are also being developed that will enable the food or food ingredients to be traced back to the source of origin.

25.3.10 Low calorie foods

The current trend towards a more health- and nutrition-conscious lifestyle has encouraged the development of low calorie foods. The non-nutritive sweetener market has been predicted to reach \$500 million by the year 2000. A new class of compounds called taste-active proteins functions as sweeteners and flavor modifiers and includes compounds such as aspartame, thaumatin, and monellin. The gene which codes for the protein thaumatin has been isolated and characterized. Transfer of this gene into bacteria would allow the production of thaumatin via fermentation. If engineered into plants, new and unique foods could be developed. Another application of biotechnology in low calorie food production is the development of low calorie fats and oils. Genetically inducing the production of shorter chain fatty acids in soybean or rapeseed would speed the development of a low calorie vegetable oil. The market for this oil could reach \$2 billion a year by the end of the next decade.

25.3.11 Diagnostic tests for food safety and quality assurance

Detection and identification of pathogenic bacteria in foods is extremely important for ensuring the safety of food supplies as well as for confirming food-related outbreaks. However, microbial detection and identification is a challenge. First of all, high sensitivities are required for preliminary enrichment and subsequent isolation steps that separate the microorganisms from the foods. Secondly, high specificities are needed for microbial identification to rule out the possibility of false positive and false negative results. The identification step can effectively separate the target pathogens from the background

microflora. In this context, the conventional methods based on microbiological culturing and metabolic activation have been traditionally used for analysis of foods in dairy industry across the world. The conventional methods of pathogen identification and confirmation based on culturing on selective medium, biochemical tests and immunological assays are extremely laborious, cumbersome and many times remain inconclusive and results are invariably delayed to make them virtually redundant for any follow up corrective actions. However, with the advancements in Biotechnology and Molecular Biology, new innovative molecular and immunological techniques have also been developed and applied in specialized food laboratories in advanced countries like USA and Europe for assessing the microbiological quality and safety of foods. Some of these rapid assays based on molecular techniques such as PCR and Real Time based diagnostics, Immunological assays, biosensors and enzyme based kits etc. are considered to be more reliable and rapid for quick detection of pathogens.

25.3.12 Biosensors

Biosensors represent analytical new generation of powerful tools incorporating biologically derived material or biomimic with a physiochemical transducer or transducing microsystem. Biosensors are currently being explored for a wide range of applications in food industry. The techniques based on Biosensors are being developed for rapid direct or indirect detection of foodborne microorganisms, toxins, or undesirable metabolites or other compounds. These systems have a potential application in real-time validation of critical control points. Sensitive, specific and rapid processes have been developed that require minimal culture enrichment and utilize immuno-based biosensors, such as immunomagnetic-electrochemiluminescence to detect pathogenic microorganisms in food systems. Immuno-based biosensors to detect low levels of *E. coli* 0157 and *Salmonella* within 2-8 hrs are being used in meat and poultry plants as well as in dairy industry. New technologies such as acoustic wave biosensors and radio frequency identification (RFID) sensor tags promise to greatly improve food safety. Research to develop a single computer chip that will automatically assess food safety at any point from source to consumption is ongoing. The advanced biosensor based systems have the advantage to be integrated into the processing line for monitoring the possible contaminants and pathogens on line during different food processing stages so that follow up action could be taken immediately.

25.4 Impact of Biotechnology in Food Industry

In the backdrop of growing human population at an alarming rate in third world countries including India and the ensuing poverty that continue to daunt the countries, the demand for food and nutritional security has increased dramatically. As a result of this, the role of food industry has become extremely pertinent in producing high quality nutritious and wholesome foods which are safe and cost effective to cater to the needs of their vast respective populations. The application of traditional biotechnology in food industry has been in vogue for quite some time and has made a significant impact on commercial production and processing of foods by improving the fermentation efficiency of the micro-organisms through optimization of processing parameters to produce the desired quality of food products. The most recent application of modern biotechnology to food industry is the genetic modification (GM) also known as genetic engineering/genetic manipulation/gene technology or recombinant DNA technology. The aims are to increase the range and quality of products available, to reduce their price and to protect the environment. Biotechnology has already made a strong impact on food and dairy industry by improving the nutritional quality, shelf life and safety of processed foods with lot of value addition for different applications including health benefits. By adopting new advancements and innovations in modern

biotechnology such as rDNA technology, transgenics, animal/plant cloning, tissue culture and improved bioprocess engineering tools, food industry can benefit immensely through not only improving the yield and quality of the processed foods but also bringing in lot of product diversification by producing novel foods customized for specific consumers. Application of biotechnology in true letter and spirit is likely to revolutionize the concept of food processing in the Indian food industry and hence can make a dramatic influence on our lives and that of future generations if used properly and judiciously. However, preceding the advent of such products onto the market, questions were being raised about their safety, labeling, need and ethics. The use of modern biotechnology (recombinant DNA technology) to produce foods and food ingredients is a subject of heightened discussions and controversy among consumers and public policy makers, and within the scientific community and hence can have lot of impact on the industry and the consumers from the health benefits and safety perspectives.

25.4.1 Biotechnological interventions in food processing

Since proteins and vitamins are often lost in traditional food processing, fermentation processes may offer a way to preserve them. Biotechnology can be used for the upgrading of traditional food processing based on fermentation such as the procedures used to produce high quality, nutritious and wholesome fermented foods such as traditional dairy based products like dahi, lassi, shrikhand and nondairy products like gari, a fermented, gritty and starchy food derived from cassava. Biotechnology can also help to eliminate toxic components, either by genetic engineering or through food processing. In addition to eliminating unwanted components, biotechnology can be used for the inexpensive production of additives that increase the nutritive value of the final product or that improve its flavour, texture or appearance. Present-day applications of biotechnology in food processing are far more advanced than applications in the field of plant genetic engineering. The genetic manipulation of micro-organisms used in food processing is considerably easier than the manipulation of more complex plants. It is, therefore, intriguing that research centers primarily on plant genetic engineering, where there are still many obstacles to overcome, while the chance to improve food processing is largely neglected.

25.4.2 Current status of biotechnology in food processing - food fermentations

Microorganisms are an integral part of the processing system during the production of fermented foods. Microbial cultures can be genetically improved using both traditional and molecular approaches, and improvement of bacteria, yeasts and moulds is the subject of much academic and industrial research. Traits which have been considered for commercial food applications in both developed and developing countries include sensory quality (flavour, aroma, visual appearance, texture and consistency), bacteriophage resistance in the case of dairy fermentations, and the ability to produce antimicrobial compounds (e.g. bacteriocins, hydrogen peroxide) for the inhibition of undesirable microorganisms. In many developing countries, the focus is on the degradation or inactivation of natural toxins (e.g. cyanogenic glucosides in cassava), mycotoxins (in cereal fermentations) and anti-nutritional factors (e.g. phytates).

Biotechnology has also been extensively explored in the production of enzymes for application in raw and processed foods. In the past, enzymes were isolated primarily from plant and animal sources, and thus a relatively limited number of enzymes were available to the food processor at a high cost. Today, bacteria and fungi are exploited and used for the commercial production of a diversity of enzymes. Several strains of microorganisms have been selected or genetically modified to increase the efficiency with which they

produce enzymes. In most cases, the modified genes are of microbial origin, although they may also come from different kingdoms. For example, the DNA coding for chymosin, an enzyme found in the stomach of bovine and buffalo calves, that causes milk to curdle during the production of cheese, has been successfully cloned into yeasts (*Kluyveromyces lactis/Pichia pastoris*), bacteria (*Escherichia coli*) and moulds (*Aspergillus niger* var. *awamori*). Chymosin produced by these recombinant microorganisms is currently commercially produced and is widely used in cheese manufacture. Genetic technologies have not only improved the efficiency with which enzymes can be produced, but they have increased their availability, reduced their cost and improved their quality. This has had the beneficial impact of increasing efficiency and streamlining processes which employ the use of enzymes as processing aids in the food industry.

25.4.3 Some issues relevant to developing countries

Biotechnological research as applied to bioprocessing in the majority of developing countries, targets development and improvement of traditional fermentation processes. In this context, some areas specifically relevant to developing countries as listed below need to be looked into before adopting advanced biotechnological tools in the food processing industry.

25.4.3.1 Socio-economic and cultural factors

Traditional fermentation processes employed in most developing countries are low input, appropriate food processing technologies with minimal investment requirements. They make use of locally produced raw materials and are an integral part of village life. These processes are, however, often uncontrolled, unhygienic and inefficient and generally result in products of variable quality and short shelf lives. Traditional fermented foods like dahi, srikhand and butter milk etc., nevertheless, find wide consumer acceptance in developing countries and contribute substantially to food security and nutrition. Applications of biotechnology to fermented foods can have a strong impact on these socio-economic and cultural factors.

25.4.3.2 Infrastructural and logistical factors

Physical infrastructural requirements for the manufacture, distribution and storage (e.g. by refrigeration) of microbial cultures or enzymes on a continuous basis is generally available in urban areas of many developing countries. However, this is not the case in most rural areas of developing countries. Should research be oriented to ensure that individuals at all levels can benefit from applications of biotechnology in food fermentation processes, i.e. should logistical arrangements for starter culture development be integrated into biotechnological research targeting improvement of traditional fermentations? What is required for the level of fermentation technologies and process controls to be upgraded in order to increase efficiency, yields and the quality and safety of fermented foods in developing countries?

25.4.3.3 Nutrition and food safety

Fermentation processes enhance the nutritional value of foods through the biosynthesis of vitamins, essential amino acids and proteins, through improving protein and fibre digestibility; enhancing micronutrient bioavailability and degrading antinutritional factors. Many bacteria in fermented foods also exhibit functional properties (probiotics). The safety of fermented food products is enhanced through

reduction of toxic compounds, such as mycotoxins and cyanogenic glucosides, and production of antimicrobial factors, such as bacteriocins, carbon dioxide, hydrogen peroxide and ethanol, which facilitate inhibition or elimination of food-borne pathogens. Are the nutritional characteristics (and safety aspects) of fermented foods adequately documented and appreciated in developing countries? Is there a need for consumer education about the benefits of fermented foods?

25.4.3.4 Intellectual property rights (IPRs)

The processes used in the more advanced areas of agricultural biotechnology tend to be covered by IPRs and these rights tend to be owned by parties in developed countries. This applies also to biotechnology processes used in food processing. On the other hand, many of the traditional fermentation processes applied in developing countries are based on traditional knowledge. In addition to biotechnology processes, microbial strains may also be the object of IPRs. For example, an era of massive private investment in biotechnology was initiated when the United States Supreme Court ruled in 1980 (in the *Diamond versus Chakrabarty* case) that a live GM bacterium (of the genus *Pseudomonas*, modified to degrade components of crude oil) could be patented. Many of the microorganisms associated with traditional fermentation processes in developing countries are unique. Issues of ownership will become increasingly important as bacterial strains are characterized and starter cultures are developed in developing countries.

25.5 Commercial Opportunities

Biotechnological innovations have greatly assisted in industrializing production of certain indigenous fermented foods. Indonesian tempe and Oriental soy sauce are well known examples of indigenous fermented foods that have been industrialized and marketed globally. The results of biotechnology research will lead to fermented foods of improved quality, safety and consistency. Should biotechnology developments in developing countries target commercialization? Should they target diversification into new value-added products? Should biotechnology development be linked to technological developments in food processing? Can the application of biotechnology to food processing allow farmers in developing countries to add value to their agricultural products (for export or for local consumption) and improve their revenues?

Biotechnology has already made a strong impact on food and dairy industry by improving the nutritional quality, shelf life and safety of processed foods with lot of value addition for different applications including health benefits. By adopting new advancements and innovations in modern biotechnology such as rDNA technology, transgenics, animal/plant cloning, tissue culture and improved bioprocess engineering tools, food industry can benefit immensely though not only improving the yield and quality of the processed foods but also bringing in lot of product diversification by producing novel foods customized for specific consumers. Application of biotechnology in true letter and spirit is likely to revolutionize the concept of food processing in the Indian food industry and hence can make a dramatic influence on our lives and that of future generations if used properly and judiciously.

Biotechnology undoubtedly has a potential role in food processing industry in India and other developing countries and hence can help in meeting the food and nutritional security effectively. Judicious use of modern biotechnology tools and strategies could be extremely valuable not only to increase the food production for the growing population but also can aid in improving the processing quality, taste,

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nutritional value, texture, shelf life, marketability and added advantages of having medicinal properties for various ailments, thereby, enhancing the commercial value of these foods considerably. The resurgence of concept of functional foods and nutraceuticals for health applications gained momentum at the global level through biotechnological applications. These value added biotech based farm products tailored to processing industries certainly can increase farmers and processors revenue, at the same time satisfying the consumer preferences. Biotechnology has tremendous potential for increasing food production and improving food processing although the real impact will only be felt after a few decades and it will differ from country to country. Nevertheless, biotechnology can have a dramatic impact on the food processing industry in developing countries like India by not only improving the efficiency of food processing but also through value addition and product diversification for catering to the needs of both domestic market and their exports. Additionally, biotechnology interventions in the food chain of agriculture and food processing sectors can generate lot of employment opportunities in the country. By producing safe, high quality, nutritious wholesome and healthy foods within reach of common consumers, biotechnology can help in creating a healthy society and can tremendously boost the growth, productivity and economic status of India at the global level.



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Lesson 26

APPLICATION OF BIOTECHNOLOGY FOR DISPOSAL OF WHEY AND DAIRY EFFLUENTS

26.1 Introduction

The dairy effluents including whey are quite rich in degradable organic matter and exert a high oxygen demand. The dairy effluents are peculiar as compared to other industrial wastes, because of relatively high concentrated effluent, particularly whey and butter washings. The processing of one litre of milk, yields about 8-10 litres of waste water depending on the type of products manufactured. More than 90 per cent of a dairy waste consists of milk components (lactose, proteins, and butterfat) that are lost and flow into floor drains during processing.

Whey is the major by-product, along with dairy effluents produced during the manufacture of cheese, paneer or casein from milk, representing 80 to 90% of the volume of milk transformed. It contains approximately 4.5% (w/v) lactose, 0.8% (w/v) protein, 1% (w/v) salts, and 0.1% to 0.8% (w/v) lactic acid. Proper disposal of whey is extremely important as it is considered as a pollutant due to its high biological oxygen demand (32, 000 to 60,000 ppm). Contaminating whey in natural water systems can quickly deplete oxygen levels due to the metabolic degradation of the organic constituents by the microbial population and hence disposing of whey has always been a major problem. Whey cannot be discharged into lakes or rivers for environmental reasons and at the same time, it is also not desirable to simply dump it to waste treatment facilities for economic reasons. Biotechnology offers ample opportunities in converting whey into numerous useful ingredients or product formulations reducing the burden on the effluent disposal.

26.2 Biotechnological approaches in treatment of whey and dairy waste

The strength of whey pollution in dairy waste is determined by two parameters *viz.* **Biochemical oxygen demand (BOD)** and **Chemical oxygen demand COD** which are taken into consideration while designing the strategy for waste disposal.

Biochemical oxygen demand (B.O.D) is the amount of dissolved oxygen needed by aerobic biological organisms in a body of water to break down organic material present in a given water sample at certain temperature over a specific time period. The BOD₅ value is most commonly expressed in milligrams of oxygen consumed per litre of sample during 5 days of incubation at 20°C.

Chemical oxygen demand (COD) is the amount of oxygen (in mg) required for the complete chemical oxidation of organic and inorganic material in 1 litre of an effluent

Before visualizing the application of biotechnological methods for treatment of whey and dairy effluents, it is important to be acquainted with the various processes involved in dairy product manufacturing and the pollution potential of different dairy products (Table 26.1)

Table 26.1 BOD and COD values for typical dairy products

Product	BOD ₅ mg/l	COD mg/l
Whole milk	114,000	183,000
Skim milk	90,000	147,000
Butter milk	61,000	134,000
Cream	400,000	750,000
Evaporated milk	271,000	378,000
Whey	42,000	65,000

26.2.1 Whey utilization and disposal

Whey is a complete protein, lush with amino acids and so it can be processed to produce a wide range of commercial products. In recent times the perception of whey from a “waste material” to an “opportunity” for further processing is rapidly changing as recombinant DNA technology have offered a more direct way of manipulating the cell's metabolism for the production of specific biochemical products from whey. (Table 26.2)

Table 26.2 Some of the organisms used for utilization of whey for reducing the BOD

Biotechnologically identified or modified Organism	Enzyme / property	Function
<i>Kluyveromyces sp.</i>	β -galactosidase	Reducing the lactose content
<i>Bacillus licheniformis</i>	Proteases	Soluble whey proteins can be hydrolyzed into amino acids and peptides
<i>Kluyveromyces fragilis</i> , <i>Kluyveromyces marxianus</i> , and recombinant <i>Saccharomyces cerevisiae</i> strain	Lactose utilizing enzyme	Reducing the lactose content and producing bioethanol
<i>Lactobacillus helveticus</i> , <i>Lactococcus lactis</i> subsp. <i>cremoris</i> <i>Kluyveromyces fragilis</i> , <i>Kluyveromyces lactis</i> M2 <i>Saccharomyces cerevisiae</i> <i>Candida sp.</i> ,	Exo-polysaccharides (EPS) Converting lactose to protein	improve the solubility and reduce the gritty mouth feel of whey mineral concentrate Production of Single cell proteins and BOD reduction in whey

26.2.2 Dairy waste disposal

Dairy waste may be treated physically, biologically and chemically. The physical methods include, include screening, sedimentation, filtration, or flotation. Chemical processes comprises of disinfection, adsorption, or precipitation. Biological methods consist of aerobic systems such as activated sludge, trickling filter, oxidation ponds, lagoon technology and anaerobic systems.

However, genetically modified microbes can be produced by applying genetic engineering tools and used in several ways while dealing with different biological waste treatment methods for improving their efficiency and also generating useful end products for commercial use.

Recombinant DNA technology offers a more direct way of manipulating the cell's metabolism for the production of specific biochemical products. The technology has proved extremely useful for determining the nucleotide sequences of the DNA in and around genes and identifying those segments that are needed for the control of the genes or their products. These segments constitute prime targets that might be specifically mutated or deleted to alter the control of the genes. New genes might also be introduced into bacterial cells to give them novel synthetic capacities.

Application of recombinant DNA technology to waste treatment procedures involve two steps

- Finding a microorganism that has the desirable function (e.g., ability to degrade a pesticide)
- Transferring this desirable function to a suitable host, preferably a microorganism with some relevance from an environmental viewpoint

Some of the notable areas of applications of specially designed or genetically manipulated strains of microbes for utilization of whey or disposal of dairy waste in more economical way are summarized below:

1. BOD Reduction

Lactose utilizing yeasts such as *Kluyveromyces sp.* are important sources for the production of β -galactosidase enzyme. It is one of the most promising enzymes for biotechnological applications for treating whey and dairy waste for reducing the lactose concentration and BOD content

2. Single cell proteins

Conversion of whey and useful components form dairy waste into single cell protein serves two functions (i) for reduction in pollution and (ii) creation of edible protein. The *Kluyveromyces species* have been most widely studied for SCP production by converting lactose to protein into microbial biomass. The mixed culture of *K. lactis* and *K. marxianus* with *S. cerevisiae* was reported to be viable and an attractive alternative for removal of BOD and obtaining a valuable biomass yield.

3. Bio-ethanol

Several metabolic engineering approaches have been used to construct lactose-consuming *S. cerevisiae* strains, specifically involving the expression of the lactose genes of the phylogenetically related yeast

Kluyveromyces lactis, and also from *Escherichia coli* and *Aspergillus niger* for the production of bio-ethanol from lactose.

4. Exopolysaccharides (EPS)

Modified lactic acid bacteria (LAB) such as *Lactobacillus helveticus* or *Lactococcus lactis* subsp. *cremoris* are being successfully employed for the production of EPS for use in various food formulations

5. Removal of toxic metals

Extracellular polymers produced by microorganisms commonly found in activated sludge display a great affinity for metals. Several bacterial types (e.g., *Zooglea ramigera*, *Bacillus licheniformis*) produce extracellular polymers that are able to complex and subsequently accumulate metals such as iron, copper, cadmium, nickel, or uranium. The accumulated metals can be easily released from the biomass by treatment with acids.

6. Biological fuel cells

Microorganisms can be used as electron donor in biological fuel cell (BFC) for the conversion of organic matter into power. One of the microbes exploited in this area is *Rhodospirillum rubrum*. BFC generates electrical energy through the oxidation of biodegradable organic matter in the presence of either fermentative bacteria or enzyme under mild reaction conditions like ambient temperature and pressure

7. BOD Sensors

Biofilm-based biosensors consist of immobilized microorganisms trapped between a porous membrane and a gas-permeable membrane. Biosensors using pure microbial cultures (e.g., *Bacillus subtilis*, *Klebsiella oxytoca*, *Clostridium butyricum*, *Pseudomonas putida*, *Trichosporon cutaneum*) or mixtures of activated sludge microorganisms have been considered and are commercially available.

8. Biodegradation of oil

Biodegradation of oil spills is a major problem. Moreover, a single bacterium cannot degrade all the components of oil which are petroleum products. Anand Chakrabarty, an Indian scientist, genetically engineered a strain of *Pseudomonas putida* that can degrade more than 3-4 compounds of petroleum.

9. Application of Immobilized cell technology can be used in the treatment of supernatant liquor which is a by-product of sewage sludge processing having high concentrations of ammonia. This high load of ammonia can be reduced by nitrifiers which convert ammonia to nitrate and this ammonia is oxidised to nitrite by *Nitrosomonas*, and then the nitrite is oxidised to nitrate by *Nitrobacter*.



Lesson 27

BIOINFORMATICS AND GENOMICS, TRANSCRIPTOMICS AND PROTEOMICS

27.1 Definition of Bioinformatics

As per Oxford dictionary, Bioinformatics is defined as conceptualizing biology in terms of molecules (in the sense of physical chemistry) and applying "informatics techniques" (derived from disciplines such as applied maths, computer science and statistics) to understand and organize the information associated with these molecules, on a large scale. In short, bioinformatics is a management information system for molecular biology and has many practical applications.

Bioinformatics is the application of computers in the management of biological data.

The term was coined by Paulien Hogeweg in 1978. It involves development and application of computer hardware and software to the acquisition, storage and analysis of the tremendous biological data. The data in bioinformatics pertains to nucleotide (genomics) as well as protein (proteomics) sequences and many more related to 'omics' technologies which need to be analysed by Bioinformatic analysis tools as shown in Fig. 27.1.

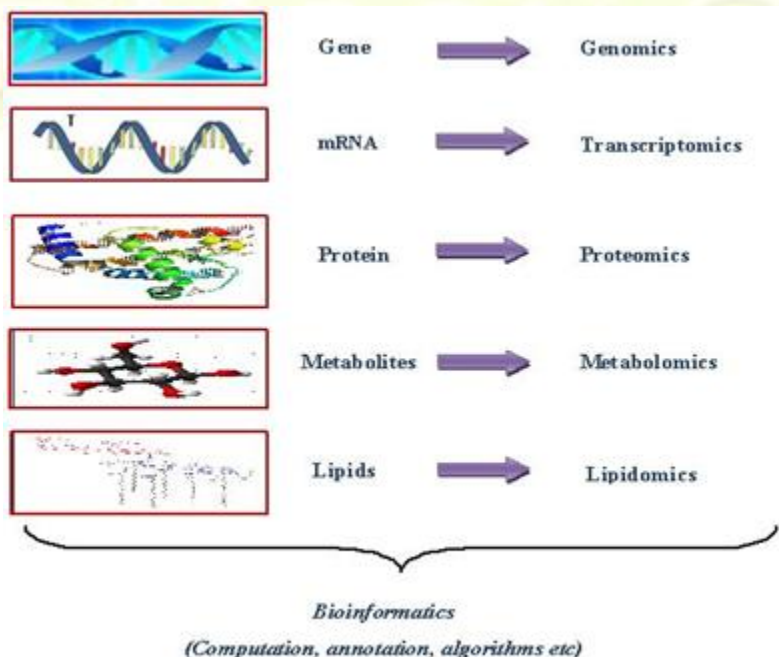


Fig. 27.1 Data analysis obtained from various 'Omics' technologies by bioinformatic tools

Bioinformatics make extensive use of mathematical and statistical models which are hidden in the computer science aspect of bioinformatics. The mathematics involved mainly deals with algorithms which is a step-wise method for solving a problem. The techniques and algorithms were specifically developed for analysis of biological data.

27.2 Aims of Bioinformatics

The bioinformatics is aimed at achieving the following targets.

1. Bioinformatics organize data in a way that allows researchers to access existing information and to submit new entries particularly in nucleotide and protein databases e.g. GenBank and Protein Data Bank.
2. Bioinformatics also develop tools and resources required in the analysis of data e.g. development of software tools to compare nucleotide and protein sequences and also their alignment with the existing sequences stored in the form of database.
3. Bioinformatics involve the application of the analysis tools to analyze the data and interpret the results in a biologically meaningful manner.

27.3 Databases

A database consists of an organized collection of vast data for one or more uses, typically in digital form that can be easily accessed, managed and updated. Initially, a database was created in USA and UK. The database includes associated tools (software) necessary for access, updating, information insertion and information deletion. The following databases are available for global access.

- Sequences (DNA / Nucleotide, protein)
- Genomics
- Mutation/polymorphism
- Protein domain/family
- Proteomics (2D gel, Mass Spectrometry)
- 3D structure
- Metabolism
- Bibliography
- Expression (Microarrays)
- PubMed
- SNP (single nucleotide polymorphism)
- Specialized i.e. Expressed Sequence Tags (EST), Sequence tagged sites (STS) etc.

27.4 National Centre for Biotechnology Information (NCBI) Resource

NCBI was established in 1988 in USA. The National Center for Biotechnology Information (NCBI) is part of the United States National Library of Medicine (NLM), a branch of the National Institute of Health. The NCBI houses genome sequencing data in GenBank and an index of biomedical research articles in PubMed Central as well as other information relevant to biotechnology. All these databases are available online through the Entrez search engine.

27.4.1 Nucleotide sequence databases

The International Nucleotide Sequence Database Collaboration (INSDC) consists of joint effort to collect and disseminate databases containing DNA and RNA sequences. It involves three major databases i.e. GenBank (www.ncbi.nlm.nih.gov/GenBank); European Molecular Biology Laboratory (EMBL, www.ebi.ac.uk/embl) and DNA Data Bank of Japan (DDBJ, www.ddbj.nig.ac.jp).

27.4.1.1 GenBank database

The GenBank database was created in 1982 with funding from National institute of Health (NIH), the National Science Foundation, the Department of Energy and the Department of Defense, USA. The NCBI has had the responsibility for making available the GenBank DNA sequence database since 1992 to all the scientific community. The GenBank sequence database is an open access, annotated collection of all publically available nucleotide sequences as well as their protein translations. Sequences can be submitted to GenBank using BankIt which is a web based form or Sequin which is a stand alone submission. GenBank also coordinates with individual laboratories and other sequence databases such as those of the EMBL and DDBJ.

27.4.1.2 EMBL nucleotide sequence database

The EMBL database constitutes Europe's primary nucleotide sequence resource. It includes direct submissions from researchers, genome sequencing projects and patents.

27.4.1.3 DNA Data Bank of Japan (DDBJ)

DDBJ began its activities in 1986 at National Institute of Genetics (NIG) with the endorsement of Ministry of Education, Science, Sport and Culture of Japan. It collects nucleotide sequences from researchers and issues internationally recognized accession numbers to submitters.

The DDBJ/EMBL/GenBank synchronization is maintained according to the published guidelines by International Advisory Board.

27.4.2 Protein databases

Some of the frequently used protein database include Protein Data Bank (PDB, www.rcsb.org/pdb); Swiss-Prot (www.expasy.ch/sprot/sprot-top.html); Protein Information Resource (PIR, www.mips.biochem.mpg.de/proj/protsedb) and Uniprot (www.uniprot.org).

27.4.3 Genome database

The Genome database consists of sequences of complete or on-going genomes, sequence maps with contigs and is organized into six main groups i.e. Archaea, Bacteria, Eucaryote, Viruses, Viroids and Plasmids as well as draft genome assemblies.

27.5 Software Tools For Data Analysis

The NCBI has software tools that are available by www browsing or by FTP. The application of software tools is in three major areas i.e. sequence, structure and function analysis.

27.5.1 Sequence analysis

27.5.1.1 BLAST (*Basic Local Alignment Search Tool*)

BLAST is a sequence similarity searching program. BLAST can do sequence analysis of both nucleotide or protein, for comparison against the GenBank DNA/protein database in less than 15 seconds. Types of sequence alignments are pairwise or multiple. Pairwise alignment considers one pair of sequence at a time whereas multiple alignment takes into consideration more than one sequence at a time. It is a progressive, linear, pairwise alignment which provides information about conserved sequences in a group of closely related or distantly related organisms. BLAST can be used to infer functional and evolutionary relationships between sequences as well as to help identify members of gene families.

27.5.1.2 BLAST *microbial genomes*

It performs a BLAST search for similar sequences from selected complete eukaryotic and prokaryotic genomes.

27.5.1.3 *Open reading frame finder (ORF finder)*

ORF analysis tool finds all open reading frames in a user's sequence or in a sequence already in the database. Sixteen different genetic codes can be used. The deduced amino acid sequence can be saved in various formats and searched against protein databases using BLAST.

27.5.1.4 *Primer blast*

The Primer-BLAST tool uses Primer 3 to design PCR primers to a sequence template which are then automatically analyzed with a BLAST search against user specified databases, to check the specificity of the primers.

27.5.1.5 *VecScreen*

VecScreen identifies segments of a nucleic acid sequence that may be of vector origin. It searches a query sequence for segments that match any sequence in a specialized non-redundant vector database (UniVec).

27.5.2 Structural analysis

Structural analysis involves tools to compare and analyse new protein structures with the known protein structures available in the databases.

27.5.3 Functional analysis

Functional analysis tools are used for gene expression profiling, protein protein interaction and prediction etc.

27.6 Bio informatics for Next Generation Sequence Analysis

Next generation sequencing technologies such as Solexa, Pyrosequencing and SoLiD are currently being used for genome sequencing which generate enormous data. Hence, a variety of software tools are being made available for analyzing the vast data which mainly include *de novo* sequence assembly and associated tools. The *de novo* sequence assembly tools in fact provide a better platform for analysis of whole genome sequence. Algorithms have been developed for assembly of very short reads. Genome annotation tools comprising of structural and functional analysis have also been developed which assigns biological function to the genes.

27.7 Applications of Bio informatics

1. With the large surge of data, the computation tools have become indispensable in all branches of life sciences.
2. The drug design process has become much faster and the cost of drug design also has decreased. A new field pharmacogenomics allows scientists to use bioinformatics tools to design and prescribe personal medications to individuals.
3. Drug targets in infectious organisms can be revealed by whole genome comparisons of infectious and non-infectious organisms.
4. Bioinformatics could also be immensely useful in clinical diagnostics as it helps to diagnose genetic disorders and other health problems easily.
5. *In silico* screening for small molecule ligands to develop them as potential drugs against infectious agents and cancer.

27.8 Genomics

With the development of 'Omics' era (Fig 27.2) structure and function of the genes can be deciphered. The basic principle of 'Omics' technologies has been given in Fig. 27.3.

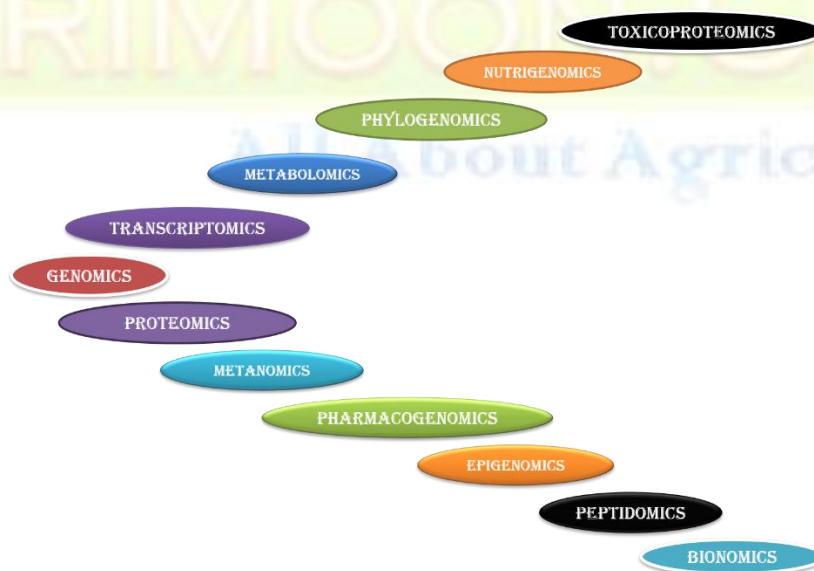


Fig. 27.2. Progression of 'Omics' era

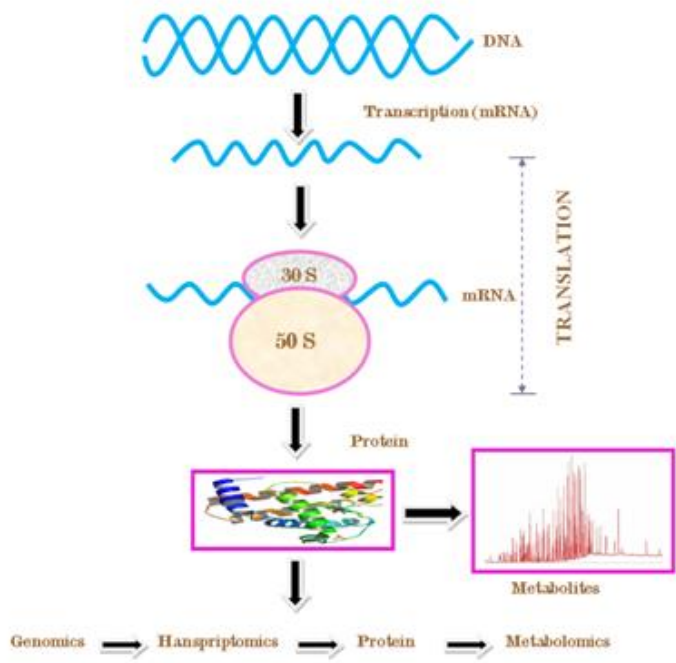


Fig. 27.3 Principle of “Omics’ technologies from genes to proteins

'Omics' is a general term for a broad discipline of science and engineering for analyzing the interactions of biological information objects in various 'OMES'. These include genomics, proteomics, metagenomics, metabolomics, expressomics and interactomics etc.

'Omics' Sciences generate massive amount of data which require powerful statistical tools for analysis. These sciences have enormous role in understanding the normal biological function, disease and personalized health care. One of the first omic sciences “Genomics” is the study of the genomes of the organisms or can be defined as the comprehensive study of the genetic information of the cell or an organism. It can also be referred to as the ‘omics’ study of genes of individual organisms, populations, and/or species. A genome is the sum total of all an individual organism's genes. The term 'genomics' has been coined by Dr. Tom Roderick, a geneticist at the Jackson Laboratory (Bar Harbor, Maine) at a meeting held in Maryland on the mapping of the human genome in 1986. Genomics integrate intensive efforts to determine the entire DNA sequence of organisms and fine-scale genetic mapping. The first genome to be sequenced was that of a virus and mitochondrion by Fred Sanger and his group during 1970-1980. Frederick Sanger sequenced the first DNA based genome of bacteriophage Φ -X174; (5,368 bp) in 1977. The first free-living organism to be sequenced was that of *Haemophilus influenzae* (1.8Mb) in 1995, and since then genomes are being sequenced at a rapid pace.

The importance of deciphering the entire genome sequence of humans was recognized more than two decades ago and it was the very first step in ushering into the field of genomics. Human Genome Project was initiated at National Institutes of Health (NIH) in the United States with an International consortium and the project was funded by Department of Energy. Simultaneously, Dr. J. Craig Venter of The Institute for Genomics Research (TIGR) and Celera Genomics also took an initiative to map human genome. NIH-led consortium used tiling array of large insert clones to sequence the genome. Venter group employed a random shotgun sequencing method followed by computer analysis of sequences obtained to assemble the overlapping sequences. On June 26, 2000, it was announced that human genome had been successfully deciphered. Thus, finally, the draft human genome sequence was completed ahead of schedule in 2001 and the project was completed in 2003. This discovery opened thousands of new doors for scientific research, offering vast opportunities for better health, longer lives and richer human understanding. However, with the fast advancements in sequencing technologies, the entire human genome can be sequenced in less than a month's time. With the advancements in the second (next) generation sequencing technologies, the cost of genome sequencing is becoming so low to make personal genomics a reality. The cost of sequencing an entire human genome will be approximately \$1000 in the near future and a single prokaryotic genome sequence will cost only around \$1. The 1000 Genomes Project (www.1000genomes.org), an international public-private consortium announced the completion of three pilot projects and the deposition of the final resulting data in freely available public databases for use by the research groups world over and is the largest one to build the most detailed map of human genetic variation to date. The three industrial participants included 454 Life Sciences (Roche), Applied Biosystems (CA, USA) and Illumina Inc. (San Diego). Work has also begun on the full-scale effort to build a public database containing information from the genomes of 2,500 people from 27 populations around the world.

Recently, Council of Scientific and Industrial Research (CSIR), India, has been successful in achieving completion of first ever human genome sequencing in India. Sridhar Sivasbhu and Vinod Scaria of IGIB, New Delhi successfully sequenced the first India's genome of a 52 year old Jharkhand resident by cracking the 310 crore base pairs. DNA mapping has revealed marked genetic variations suggesting vulnerability of Indians to cardiovascular diseases (CVD), colorectal cancer and schizophrenia. However, sequencing needs to be performed on large number of people across the regions to draw any conclusion. IGIB plans to map the DNA of ten Indians from different states. With the completion of the first human genome sequence in India, India is now in the league of select few countries like the US, China, Canada, UK, and Korea who have demonstrated the capability to sequence and assemble complete human genomes. CSIR could achieve completion of human genome in 45 days by adopting next generation sequencing technology, resulting in over 13x coverage of the human genome and by effectively integrating complex computational tools with high throughput analytical capabilities using supercomputer. The sequencing of the first human genome in India in conjunction with Indian Genome Variation program opens newer possibilities in disease diagnostics, treatment and low-cost drugs for healthcare. Many microbial sequencing projects have also been already completed or are being carried out. A total of 1525 microbial genomes and 1112 eucaryotic genomes have been sequenced. Entrez Genomes currently contains 3805 reference sequences for 2621 viral genomes and 41 reference sequences for viroids. A number of comparative genome studies are under way to link genotype and phenotype at the genomic level.

Genome projects and sequences are available at:

- DOE Joint Genome Institute: Human, plant, animal, and microbial sequencing.
- GOLD -- Genomes Online Database provides comprehensive access to information regarding complete and ongoing genome projects around the world.
- Comprehensive Microbial Resource -- A tool that allows the researcher to access all of the bacterial genome sequences completed to date.
- Entrez Genome project -- A resource from the National Center for Biotechnology Information (NCBI) for accessing information about completed and in-progress genomes.

27.8.1 Applications

1. Analysis of genes at the functional level is one of the main uses of genomics (Functional genomics)
2. With the help of genomics, we can study evolutionary relationships between populations, species and genera.
3. Molecular diagnostics
4. Improving quality and efficiency of next generation technologies in terms of simplicity, time and cost.

27.9 Comparative Genomics

Comparative genomics is the analysis and comparison of genomes from different species. The idea is to gain a better understanding of evolution of different species and also to determine the function of genes and non-coding regions of the genome. Comparative genomics involves the use of computer programs that can align multiple sequences and look for regions of similarity among them. The sequence similarity tools like BLAST are accessible from the National Center for Biotechnology Information (NCBI) and ClustalW. BLAST is a set of programs designed to perform similarity searches on all available sequence data.

27.10 Metagenomics

Metagenomics is the study of metagenomes - the genetic material recovered directly from environmental samples i.e community analysis. The underlying strategy for metagenomic study is given in Fig. 27.4. Advancements in sequencing technologies involving pyrosequencing, illumina etc. can be exploited to get information about the genes from all the members of sampled communities. The power of genomic analysis is applied to entire communities of microorganisms for discovery of new micro-organisms or their functional properties by bypassing the need to isolate and culture individual microbial species. Metagenomics find application in almost all the frontier areas of science including agriculture, environment, energy and human health etc.

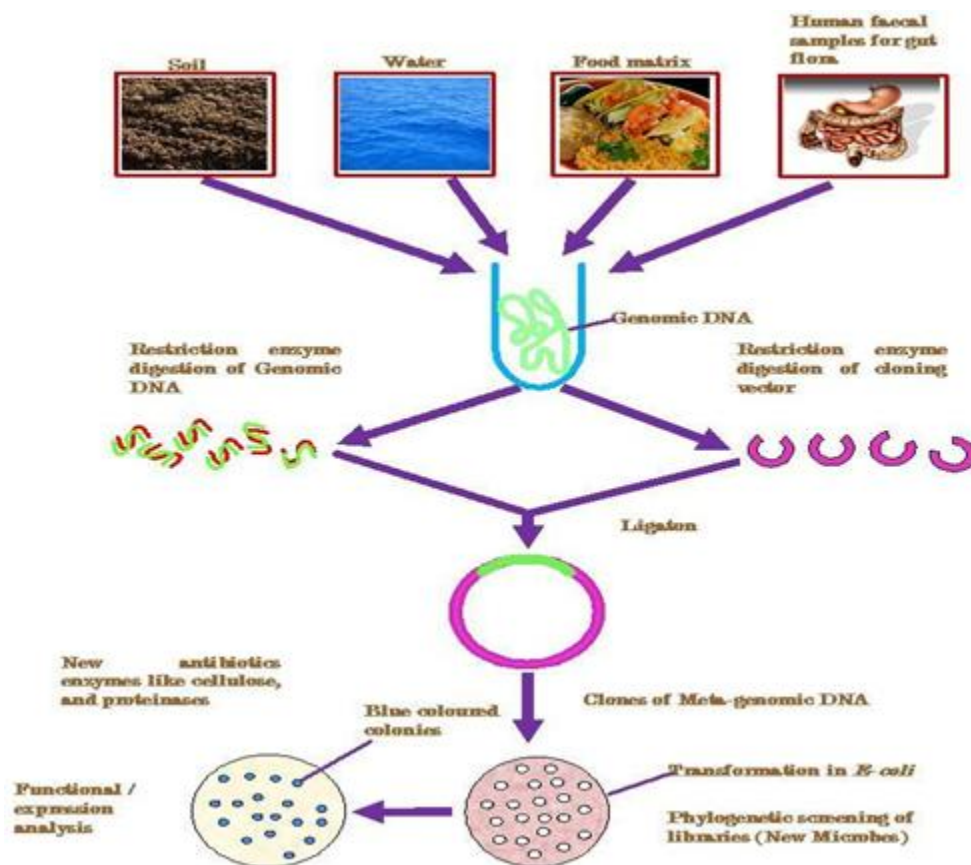


Fig. 27.4 Meta genomic library preparation from different environmental samples

Till now we have very little information about the bacterial diversity present on this earth since only less than 1% of bacteria have been cultured due to lack of knowledge of physiological and cultural conditions required for cultivation of such unknown organisms using laboratory media. The term ‘metagenomics’ was coined by Jo Handleman and others in the University of Wisconsin and first appeared in a publication in 1998 and relied on 16S rRNA sequences. The gold standard for molecular identification of microbial species is the phylogenetic analysis of small-subunit rRNA genes (SSUrDNA), which are present in all cellular organisms from the environmental samples using DNA extracted from the composite samples. With recent advancement in sequencing technologies, job has been simplified to a great extent and has enabled the researchers to sample all genes from all the members of sampled communities to study their diversity. The information obtained from metagenomics provide the information both on the type of organism and the metabolic process. Powerful molecular microbiota analyses methods, including 16S rRNA sequencing through a massively parallel barcoded pyrosequencing approach, facilitate our ability to analyze microbiota in environmental samples comprehensively and in an efficient manner.

27.11 Functional Genomics

Functional genomics is the study of function-related aspects of the genome which encompasses transcriptomics, proteomics and metabolomics which we will study in the next section. Although, the complete sequence of human genome is available, the determination of function of various genes is still to be worked out in detail. With the development of several high through put molecular technologies besides mutagenesis and gene knock out techniques, it is now possible to decipher the gene function at a faster pace. Only 60% of the genes have been annotated in *E. coli* and humans for their functions. Rest 40% include genes that are unique to the organism.

27.12 Transcriptomics

After completion of the sequencing of human genome, efforts are now made to determine the function of the genes located therein. With the advancements in Bioinformatics, the researchers predicted that there were only 20,000 – 25,000 transcripts encoded by the entire human genome. This indicated that all of the genes did not express themselves. Transcriptomics is the study of transcripts encoded by a particular gene. Transcriptome is the set of all the RNA molecules, including mRNA, rRNA, tRNA and non-coding RNA produced in one or a population of cells. The study of transcriptomics is also referred as expression profiling which examines the expression level of mRNAs in a given cell population under a given set of conditions since the expression level of genes may vary under different conditions. The genome is static but the transcriptome is highly dynamic and changing, due to varying patterns of gene expression. In any organism, the transcriptome of different cells is never identical. DNA microarray technology is a powerful tool to obtain a transcriptome and helps in studying the gene that are turned 'on' in a particular environment. Currently, the most widely used method for the analysis of transcriptomics is DNA microarray and RNA seq using Next generation sequencing technologies. The mRNA from the cells under study is extracted and then labeled with a fluorescent dye and placed in a DNA array slide spotted with a large number of DNA probes (as discussed under DNA microarray Chapter 3.7). The mRNA will attach to its complementary DNA on the microarray and gives fluorescence. This can be successfully used in identification of genes which are expressed under normal and diseased conditions. The science of transcriptomics is important for identifying the set of genes that are differentially expressed in distinct cell populations or subtypes, to obtain data on the likely proteins that will be found in a particular cell.

Global transcriptome analysis is an emerging area to investigate the role of genetic variants in several of the diseases like cancer, metabolic diseases like diabetes, CVD etc. In a recent study conducted in India, it has been revealed that Indians are more susceptible to diabetes because of genetic variants. Asian Indians are more prone to obesity and diabetes since the variants found in gene FTO and near MC4R cause a 2cm expansion in waist circumference which make them resistant to insulin leading to development of Type 2 diabetes.

27.12.1 Tools

Global analysis: high-density DNA microarrays (Please see Lesson 15 for DNA microarray)

Real Time PCR (RT-qPCR, Molecular Beacons)

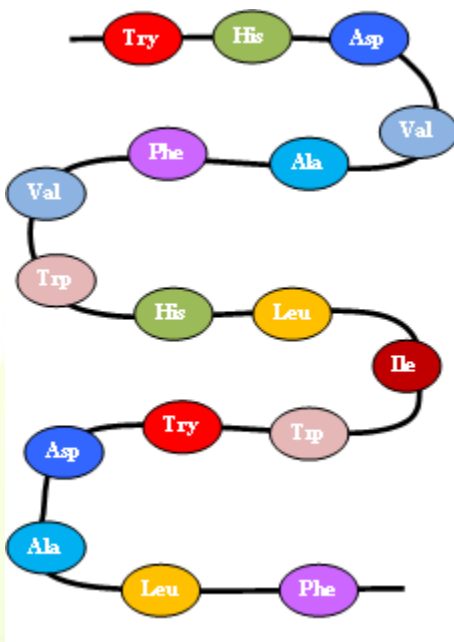
RNA-Seq – Uses deep sequencing technologies (next generation technologies) to study the transcriptome at the nucleotide level. RNA-Seq provides a far more precise measurement of levels of transcripts and their isoforms than other methods.

27.12.2 Applications

1. Transcriptomics find role in exploratory studies in order to elucidate the type of genes which are differentially expressed (normal versus diseased status) or co-expressed or interact which can enhance the knowledge concerning gene function, regulation and interaction.
2. Prognostic studies to decipher the effect of drug to find the best treatment regimen.

27.13 Proteomics

Proteomics is the study of proteins, particularly their structure and functions. Proteins exist in several structures including primary, secondary, tertiary and quaternary. Tertiary structure of protein is important for its functionality. The following Fig. 27.5 shows the various structures of proteins e.g. primary, secondary, tertiary and quaternary.



Primary structure of a protein(Amino-acid sequence of the polypeptide chain by peptide)



Secondary structure of a protein Alpha helices & Beta sheets, Loops



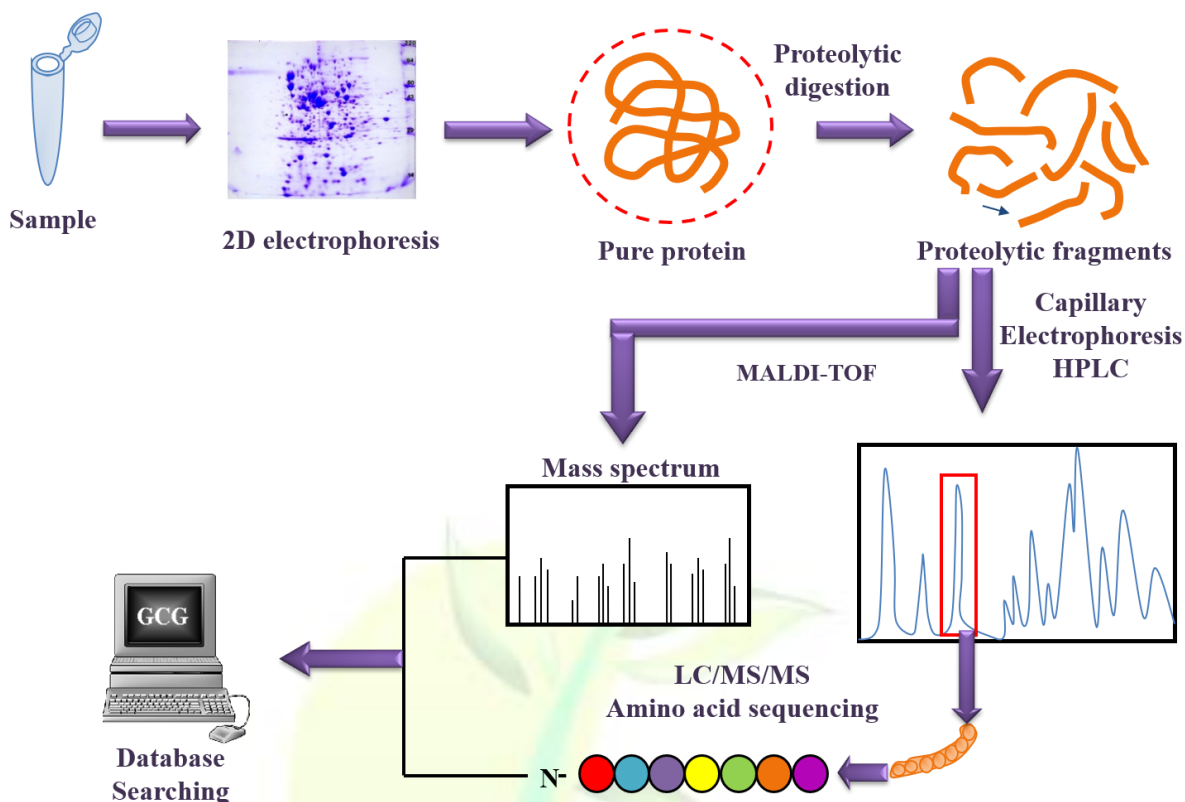
Tertiary structure of protein arrangement of secondary element in 3D space



Quaternary structure of protein Packing of several polypeptide chains

Fig. 27.5 Structure of proteins

The proteome is the entire complement of proteins including the modifications made to a particular set of proteins, produced by an organism or system. A proteome differs from cell to cell and constantly changes through its biochemical interactions with the genome and the environment and reflects the gene expression repertoire (Fig 27.6). The word "proteome" is a blend of "protein" and "genome", and was coined by Marc Wilkins in 1994. Sometimes, the mRNA level does not correlate with the protein content since amount of protein produced depends on a gene it is transcribed from or its post translational modifications which may change the function of protein. On the other hand, an mRNA produced in abundance may be degraded rapidly or translated inefficiently, resulting in a small amount of protein. Around 25- 30,000 genes code for at least 100,000 proteins in human cells, mainly due to a variety of post translational modifications such as phosphorylation, ubiquitination, methylation, acetylation, glycosylation, oxidation, nitrosylation etc. Thus, the set of proteins present in any cell at any given time can vary to a large extent and the utility of transcriptomics becomes limited. Proteomic research aims to develop markers of disease expression and find therapeutic solutions. The study of proteomics is quite complicated as compared to Genomics because the proteome varies with time and environment in the same cell.

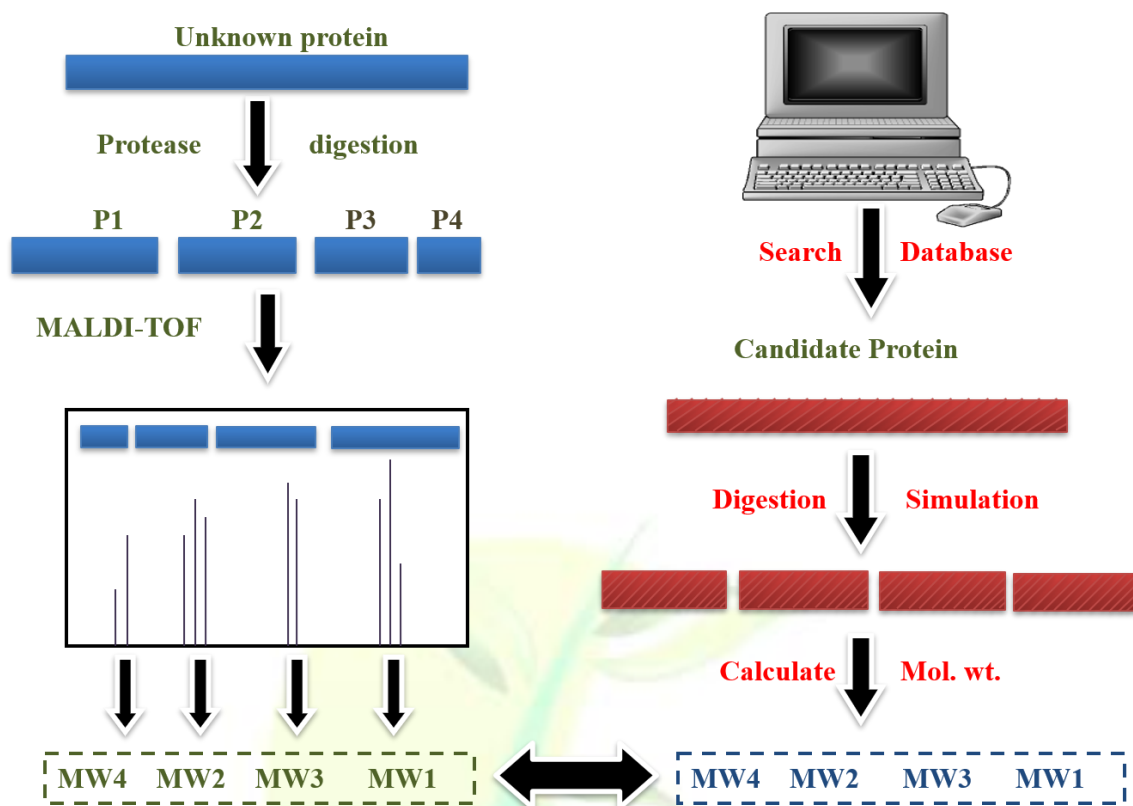


PROTEOME REFLECTS GENE EXPRESSION REPERTOIRE

Fig. 27.6 Gene expression profile as reflected from proteome

Proteomics is rapidly becoming an essential part of biological research. In conjunction with advances in bioinformatics, it will have a major impact on our understanding of the phenotypes of both normal and diseased cells. Initially, 2D PAGE (polyacrylamide gel electrophoresis) was used to construct the protein maps. Recently, mass spectrometry has been incorporated to enhance sensitivity and specificity besides providing results in a high through put format. The strategy used for identification of an unknown protein is given in (Fig 27.7). Any proteomic analysis is very costly, laborious, and time consuming. It yields huge amount of data which is difficult to interpret which requires careful designing of simple experiments.

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COMPARE PEPTIDES AND IDENTIFY THE UNKNOWN PROTEIN

Compare peptides and identify the unknown protein

Fig. 27.7 Identification of unknown protein and peptides

27.13.1 Tools

- Western blot
- Immuno-histochemical staining
- Enzyme linked immunosorbant assay (ELISA)
- Mass spectrometry
- 2 D gel electrophoresis
- MALDI TOF/TOF
- MALDI-MS
- LS-MS/MS, LC/LC -MS/MS
- Protein microarrays (or Biochips spotted with antibodies or proteins and probed with a complex protein mixture)
- Bioinformatic tools (ExPASy Proteomics Tools)

27.13.2 Applications

1. Identification of potential new drugs for the treatment of diseases. The proteins associated with the disease can be identified using information from genome and proteome. The 3D structure can provide the information to design drugs to interfere with the action of the protein.
2. Specific protein biomarkers to diagnose disease
3. Protein microarrays are used to study protein protein interactions.



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Lesson 28

METABOLOMICS, STRUCTURAL BIOLOGY, NUTRIGENOMICS AND PHARMACOGENOMICS

28.1 Introduction

Metabolomics is the comprehensive quantitative analysis of all the metabolites of an organism or specified biological sample. On the other hand, Metabonomics is the quantitative measurement of the multiparametric time related metabolic responses of a complex (multicellular) system to a pathophysiological intervention or genetic modification. Thus, where metabolomics means the global analysis of all metabolites in a sample, metabonomics is the analysis of metabolic responses to drugs or diseases. Several factors affect metabolic profile such as diet and lifestyle, environment and genetics as well as pharmaceutical effects. The metabolome is a direct reflection of the physiological status of a cell and unlike transcriptomics or proteomics studies, which only reveal part of what might be happening in a cell, metabolomics can give us an instantaneous picture of the entire physiology of the cell. The science of metabolomics can help us in generating metabolic signatures, monitor enzyme kinetics/pathways, measure metabolite flux, identify phenotypes, monitor gene/environment interactions and identify functions of unknown genes.

The measurement of such a large number of metabolites requires advanced methodologies which include nuclear magnetic resonance (NMR), functional magnetic resonance imaging (MRI) and high performance liquid chromatography (HPLC), and handling of a large amount of mathematical data which require advanced Bio-informatics tools. Mass spectrometry (MS) is used for measuring compounds with molecular weight of 70-500 Da. However, it can not distinguish compounds with similar molecular weights. Hence it needs to be combined with other techniques such as liquid chromatography (LC) and gas chromatography (GC), denoted as LC/MS and GC/MS. Analysis of data thus obtained requires sophisticated tools of information technology (IT). Recently, capillary electrophoresis (CE) which combines MS, known as CE/MS has been found suitable for obtaining the metabolome. One of the application of studying metabolome is that of normal versus diseased cells and their modulation by drugs or nutrients.

The Human Metabolome Database (HMDB) is a freely available electronic database containing detailed information about small molecule metabolites found in the human body. It is intended to be used for applications in metabolomics, clinical chemistry and biomarker discovery.

28.1.1 Samples

The clinical samples for analysis include biofluids such as urine, blood (plasma and serum), saliva, bile, cerebrospinal, digestive, seminal, amniotic etc. and also cells, their supernatants, tissue extracts and biopsies.

28.1.2 Tools

The techniques to be used include:

Mass Spectrometry (MS),

Gas chromatography (GC),

High Pressure Liquid Chromatography (HPLC),

Ultra Performance Liquid Chromatography (UPLC),

Nuclear Magnetic Resonance (NMR),

Capillary Electrophoresis (CE)

Flux analysis,

Fourier transform ion cyclotron mass spectrometers (FT-ICR-MS).

28.1.3 Applications

1. Metabolomics offer the direct measure of physiological activity, hence can be used for biomarker discovery, drug discovery, physiological exploration, diet and disease.
2. Metabolomics have tremendous applications in food as well as in livestock sector to find how various feeding strategies like concentrates affect the development of epithelial tissue from young calves during weaning.
3. Nutritionists can determine physiological response of nutrients. Metabolomic assays can be used to measure small molecule biomarkers of oxidative stress, redox potential, anti-oxidant activity, inflammation and cardiovascular disease risk.

28.2 Structural Biology

Structural biology is a branch of molecular biology, biochemistry and biophysics concerned with the molecular structure and shape of biological macromolecules particularly proteins and nucleic acids to find what causes these molecules to acquire the structures they have, and how alterations in their structures can affect the biological function. The typical three dimensional structure of macromolecules (proteins) is very important for their physiological functions. With the help of structural biology, we can understand how a macromolecule, a complex of macromolecules, or a cellular sub-section functions through elucidation of its three-dimensional structure. The field of Structural Biology was essentially born when the DNA Double Helix structure was discovered by Watson and Crick in 1953 whereby the structure of the DNA was linked to the way DNA is replicated and transcribed in the cell. The first and foremost thing a structural biologist needs to know is the systematic experimental determination of many more three dimensional structures of biological macromolecules and complexes of macromolecules (such as proteins, DNA, RNA, carbohydrates or lipids). Determination of structures will form the basis of our understanding the relationships between primary structures and the tertiary structures of biologically active macromolecules and, even more so, the quaternary structures of the multi-subunit complexes which mediate most biological activities. Next is the methods that the structural biologists require to determine the structures of macromolecules which generally include X ray Crystallography (crystal of chymosin shown in Fig. 28.1), Nuclear Magnetic Resonance (NMR), Electron Microscopy/Tomography and Circular Dichroism (CD) which have expanded the repertoire and capabilities of macromolecular imaging to unprecedented levels. With contributions from mass-spectrometry and high-resolution fluorescence microscopy, structural biology has grown to a powerful methodological arsenal for the study of macromolecules regardless of their size and complexity. Using X ray crystallography, several structures e.g. lactoferrin, lysozyme, hemoglobin etc. have been elucidated as shown in Fig. 28.2. The most

important role is played by Bioinformatics. Three dimensional structures can also be determined using *in silico* methods.

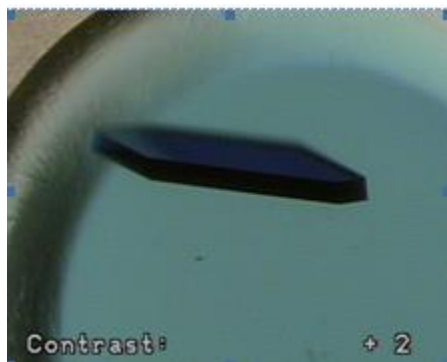
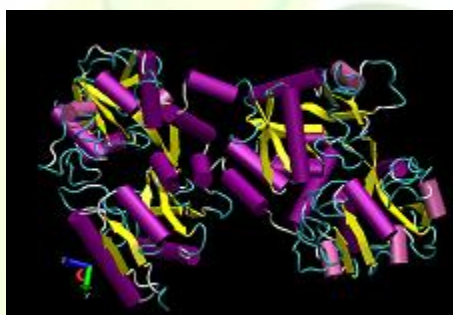
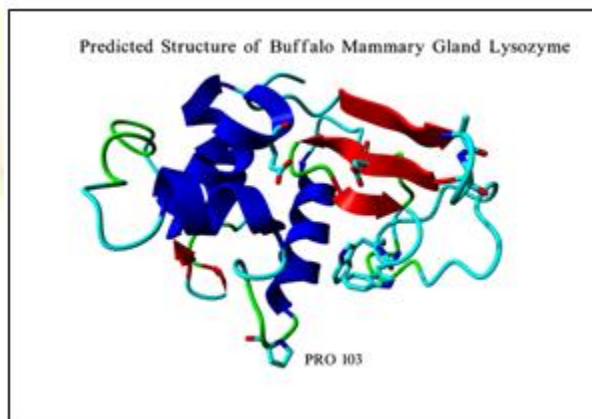


Fig. 28.1 Crystal of buffalo chymosin



Sheep lactoferrin



Buffalo Mammary gland lysozyme

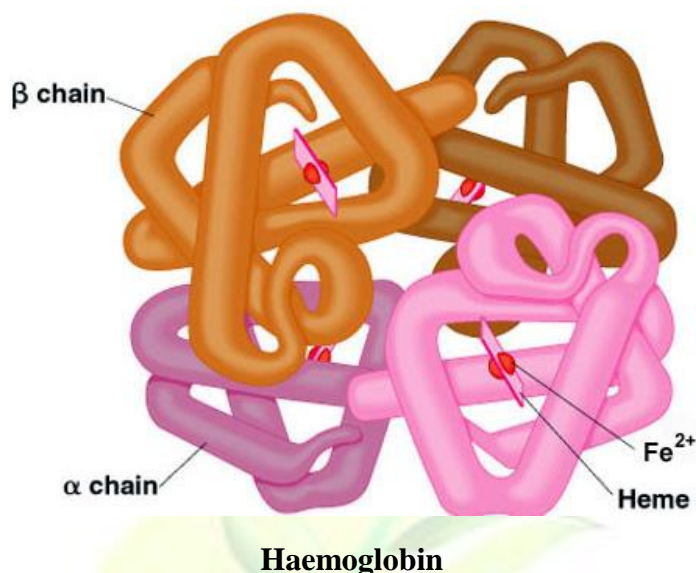


Fig. 28.2 Predicted structure of few proteins

Structural biologists compare the structure of proteins by superimposing them i.e. they use web server called ‘Superpose’ wherein the proteins structures are uploaded and compared. Fig. 28.3 shows the superimposition of buffalo and bovine chymosin. Since, structural biology plays a major role in the understanding of biological processes at the molecular level, there has been a continuing demand for faster and more cost-effective determination of protein structures and their in-depth functional, mechanistic and biological analysis. Detailed structural descriptions using structural biology can provide a better understanding of the basis by which different proteins achieve selectivity and specific binding to their cognate ligands and drugs. Several drugs have been developed using structure-based methods which target several of the diseases particularly AIDS, leukaemia and cancers.

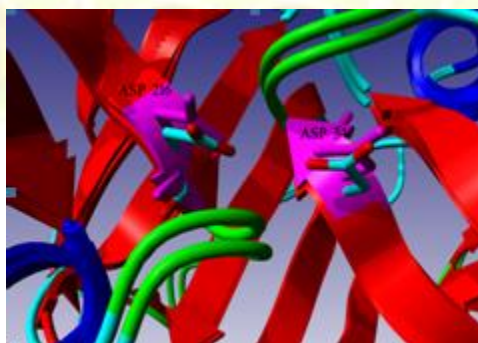


Fig. 28.3 Superimposition of buffalo and bovine chymosin structures

28.2.1 Tools

These methods include

- X ray crystallography
- NMR
- Electron Microscopy
- Single molecule scattering
- Circular Dichroism (CD)

- Cryo-electron microscopy (cryo-EM)
- Multiangle light scattering
- Ultra fast laser spectroscopy
- Dual polarization interferometry
- Bioinformatics - Protein structures can be viewed at

Jmol

– <http://jmol.sourceforge.net>

PyMOL

– <http://pymol.sourceforge.net>

Swiss PDB viewer

– <http://www.expasy.ch/spdbv>

Mage/KiNG

– <http://kinemage.biochem.duke.edu/software/mage.php>

– <http://kinemage.biochem.duke.edu/software/king.php>

Rasmol

– <http://www.umass.edu/microbio/rasmol/>

Structures can be predicted using threading. 3D threading servers predict the structure or gives models based on input sequence :

- PredictProtein-PHDacc

o <http://www.predictprotein.org>

- PredAcc

o <http://mobylye.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py?form=PredAcc>

- Loopp (version 2)

o <http://cbsuapps.tc.cornell.edu/loopp.aspx>

- Phyre

o <http://www.sbg.bio.ic.ac.uk/~phyre/>

- SwissModel

– <http://swissmodel.expasy.org/>

28.2.2 Applications

1. Based on structure predictions and spatial organization, improved biomolecules with the desired properties can be designed.
2. Structural biology has an important role to play in drug discovery.
3. Knowledge about three dimensional structural analysis of biomolecules can help in rational designing of molecules with certain desirable properties e.g. proteins with increased temperature and pH stability (protein engineering), or small molecules that inhibit or activate certain biological processes (drug design).

28.3 Nutrigenomics

Nutrigenomics is the study of the influence of foods and their constituents on gene expression profile of individuals using high-throughput genomic tools (Fig 28.4). It can also be defined as the use of genomic analysis to investigate diet-gene interactions that impact human health and disease. Several such definitions for nutrigenomics exist. Nutrigenomics make use of all the post genomic tools such as genomics, transcriptomics, proteomics, metabolomics, metabonomics, interactomics etc. Nutrigenomics helps us in understanding how our body responds to food components using ‘System Biology’. These ‘omics’ technologies use the information from genomics and study the effect of dietary constituents and

their influence on proteins and metabolites produced in our body. The two terms nutrigenomics and nutrigenetics are commonly used and are distinguished by defining them as - nutrigenomics is the identification of genes involved in physiological responses to diet and the genes in which small changes, called polymorphisms, might have significant impact on nutritional consequences. On the other hand, Nutrigenetics is the study of these individual genetic variations or polymorphisms, their interaction with nutritional factors, and linkage with health and disease. Basically, nutrigenomics explore how nutrients in foods interact with genes which will provide an understanding of how diet and genes interact. This information will help us to better manage our own health and possibly prevent, mitigate and delay the onset of chronic and age related diseases. Today's life style diseases like obesity, diabetes and cardiovascular diseases are all related to diet and such diseases can be very well managed by following nutrigenomic approach. The concept of linkage between diet and health is an ancient one. In 400 B.C. Hippocrates said "let food be thy medicine and medicine by thy food". Nutrigenomics will lead to evidence-based dietary intervention strategies for restoring health besides preventing diet-related diseases. Nutrigenomics is like a personalized medicine which means personalized dietary regimen to follow healthy life style.

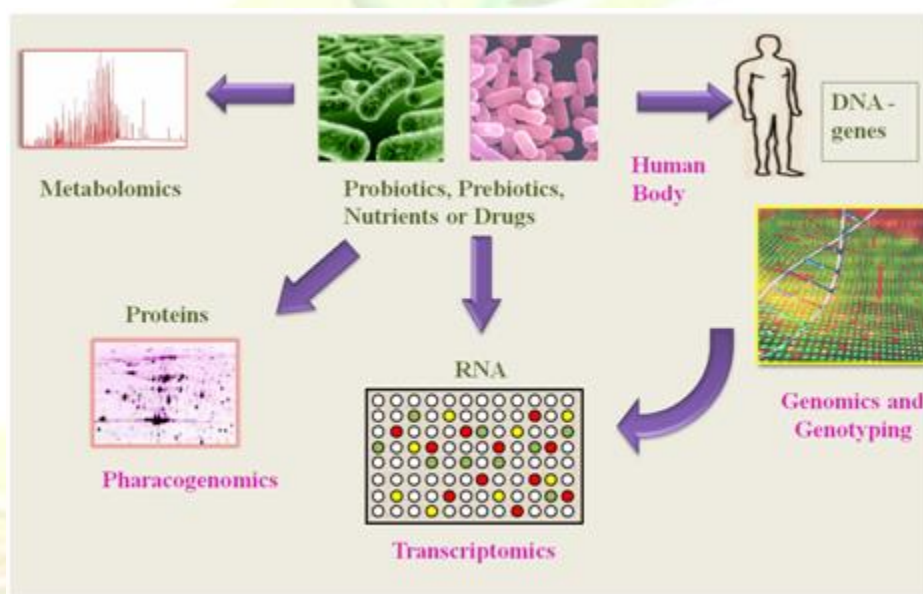


Fig. 28.4 Nutrigenomics

Nutrigenomic studies demonstrate that diets have variable effects on individuals which depend on the genetic makeup of the individual. Identification of DNA variants that contribute towards diet related disease risk is important in order to understand the causes of diseases in humans. The Human Variome project (www.humanvariomeproject.org) is an international effort to identify genes, their mutations and variants associated with disease risk. The genes examined in most of the studies are generally those that have been previously identified as genetically or biochemically involved in altering either an intermediate risk factor or the chronic disease itself. One example is the thymidine variant instead of cytosine at position 677 (C677T) in the methylenetetrahydrofolate reductase (MTHFR) gene, which is associated with neural tube defects in women with low intakes of dietary folate in certain populations. Women with TT allele are more prone to neural tube defect. The nutrients from foods can be investigated as modulators of gene expression rather than as simple nutrients for providing basic nutrition e.g. the addition of folate in the diet of a pregnant women alters gene expression in a positive way. Genistein, an isoflavone from soybeans has been shown to possess anti-cancer potential and is a good supplement for prevention of

cancer.

Gene expression can be affected by nutrient – gene interaction that involves transcriptional factors. These transcriptional factors bind to response element sequences to initiate transcription. Nutrients bind to transcription factors to modulate gene expression. The positive effect of omega – 3 fatty acids has been shown to affect lipid profile since it binds to PPAR receptors to initiate transcription of genes for fatty acid oxidation.

Diagnostics, preventive lifestyle guidelines, more efficacious dietary recommendations, health-promoting food supplements, and drugs are some of the anticipated end-products of nutrigenomics research. It has been found from the outcome of several investigations that the same dietary factors are responsible for causing disease in a person who had a genetic predisposition to that particular disease, but not in others with a different genetics. The individual's response to a food or nutrient could be traced to differences in metabolic handling of a dietary component, involving complex interactions between genotypes, metabolic phenotypes, other dietary factors, lifestyle and environmental factors. This led to the concept of developing personal diets for individuals. As a result of emerging high through put techniques associated with Human Genome Project, tools have become available which could lead to a deeper understanding of interactions among food, genes, protein structure, post-translational changes in protein structure and consequent effects on metabolism.

28.3.1 Tools

1. Microarrays - powerful tool for studying functions of food and nutrients
2. Bioinformatics tools

28.3.2 Applications

Nutrigenomics will have a significant impact on the field of dietetics and personalized diet to prevent disease risk.

28.4 Pharmacogenomics

Pharmacogenomics is the study of how variations in the human genome affect the response to various drugs (Fig. 28.5). The older term "pharmacogenetics" was created from the words "pharmacology" and "genetics" to indicate the intersection of pharmaceuticals and genetics. With the availability of human genome sequence and the introduction of new technologies particularly 'omics' have made it possible to analyze multiple genes simultaneously, rather than one at a time. The recently coined term "pharmacogenomics" describes genomewide approaches. The two terms 'pharmacogenomics' and 'pharmacogenetics' tend to be used interchangeably. Pharmacogenomics is the whole genome application of pharmacogenetics which examine single gene interaction with drugs. Pharmacokinetics is the study of uptake, conversion and breakdown of drugs in the body over time. Pharmacodynamics deals with the influence of genes on the interactions between drugs and their molecular targets. Pharmacists may use a patient's genetic profile to select the most appropriate drug for the treatment or prevention of a disease to which the patient is genetically predisposed. An individual's genetic profile may also be used to choose a medication with minimal side effects. One drug may work for one person and may not work for another. It is all due to genetic blueprint which is unique for every person and hence the concept of personalized

medicine. Pharmacogenomics is closely linked to nutrigenomics as in both cases effect of drug or a nutrient is investigated on genome profile of an individual i.e. how genetic variations influence the responses to these chemicals. Although, a person's response to medication may be influenced by environment, age, diet, lifestyle, and health status, the major factor is the genetic make up. Hence, a person's genetic make up can help in designing of a personalized drug with greater efficacy and safety.

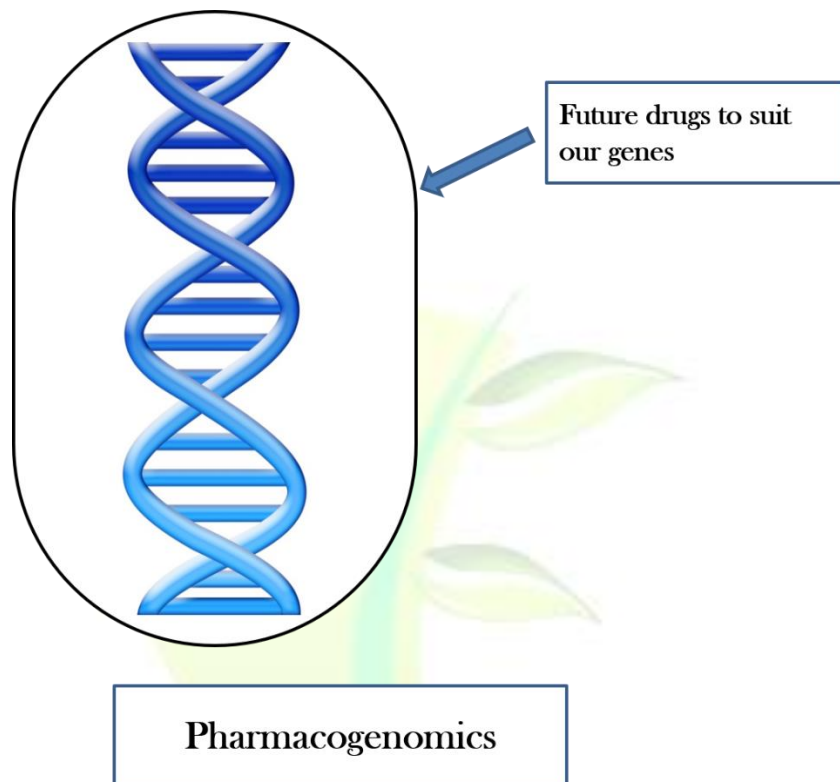


Fig. 28.5 Pharmacogenomics (AS PPT LINK)

In vitro model systems i.e. cell lines from large numbers of individuals represent an attractive and cost-effective approach to identify genes associated with variations in drug response by the application of genome-wide techniques and these laboratory based results are validated by translation in clinical trials.

One of the key emerging areas in today's 'omic' era for personalized medicine is to test the patients prior to prescribing a particular drug therapy in order to determine their ability to metabolize different classes of drugs. Pharmacogenomics is being used in some of the cases like cytochrome P450 (CYP) family of liver enzymes and TPMT (thiopurine methyltransferase). The patients are screened for TPMT deficiency and to look for variations in cytochrome P450 genes. TPMT is responsible for breakdown of drug thiopurine used for leukaemia and when not broken down, increases to toxic levels. Another example is cystic fibrosis patients. There are several such examples in which patient's genetic profile is very useful in prescribing a particular drug. One of the most important area is the tumor therapy. Looking at the molecular differences between several tumors, initial drug treatment could be improved significantly.

Pharmacogenomic test results sometimes are difficult to interpret since enzymes involved in drug metabolism arise from multiple genes which is a complex process. The results are predictions based on information about the specific gene variants, associated diseases, adverse drug reactions, and patient outcomes.

28.4.1 Tools

- Sequencing
- Next generation sequencing technologies
- RFLP (Restriction Fragment length Polymorphism)
- Common genotypic methods
- Single nucleotide polymorphisms (SNPs)
- Real Time PCR (TaqMan)
- DNA Microarray for expression studies
- Denaturing high-performance liquid chromatography (DHPLC) - uses a reverse-phase ion-pair column to discriminate between variant and non-variant alleles.
- Mass spectrometry differentiates DNA molecules using a defined mass
- Bioinformatic tools

28.4.2 Applications

1. Pharmacogenomics will determine the most effective treatment for patients prior to drug prescription.
2. Pharmacogenomics will help in drug discovery that is targeted towards a specific disease. Pharmaceutical companies will be able to discover potential therapies more easily using genome targets.
3. It will help the Doctors to prescribe the drug after looking at the person's genetic profile which will make the recovery faster and minimize the adverse effects.
4. Pharmacogenomics can be used for all critical illnesses like cancer, diabetes, CVD, obesity, tuberculosis etc.



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Module 7. Regulatory and safety aspects of biotechnology particularly in the light of GMO foods

Lesson 29

ETHICAL ISSUES RELATED TO USE OF GENETICALLY MODIFIED FOODS

29.1 Introduction

With the recent advancements in the area of rDNA (Recombinant DNA technology) and Genetic Engineering, virtually any desired trait or gene from any organism or even the synthetic gene can be introduced / expressed in any organism including bacteria, yeast, insect cells, plants and mammals. These spectacular advances made in the area of rDNA technology have been successfully exploited in different sectors viz. agriculture, health care, food processing industry as well as environment.

29.2 Definition of GMO

A genetically modified organism (GMO), referred to as a living modified organism or transgenic organism means any living organism that possesses a novel combination of genetic material obtained through the use of modern biotechnology i.e genetic engineering or rDNA or the manipulation of an organism's genetic endowment by introducing or eliminating specific genes through modern molecular biology techniques.

Genetically modified organisms (GMO) have found tremendous applications in health care e.g. therapeutic proteins, vaccines, diagnostics and gene therapy. The health care products address life threatening situations wherein risks and benefits need to be balanced and hence generally no ethical issues arise. There is not much public debate on the use of medically important GMO products since they are purified from recombinant organisms in contained environment. On the other hand, genetic modification in agriculture is a sensitive issue and thus has raised lot of concerns with regard to human health.

29.3 Genetic Engineering in Health and Medicine

Genetic Engineering (GE) has tremendous applications in health and medicine. A number of recombinant pharmaceutical proteins to be used as potential drugs have been produced using GE. GE has been used to produce several useful proteins into sheep and cow's milk e.g. factor IX, alpha-1-antitrypsin, streptokinase etc. Moreover, genetic engineering has helped in development of vaccines, gene therapy, xenotransplantation, designer babies etc.

29.4 Genetic Engineering in Food and Agriculture

The application of genetic engineering in food and agriculture to produce genetically modified (GM) foods particularly transgenic plants has been very controversial in comparison to the products for human medical importance. Genetic engineering is mainly used in agriculture to make plants with enhanced yield, high nutritional value like golden rice with increased levels of beta carotene (to meet vitamin A deficiency), disease resistance, plants that can tolerate high salt, draught resistance, pest resistance like Bt genes to kill selectively pests that eat crops. GM crops have also been designed to produce pharmaceuticals and hence are also called "pharma crops." Ventria Biosciences (Sacramento) have launched GM rice with two synthetic human genes viz. human lactoferrin and lysozyme. However, there was fear among people that GM rice could cross-pollinate other crops and introduce foreign genes and

proteins into the human food chain. INB Biotechnologies (Philadelphia) is developing a nontoxic anthrax vaccine through the transgenic modification of petunias, causing the plant to manufacture new proteins, which when eaten prompt the development of anti-anthrax antibodies.

Several countries of the world use GM crops for food production but in certain countries GM crops are not acceptable. The ethical issues with regard to GM crops are being described below.

29.5 Ethical Issues on Use Of GMO

Risks arising from the application of GMOs in agriculture include risks to human health, environment besides ethical concerns. There are concerns, doubts and fears in the mind of common men about genetic engineering. There are people who are pro and anti genetic engineering. There is no doubt that genetic engineering offers great opportunity for solving hunger, food insecurity and malnutrition problems globally. On the other hand, some people think that genetic engineering is unnatural and people are scared to buy GM foods since they think that genes will be transmitted to them. Moreover, some of the companies even do not label GM foods. Although, people in US eat GM foods but in Europe, people are not willing to accept GM foods because of fears of risks and other ethical concerns. However, GMOs need to be tested extensively for toxicity to humans and animals before their release in the market. Sometimes, newer proteins may behave as allergens. GMOs are generally produced involving antibiotic resistance genes which raises the concerns of antibiotic resistance gene transfer on consumption. All these are discussed in the following section.

29.5.1 Antibiotic resistance

There have been several issues raised at different platforms on the use of antibiotic resistance markers in selection of recombinant organisms (GMOs).

- i. It is generally assumed by people that eating foods with antibiotic resistance markers would reduce the effectiveness of an antibiotic since the antibiotic will be degraded. This issue was raised during the approval for Galgene's FlavrSavr and Ciba Giegy's Bt corn 176.
- ii. The transfer of antibiotic resistance marker gene from GMO to intestinal microflora also poses risk of horizontal gene transfer which can lead to antibiotic resistant micro-organisms, although its probability in acidic environment is extremely rare.

In view of the above issues, antibiotic resistance markers are being replaced with auxotrophic or food grade markers.

29.5.2 Eating exogenous/foreign DNA

There are also apprehensions regarding the ingestion of exogenous DNA while eating transgenic foods. However, there are no scientific reports that DNA from these transgenic crops or foods pose any risk to human health. Generally, the foreign DNA is destroyed by the body's defense mechanism. Generally, DNA present in micro-organisms, plants and animals are eaten by human beings all the time and does not pose any problem. Similarly, the exogenous DNA is not likely to pose any health problem.

29.5.3 Food safety

Consumers are sometimes wary of the safety of GM foods due to problems such as allergens, pesticide residues, microbiological contaminants and bovine spongiform encephalopathy (mad cow disease). There

is also a common notion that GM crops are unsafe for other organisms that feed on them e.g. Bt toxin may kill Monarch butterfly larvae. However, there are no scientific evidences to support this.

29.5.4 Environmental concerns

GMOs are novel products which, when released, may cause ecosystems to get polluted unintentionally and may also result from out-crossing with wild populations. GMOs may get released in the environment and pose several risks such as transgene instability, transfer to weeds, persistence of transgene in the environment, loss of biodiversity, changes in soil ecology, generation of new live viruses etc. NGOs have also raised the concern that growing genetically engineered crops will be harmful for the environment e.g. if herbicide resistance genes from canola flows into weedy relatives will make them resistant to herbicides. There are no scientific studies to support this. Moreover, the risks to birds, insects and other non-target species that come into contact with or consume GM plants is not known. Hence, the extent of post-release monitoring of GMOs is required to protect ecosystems.

29.5.5 Risk of toxicity

GMOs need to be evaluated for toxicity rigorously in animal models before their release for human consumption. Sometimes, the companies hide the controversial data in this regard for getting clearance from regulatory authorities.

29.5.6 Risk of allergies

The proteins from GMO may become allergens and hence need to be tested for allergenicity. Since GM foods are not labeled, a person could suffer a potentially fatal allergic reaction e.g. an allergenic Brazil nut gene was transferred to a soybean variety, but the resultant modified crop was never released to the public because of this problem. The FDA considers potential allergens to be a very important issue. Developers are required to systematically evaluate this possibility. Special care is taken with genes derived from foods that commonly cause food allergies. For example, about 8% of the people are allergic to foods that contain milk, wheat, some seafood, or nuts. The FDA regulations state that proteins taken from commonly allergenic foods are presumed to be allergens unless demonstrated otherwise. So far, no products on the market contain such allergens. If they do, FDA requires that they be clearly labeled biotech.

29.5.7 Exploitation of farmers

Biotechnology offers considerable possibilities to increase the food demand and help eliminate the anticipated shortfall with increasing human population. However, there is considerable concern that small-scale farmers should not be exploited by large international companies. The possible use of 'terminator' genes (that make seeds sterile) is one such example. They would help to reduce the hazards of environmental dispersal, however farmers in developing countries traditionally save seed from one season to the next and cannot afford to buy new supplies every year. GE is very expensive and needs lot of investment and it would not be available to poor farmers. Moreover, there could be potential loss of traditional farming practices such as collecting, storing, and replanting seed.

29.5.8 Perceived risks and benefits

While accepting any new technology like GMO, consumers always weigh the perceived benefits of accepting it against the perceived risks. The scientific evidence is required to prove their benefits.

29.5.9 Accountability

Consumers should be involved in local, national and international debates and in policy guidance. There are very few fora available to the public to discuss the wide range of issues related to GMOs. Consumers comprising everyone in the world (and including future generations) also have a stake in the process. Consumers choices to buy non-GM or GM foods cannot be ignored. They are not forced to buy the food that they don't want. Keeping this in mind, some of the leading GM crop producers are reconsidering their production decisions and the agrifood industry is rapidly restructuring.

29.5.10 Loss of biodiversity

There is a concern among people that extensive use of GM crops / foods will lead to loss of our biodiversity. GM crops could compete or breed with wild species threatening biodiversity. We need to retain our traditional foods.

29.5.11 Soil fertility

It has been demonstrated scientifically that GM crops transfer their genes to soil fungi and bacteria. The affected fungi and bacteria then behave in abnormal ways and diminish their function in breaking down organic material, which makes nutrients available to plants. The soil becomes progressively less fertile. After a few seasons of planting GM crops, the soil will not be able to host any other conventional crop. If farmers wish to switch back to conventional crops, it could take a whole season to rehabilitate the soil. Hence, the economic consequences are unfavorable besides the added cost of nutrients and fertilizers which are necessary to regenerate the soil.

29.6 Entry of GM Farm Animals Versus GM Crops in the Market

Although, GM crops have entered the market, products derived from GM farm animals have not reached the market. The scientific developments of transgenic farm animals has been accompanied by manifestations of perturbed physiology, including impaired reproductive performance. These have raised ethical problems of animal welfare, hence dampened the consumer's interest. So far, the prospects of foods from transgenic farm animals has not been well received by consumers. Consumers are accepting more of transgenic plants than of transgenic animals. Altering animals is a less acceptable practice and has broader implications since various cultures and religions restrict or prohibit the consumption of certain foods derived from animals. However, ingesting or injecting of certain pharmaceutical products from transgenic animals is more acceptable to the public. Pharmaceutical drugs produced using transgenic animals are acceptable since benefits overweigh the risks associated with them.

Although, all the above concerns are valid, there is no evidence that genetically engineered food is dangerous. We do not have any idea on long term consequences of eating GM foods on our health.

29.7 Potential Benefits of GMOs

Benefits of GMOs include:

1. There are potential benefits to agricultural productivity through the development of crops which are more resistant to disease, pests and also to harsh weather conditions which decrease the risk of devastating crop failure.
2. Genetically engineered pest and disease resistant crops could reduce the need for hazardous pesticides and other chemicals, thereby decreasing the environmental load and farmer exposure to such toxic chemicals which lead to human health risk to various dreadful diseases.

3. GM crops with extended shelf life could decrease the gross wastage associated with transportation and storage.
4. Allergenicity problem could be tackled through GE which could be used to remove genes associated with allergies, e.g., the blocking of the gene that produces the allergenic protein in peanuts.
5. The GM crops with high nutritional value or healthier foods (eliminating trans fats or caffeine) could be produced through GE e.g. Golden rice and rice with human lactoferrin and lysozyme.
6. GE could be used to develop pharmaceuticals and vaccines in plants, decrease the risk of adverse reactions and enable faster vaccination of large populations.

29.8 GM Crops in India

In India, field trials of GM crops have been carried out extensively and GM crops like the Golden Rice (which is rich in proteins) have been used. Unfortunately, the GM business is owned by top multinational companies and agribusiness is only for vested interests. India is the third largest producer of cotton after China and the U.S. GEAC gave approval for commercial plantation of the genetically engineered Bt cotton (to enable it to resist bollworm, a major pest for cotton) in four states of southern and central India in 2002 following which farmers decided to grow GM cotton. However, Bt cotton failure led to loss and Monsanto declined to even acknowledge. India is becoming a dumping ground for GE Crops As the world is waking up to human health and environment nuisance from the GM crops, International companies want to dump their products in India. In India on the other hand, besides cotton, genetic engineering experiments are being conducted on maize, mustard, sugarcane, sorghum, pigeonpea, chickpea, rice, tomato, brinjal, potato, banana, papaya, cauliflower, oilseeds, castor, soyabean and medicinal plants.

29.8.1 Bt Brinjal in India

The Bt brinjal is a transgenic brinjal (egg plant or aubergine, Fig. 29.1) created by inserting a crystal gene (Cry1Ac) from the soil bacterium *Bacillus thuringiensis* into the genome of various brinjal cultivars. The Bt brinjal has been developed to give resistance against lepidopteron insects, in particular the Brinjal Fruit and Shoot Borer (*Leucinodes orbonalis*) (FSB). It has been developed by global agri giant Monsanto's Indian partner, Maharashtra Hybrid Seed Company (Mahyco). India grows over eight million tonnes of brinjal annually and is the second largest grower of this vegetable in the world after China. The annual market for brinjal is estimated to be around Rs 8,000 crore (\$1.8 billion) and nearly 40 percent of the crop is lost due to attack by a major pest, Fruit and Shoot Borer. The Bt variety would have offered resistance and killed this major pest. Seven major states which together account for more than 70 percent of brinjal cultivation in the country have declined to introduce Bt brinjal. Hence, Indian government has not permitted commercial cultivation of the country's first GM food product.

The anti-GM (genetically modified) groups have been successful in not allowing the introduction of the country's first GM food crop although the GEAC approval was accorded on October 14, 2009 based on the issues that it threatens biodiversity and is unsafe for human consumption as well as ethical concerns such as corporate control of the food supply and intellectual property rights.



Fig. 29.1 Bt Brinjal

However, proponents of GM technology believe that Bt brinjal will have positive effects for the Indian economy and the health of the farmers. Field trials conducted on research-managed farms carried out by Mayhco and the Indian Council of Agricultural Research have indicated a 42% pesticide reduction with a doubling of the yield.. The economic gain for consumers, developers and farmers was estimated to potentially be US\$108 million per year with an additional \$3–4 million saved due to health benefits associated with decreased pesticide use.

The Foundation for Biotechnology Awareness and Education (FBAE), a non-profit organization formed to promote public awareness of scientific issues of biotechnology and to enhance levels of biotechnology education and training based in Bangalore has called for an immediate lift on the moratorium on the commercial release of Bt brinjal citing recent publications on the socio-economic benefits such as better living standards, health and education, and reduced tension, among the farming community and a healthier product to the consumer, that would accrue from the adoption of Bt brinjal.

29.9 Conclusion

Although weighing risks and benefits is necessary, it is neither easy nor the sole concern in considering the ethics of GM crops. Both human health and environment safety are of primary concern. All such issues should be dealt with before releasing GM crops. Scientists claim that GM foods will solve the problem of world hunger and malnutrition. Food scientists and technologists can support the introduction of GM technologies provided that issues of product safety, environmental concerns, ethics and information are satisfactorily addressed to so that the benefits that this technology can confer become available both to improve the quality of the food supply and to help feed the world's escalating population in the coming decades.



Module 7. Regulatory and safety aspects of biotechnology particularly in the light of GMO foods

Lesson 30

BIOSAFETY LEVELS AND BIOSAFETY BOARDS OPERATING IN INDIA

30.1 Introduction

With the recent advancements made in recombinant DNA technology and growing awareness in the society about the tremendous potential of Biotechnology in all the fields including agriculture, dairying, medical, diagnostics, forensic, food and industrial applications, National Biotechnology Board issued a set of safety guidelines for India in 1983 to ensure the safety of recombinant products along with the safety of workers in the laboratory environment. In order to fully understand the long term implications of recombinant DNA based Biotech products, it is necessary to familiarize with some of the following commonly used terms in this context.

30.2 Containment

The term “Containment” is used to describe various protocols for handling different infectious agents in the laboratory in order to reduce the risk of their release into the environment and also to reduce risk to the workers. As per WHO, following containment facilities are required for different risk groups.

30.2.1 Biological containment (BC)

Biological containment implies construction of strains including vectors and hosts which are disabled so that such recombinant vectors do not multiply in other hosts and also limit their survival in the environment.

30.2.2 Physical containment (PC)

Physical containment includes both primary and secondary containment facilities, proper laboratory practices and equipments as well as special laboratory design in order to prevent exposure to workers and environment. The primary containment facilities require good microbiological practices and safety equipments. On the other hand, secondary containment facility meant for high risk infectious agents requires safe designing of containment facility and safe operational practices in order to protect workers and the environment from infectious agents which can be hazardous.

30.3 Bio-Safety Levels

Biosafety levels are defined in terms of using specific laboratory practices and techniques, safety equipment and laboratory facilities required for different category of infectious agents based on their hazardous nature. The guidelines for Microbiological and Biomedical Laboratories suggest four Biosafety levels in incremental order depending on the nature of work. The proposed safety levels for projects involving recombinant DNA techniques take into consideration the source of the donor DNA and its disease-producing potential. Based on this, four levels are defined which correspond to (P1<P2<P3<P4) or BSL1 to BSL4 facilities.

30.3.1 Biosafety level 1 (BSL-1)

BSL-1 level is suitable for working with well characterized agents which are not known to cause any disease in healthy human beings and are of minimal hazard to workers in the laboratory as well as to the environment e.g. non-pathogenic *E. coli*. No special equipment is required. The safety precautions include following of good microbiological practices i.e using laminar flows, washing hands with anti-bacterial soap, cleaning working benches of the lab with disinfectants, decontamination of bacterial cultures by autoclaving etc. The laboratory personnel should be imparted specific training and also be supervised by a scientist with general training in microbiology.

30.3.2 Biosafety level 2 (BSL-2)

BSL-2 level is suitable for working with agents of moderate potential hazard to laboratory personnel and the environment e.g. *Salmonella* spp., *E. coli* 0157:H7, *Listeria monocytogenes*, mumps, measles, influenza etc. including genetically modified organisms. BSL-2 facility limits the release of modified organisms in the environment. Class II safety cabinets are required to be used in handling the high risk organisms under this category. They offer the additional capability and advantage of protecting materials inside the cabinet from extraneous airborne contaminants. It is an open-front cabinet which provides a HEPA-filtered, recirculated airflow within the work space. The exhaust air from the cabinet is also filtered by HEPA filters. Thus, the Class II biosafety cabinet provides personnel, environment and product protection. HEPA filters are effective for trapping particulates and infectious agents. Laboratory personnel are to be provided with specific training in handling pathogenic agents and to be supervised by competent scientists. The access to the laboratory is limited where work is being conducted. Each and everything used should be decontaminated either by autoclaving or putting them in disinfectants. A typical Biosafety Class II cabinet is shown in the following picture Fig. 30.1.



Fig. 30.1 Biosafety Cabinet Class II

30.3.3 Biosafety level 3 (BSL-3)

BSL-3 level facility is required for working with agents such as bacteria and viruses which can cause severe to fatal disease in humans on inhalation e.g. *Mycobacterium tuberculosis*, *Bacillus anthracis*, Q fever, and SARS coronavirus. However, such diseases can be treated with vaccines or other treatments. BSL-3 laboratory has special engineering and design features e.g. double door access zone and sealed penetration. However, all the laboratories may not have such facilities. In that case, BSL-2 should be upgraded to achieve BSL-3 containment level by providing facilities viz. discharge of filtered exhaust air from the laboratory to outside, restricted access to the lab and rigorously following safety precautions. Laboratory personnel need to be specifically trained in handling pathogenic and potentially lethal agents and should be supervised by competent scientists having adequate expertise in working with these agents. It is mandatory to conduct all procedures involving the manipulation of infectious materials within biological safety cabinets or other physical containment facilities or by personnel wearing appropriate personal protective clothing and equipment. Specially designed laboratories and precautions including the use of safety cabinets are prescribed and the access is strictly controlled. Class III cabinets are generally used for working with the pathogens falling in this category. It is a totally enclosed ventilated cabinet of gas-tight construction. The work within this cabinet is conducted through attached rubber gloves. When in use, the Class III cabinet is maintained through negative air pressure of at least 0.5 inches water gauge. The supply air is drawn into the cabinet through HEPA filters. The cabinet exhaust air is filtered by two HEPA filters, installed in series, before its discharge outside the facility. The exhaust fan for the Class III cabinet is generally separate from the exhaust fans of the facility's ventilation system.

30.3.4 Biosafety level 4 (BSL-4)

BSL-4 level is required for working with highly dangerous agents that pose a high risk to the workers through transmission by aerosols and lead to fatal diseases for which no treatment or vaccines are available e.g. Bird flu, swine flu, hemorrhagic fever, Ebola virus, Foot and Mouth Disease virus etc. It requires the use of 'Hazmat suit' and a self-contained oxygen supply. The entrance and exit contain multiple showers, a vacuum room, ultraviolet room as well as all the precautions designed to destroy the biohazardous waste. Multiple locks are employed which are electronically secured to prevent both doors opening at the same time. The air and water services to BSL-4 has to undergo decontamination procedures to eliminate the possibility of an accidental release. BSL-4 facility has to be created in a controlled area within the premises of the institute / industry or as a separate facility outside the building which is located away the other areas. The building protocols should use negative pressurized facilities. Airlocks should be provided during entry and exit of the personnel working in lab. Specific facility operation manual has to be prepared.

The researchers / workers / personnel working in the BSL-4 facility should be given specialized training in handling hazardous infectious agents and should be well versant with the containment equipments and laboratory design so that they follow all practices religiously.

A Hazmat suit is a garment worn as protection from hazardous materials or substances or substances duly backed up with an appropriate breathing apparatus

One such Class IV facility operating in India is “High Security Animal Disease Laboratory” (HSADL), the country's premier facility under the auspices of Indian Veterinary Research Institute (IVRI), an Indian Council of Agricultural Research (ICAR) Institute. This facility created in June 2000, is meant for handling exotic and emerging pathogens of animals by virtue of its bio-safety level-IV containment laboratory and animal experimentation facility.

30.4 Biosafety Boards Operating In India

With the advent of recombinant DNA technology and its safe applications in different fields like agriculture and animal husbandry across the world, an International meeting was held at Asilomar, California wherein scientists working in the field of genetic engineering made certain recommendations to manage the safety of recombinant DNA technology experiments. These formed the basis of subsequent biosafety guidelines and regulations in USA followed by other countries. India too developed her own regulatory guidelines for genetically modified organisms (GMOs) and recombinant products. There are at present two apex regulatory bodies *viz.* Department of Biotechnology (DBT) and Ministry of Environment and Forest (MEF) which are functioning in the country to regulate rDNA products.

MEF has developed guidelines for manufacture, import, use, research and release of GMOs as well as recombinant products produced from genetically modified organisms in order to ensure that GMOs or their products are safe to human beings. Safety guidelines were developed by DBT in 1990 for carrying out research in the field of Biotechnology, field trials and commercial applications. DBT has developed separate guidelines for research in transgenic plants in 1998 and for clinical products in 1999. Activities involving GMOs are also covered under other policies such as the Drugs and Cosmetics Act (8th Amendment), 1988, the Drug Policy, 2002, and the National Seed Policy, 2002.

Presently, there are six competent authorities under the auspices of Department of Biotechnology (DBT) and State Governments for implementation of regulations and guidelines in the country as listed below:

- i) Recombinant DNA Advisory Committee (RDAC) - DBT
- ii) Institutional Biosafety Committees (IBSC) attached to every organization engaged in rDNA research – DBT
- iii) Review Committee on Genetic Manipulation (RCGM) - DBT
- iv) Genetic Engineering Approval Committee (GEAC) - DBT
- v) State Biosafety Coordination Committees (SBCC) – State Government
- vi) District Level Committees (DLC) - State Government

30.5 Structure And Functions of Regulatory Bodies

30.5.1 Recombinant DNA Advisory Committee (RDAC)

RADC meets once in six months and monitors the developments at National and International levels for safety regulation in India on recombinant research and applications.

The functions of Recombinant Advisory Committee include:

- a) To develop long term policies for research and development in Recombinant DNA research

- b) To formulate the safety guidelines for Recombinant DNA Research to be followed in India
- c) To recommend the type of training programme for technicians and research fellows for making them adequately aware of hazards and risks involved in recombinant DNA research and also to tackle them.

30.5.2 Institutional Biosafety Committee (IBSC)

DBT has issued guidelines to all the Institutes engaged in rDNA/ genetic engineering research both in Government and Private sectors to constitute their Institutional Biosafety Committee (IBSC) comprising of following members.

- i) Head of the Institution or his nominee as Chairman
- ii) Three or more scientists engaged in rDNA work / molecular biology / genetic engineering
- iii) An outside expert in the relevant discipline
- iv) A member with medical qualifications - Biosafety Officer (in case of work with pathogenic agents/large scale use)
- v) One member nominated by DBT

IBSC is the nodal body at Institute level responsible for implementation of biosafety guidelines. The projects involving rDNA work are required to be submitted to IBSC for getting clearance. IBSC is responsible for implementation of proper safety guidelines for running the projects at Institute level. The functions of IBSC are as follows:

- a) IBSC gives clearance to rDNA projects submitted by investigators at Institute level based on different Biosafety levels
- b) IBSC meets twice in a year to review the progress and follow up of the recommendations
- c) IBSC provides half yearly report on the ongoing projects to RCGM regarding the observance of the safety guidelines on accidents, risks and on deviations, if any.
- d) IBSC is responsible for training of personnel on biosafety.
- e) IBSC is also responsible for health monitoring programme for laboratory personnel Complete medical check-up of personnel working in projects involving work with potentially dangerous microorganisms are required to be carried out on regular basis prior to start of such projects. The medical checkups including pathological tests need to be followed periodically, at least annually for scientific workers involved in such projects.
- f) Adopting emergency plans IBSC is also involved in creating awareness amongst the workers / students, faculty and technicians involved in RDNA research projects related to rDNA through popular lectures, seminars and workshops from time to time.

30.5.3 Review committee on genetic manipulation (RCGM)

RCGM is comprised of members from following National bodies.

- a) Department of Biotechnology

- b) Indian Council of Medical Research
- c) Indian Council of Agricultural Research
- d) Council of Scientific & Industrial Research
- e) Three Experts in Individual capacity
- f) Department of Science and Technology

RCGM performs the following functions:

- a) To establish procedural guidance manual / procedure for regulatory process with respect to activity involving genetically engineered organisms in research, production and applications related to environmental safety.
- b) To review the reports of all the approved ongoing research projects involving high risk category and controlled field experiments to ensure that safeguards are maintained at every step as per guidelines.
- c) To recommend the type of containment facility required and the special containment conditions to be followed for experimental trials and for certain experiments on case to case basis
- d) To advise custom authorities on import of biologically active material, genetically engineered substances or products and on excisable items to Central Revenue and Excise
- e) To assist Department of Industrial Development, Banks towards clearance of applications in setting up industries based on genetically engineered organisms
- f) To assist the Bureau of Indian Standards to evolve standards for biologicals produced by rDNA technology
- g) To advise on intellectual property rights with respect to rDNA technology on patents.

RCGM has a Research Monitoring function by a group consisting of 3 - 4 individuals and the committee is empowered to visit experimental facilities in any laboratory in India where experiments with biohazard potential are being pursued in order to determine the Good Laboratory practice and conditions of safety and can also recommend any alterations required in the course of experiments based on hazard considerations.

30.5.4 Genetic engineering approval committee (GEAC)

Genetic Engineering Approval Committee (GEAC) functions under the preview of Department of Environment (DOEn) as an apex body for review and approval of activities involving large scale application of genetically engineered organisms and their products in research and development, industrial production, environmental release and field applications. It acts as a legal and statutory body with judicial powers to inspect, investigate and take punitive action in case of violations of statutory provisions under Environment Protection Act.

The constitution of GEAC is as follows.

1. Chairman - Additional Secretary, Department of Environment

Dairy Biotechnology

2. Co-Chairman - Expert Nominee of Secretary, DBT
3. Representatives of concerned Agencies and Departments

Ministry of Industrial Development
Department of Science & Technology
Department of Ocean Development
Department of Biotechnology

4. Expert Members:

Director-General, Indian Council of Agricultural Research
Director General, Indian Council of Medical Research
Director-General, Council of Scientific & Industrial Research
Director-General, Health Services (Ministry of Health & Family Welfare)
Plant Protection Adviser (Ministry of Agriculture)
Chairman, Central Pollution Control Board
Three outside experts in individual capacity

5. Member Secretary - Official of, DOEn

30.5.5 State biosafety coordination committees (SBCC)

The SBCC is responsible for performing following functions at state level.

- a) To inspect, investigate and take action in case of violations of statutory provisions through the State Pollution Control Board or the Directorate of Health etc.
- b) To periodically review the safety and control measures in various institutions handling GMOs.
- c) To act as nodal agency at State level to assess the damage, if any, due to release of GMOs and to take site control measures.

30.5.6 District level committees (DLC)

The main functions of DLC are

- a) To monitor the safety regulations in installations
- b) To inspect, investigate and report to the SBCC or the GEAC about compliance or non compliance of r-DNA guidelines or violations under EPA.
- c) To act as nodal agency at District level to assess the damage, if any, due to release of GMOs and to take on site control measures



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