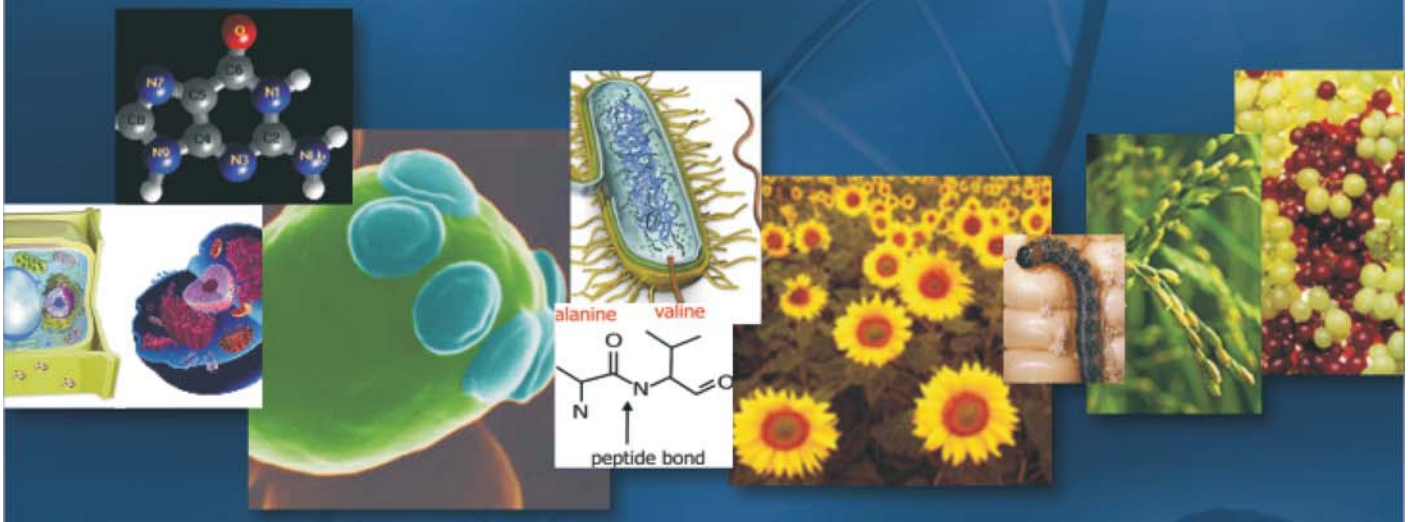
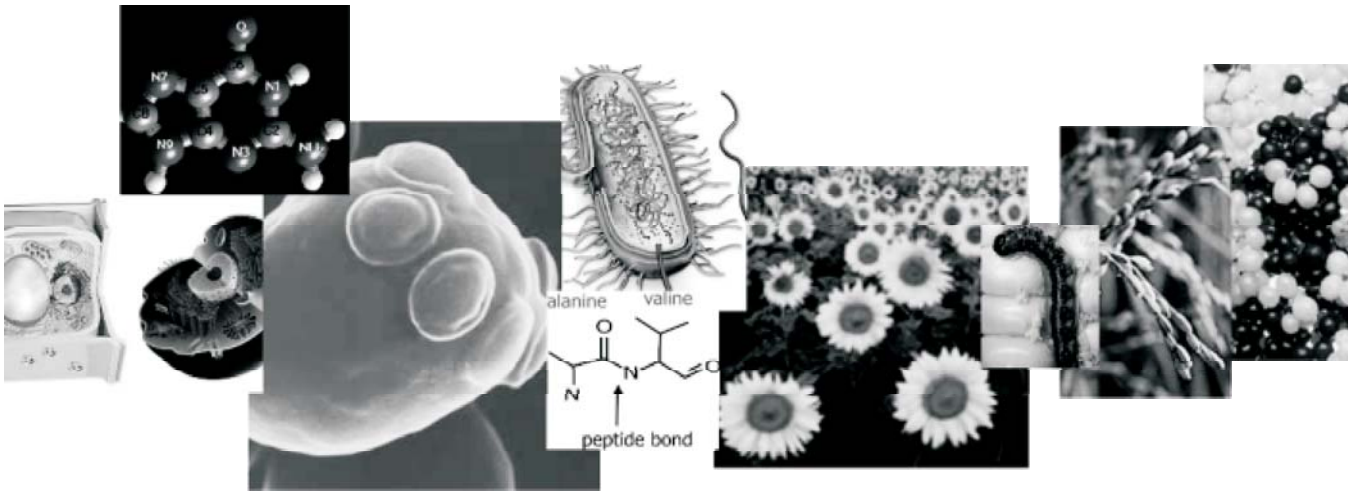
BIOTECHNOLOGY



Commission on Science and Technology for
Sustainable Development in the South (COMSATS)

BIOTECHNOLOGY



Commission on Science and Technology for
Sustainable Development in the South (COMSATS)

Biotechnology

By: Prof. Dr. Ikram-ul-Haq

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Commission on Science and Technology for Sustainable Development in the South

FOREWORD

Biotechnology is a revolutionary and accessible concept that can boost agriculture and modernize overall economy of a country. The development and dependence on biotechnology has largely increased in countries all over the world especially in the last few years. The human Genome, production of antibiotics and improvement of crops by agricultural biotechnology are some of the major breakthroughs provided by this technology.

The capacity to search, assess, develop, and utilize biotechnology is one of the most important factors accounting for differences in nations' competitiveness. The capacity is articulated in human skills, the existence of dedicated institutions and their scope of programs, availability of adequate financial resources, formulation of systemic and long-term policies, building up of scientific and technological infrastructure, and appropriate institutional networks and linkages for biotechnology R&D.

COMSATS is an international organization, aiming at socio-economic uplift of the third-world through useful applications of science and technology. Keeping in view the importance of Biotechnology, it had become essential to develop a platform where information would be easily available. In the field of Biotechnology, COMSATS has taken many steps such as to, collaborate with the National Commission on Biotechnology for the promotion of biotechnology across Pakistan; establish a Biotechnology Cell in October 2001 for development in the sectors of health, agriculture and Biotechnology; establish a web-portal and has the plans to hold a series of conferences on various themes of biotechnology for mass promotion and awareness. The main objectives of the Biotechnology cell has been to develop biotechnology related research-institutes in the South for sharing of knowledge; to carry out collaborative programs to enhance indigenous research for increasing productivity through the use of biotechnology and developing a network of scientists for capacity building within the country; to develop databank of human and technical resources; create awareness and that through a web-portal for providing quick access to the information about institutions involved in the subject and on-going research projects as well as the expertise available in Pakistan.

This book discusses the key areas of biotechnology and its role in shaping the world today. It has been written in an easy-to-understand manner especially for young minds of students to make them understand how biotechnology would bring about a radical change in global economic and industrial context. It also highlights some of the recent and ongoing developments that have or are taking place in various areas of biotechnology.

The contents of the book have been very carefully and comprehensively structured and it has a systematic approach and sequencing in which the topics and chapters have been

written. Taking account of the fundamentals and historic background of the evolution of biotechnology the book unwraps and leads to elaborate genetics engineering, culture techniques and practical applications of biotechnology with help of nicely presented diagrams and figures. Number of pictures and diagrams are presented in this book in order to provide clear understanding and explanation of various concepts and principles.

We know that in the span of a few short decades, the powerful tools of biotechnology have revolutionized medicine, agriculture and environmental protection, but full potential of biotechnology will only be realized if we continue to invest in a strong national research base and make an all out efforts to build and maintain a critical mass of young and energetic scientists to lead us in a prosperous future.

This book has been written with the same spirit and fervor as stated above and I must present my gratitude to Dr. Anwar Nasim who is the torchbearer as far as biotechnology in Pakistan is concerned, he took personal interest in getting this job done. Also special thanks are due to author of this book, Prof Dr. Ikram-ul-Haq from Biotechnology Research Center, Govt. College University, Lahore. The well balance chapters and selection of topics speak of the years long teaching experience and academic skillfulness that Prof Ikram has. Not only is he the author of various text books taught in the field at graduation level but has also earned national honors by winning the awards of 'Productive Scientist of the Year' for the two consecutive years, i.e 2002-3 and has to his name Sitara-e-Imtiaz (S.I.).

Finally, my sincere gratitude goes to peer reviewer scientists Dr. Azra Khannum and Mrs. Ghazala Zahidi as well as to the dedicated team of COMSATS that involve the names of Dr. M. M. Qureshi, Mr. Tajammul Hussain, Mr. Irfan Hayee, Mr. Imran Chaudhry and Ms. Nageena Safdar that got this book edited, proof read and finally printed. I hope this book will be widely applied and studied to deliver the essence of knowledge-sharing and technology-development.

Dr. Hameed Ahmed Khan H.I., S.I.
Executive Director - COMSATS

PREFACE

The scientific and technological revolution of the past century has given mankind prosperity and power. The invention of the wheel, explosion of the atom, landing of man on the moon are major breakthroughs in the history of science. However, the biotechnological revolution is the milestone, which is going to herald the future of mankind. The discovery of the structure of DNA in the early 1950s by James Watson and Frances Crick opened the door to extraordinary advances in biotechnology. Scientists soon learnt to identify genetic sequences and their functions, which ultimately led to recombinant DNA technology.

Of all technologies, biotechnology has the most direct influence on human life as it provides many benefits in fields such as human health, drug industry, improved post-harvest characteristics, increased resistance to insect pests and microbial diseases, improved yield, etc. Many of the benefits of biotechnology may be necessary to feed the world's growing population and to preserve biodiversity and natural habitats. Recognizing its importance every major institute around the world is concentrating on the development of biotechnology professionals. The main purpose of this book is to create awareness about biotechnology amongst the students by introducing it as a subject for graduate classes, familiarizing them with the methodologies and applications of biotechnology and encouraging them to consider the ramifications of this technology on society and the environment. Presently, there is an alarming shortage of trained manpower in biotechnology and it is very important that immediate steps should be taken to overcome this weakness.

Since biotechnology is a vast subject, it is not possible to cover all aspects in one book. However, an attempt has been made to include some basic topics like an overview of the subject, Genetic Engineering, Culture Techniques, Downstream Processing and Practical Applications of Biotechnology. Main focus of the book is on underlying Principles and Applications.

It is indeed gratifying that a number of young and dedicated students took an active part in the preparation of this book. Such efforts are greatly appreciated. I take this opportunity to thank our wonderful colleagues who have given me new ideas, inspired me to look further and deeper and provide me with the much needed feedback.

I am very grateful to COMSATS for publishing Biotechnology with their customary excellence. Special thanks are due to Dr. Hameed Ahmed Khan and Mr. Tajammul Hussain, without whose constant efforts this volume could not be published. Finally the editors also wish to thank the members of the Advisory board for their encouragement, helpful suggestions and their constructive criticism. This book is a result of the joint responsibility and the efforts of many people.

Prof. Dr. Ikram-ul-Haq

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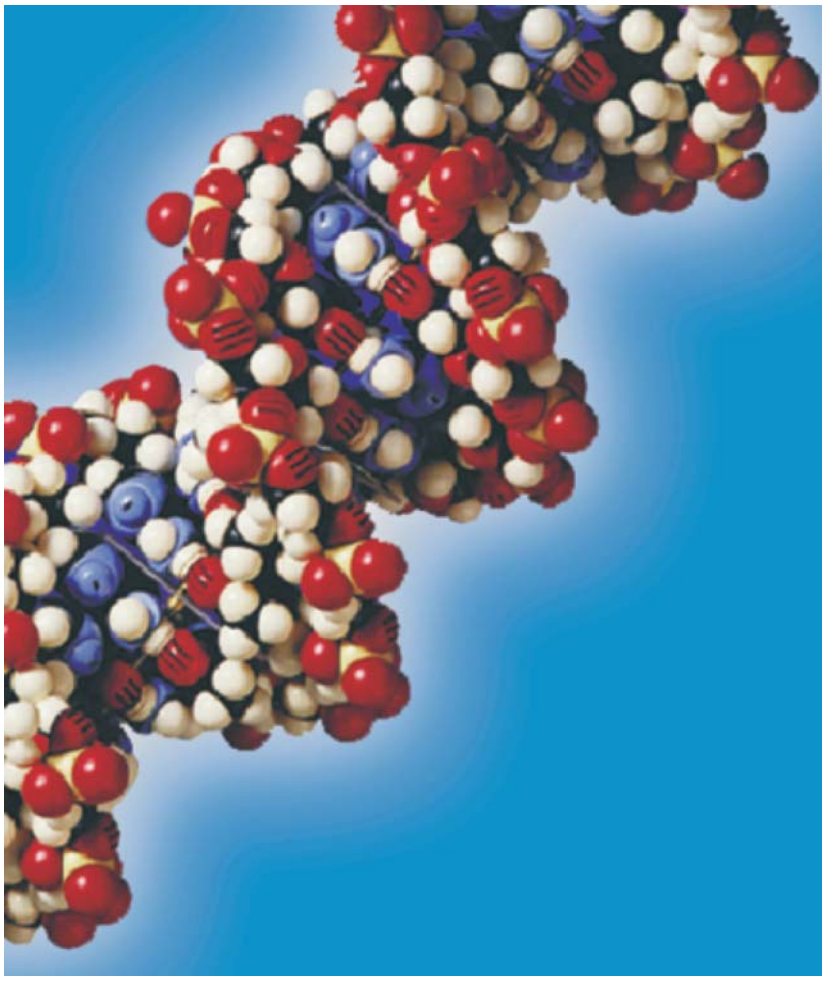
Chapter 1

Overview and History of Biotechnology



Chapter 1

OVERVIEW AND HISTORY OF BIOTECHNOLOGY

Biotechnology can be defined as any technique that uses living organisms (or parts of organisms) to make or modify products, to improve plants and animals or to develop microorganisms for specific uses. This is a newly acquired biological knowledge, which has already made important contributions vastly to health and welfare of mankind. Yet few people fully recognize that the life sciences affect over 30 % of global economic turnover through health care, food and energy, agriculture and forestry and that this economic impact will grow more and more as biotechnology provides new ways of influencing the raw material processing. Biotechnology will increasingly affect the efficiency of all fields involving the life sciences. It is now realistically accepted that in twenty-first century it will be contributing trillions of dollars to world markets. Biotechnology is developing at a phenomenal pace and will increasingly be seen as necessary part of the advance of modern life and not simply a way to make money.

Biotechnology covers a multitude of different applications ranging from the very simple to traditional, such as the production of beer, wine and cheese, to highly complex molecular processes, such as the recombinant DNA (deoxy ribonucleic acid) technologies to yield new medicines/therapeutics or to introduce new traits into commercial crops and animals. The association of traditional industries such as brewing with modern genetic engineering is gaining momentum and it is not for nothing that industrial giants such as Guinness, Carlsberg and Bass are heavily involved in biotechnology research.

While biotechnology has been defined in many forms in essence it implies the use of living organisms / their products to enhance human health and human environment. The European Federation of Biotechnology (EFB) considers biotechnology as the integration of natural sciences and organisms, cells, parts thereof and molecular analogues for products and services. The definition of EFB is applicable to both traditional and modern biotechnology. Traditional biotechnology refers to the conventional techniques that have been used for many centuries to

produce beer, wine, cheese and many other foods while modern biotechnology embraces all methods of genetic modifications by recombinant DNA and cell fusion techniques, together with the modern development of these processes. This difficulty of defining biotechnology and the resulting misunderstandings have led some people to suggest the abandonment of this term as too general and to replace it by the precise term of whatever specific technology or application was being used. Biotechnology is not itself a producer like microelectronics: rather it should be regarded as a range of enabling technologies that will find significant application in many industrial sectors. As will be seen in later sections, it is a technology in search of new applications and main benefits lie in the future. New processes will, in many instances, function at low temperature, consume little energy and rely mainly on inexpensive substrates for biosynthesis.

The history of biotechnology begins when primitive man became domesticated enough to breed plants and animals, gathered and processed herbs for medicine, make bread and wine and beer, created many fermented food products including yogurt, cheese and various soy products, created septic systems to deal with their digestive and excretory waste products, and to create vaccines to immunize themselves against diseases. Archeologists keep discovering earlier examples of each of the uses of organisms by man and examples of most of these processes go back to 5000 to 10,000 B.C.

Karl Ereky, a Hungarian engineer, coined the term biotechnology in 1919. At that time, the term meant all the lines of work by which products are produced from raw materials with the aid of living organisms. Ereky envisioned a biochemical age similar to the stone and iron ages.

Biotechnology seems to be leading a sudden new biological revolution. It has brought us to the brink of a world of "engineered" products that are based in the natural world rather than on chemical and industrial processes. Biotechnology has been described as "Janus-faced". This implies that there are two sides. On one side, techniques allow DNA to be manipulated to move genes from one organism to another while on the other side, it involves relatively new technologies whose consequences are untested and should be met with caution (Figure 1.1).

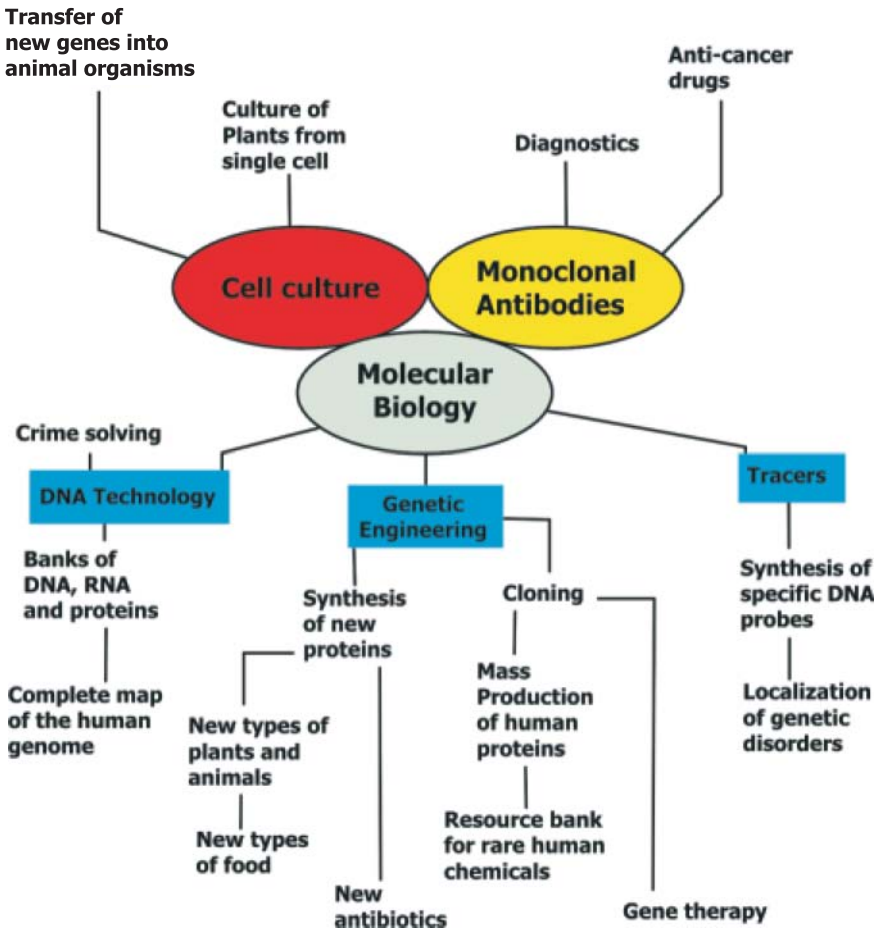


Figure 1.1: Areas of Applied Biotechnology

The History of Biotechnology

(i) 6000 BC-1700 AD: Early Applications and Speculations

Biotechnology as a way of life in ancient and pre-modern times:

6000 BC:

Sumerians and Babylonians used yeast to make beer.

4000 BC:

The Egyptians discovered how to bake leavened bread using yeast. Other fermentation processes were established in the ancient world, notably in China. The preservation of milk by lactic acid bacteria resulted in Yogurt. Molds were used to produce cheese. Vinegar and wine were also manufactured by fermentation.

1000 BC:

Babylonians celebrated the pollination of date palm trees with religious rituals.

1000 AD:

Hindus observed that certain diseases may "run in the family." Moreover, they came to believe that children inherit all their parents' characteristics.

1100-1700 AD:

Spontaneous Generation was the dominant explanation that organisms arise from non-living matter. Maggots, for example, were supposed to arise from horsehair.

1300 AD:

The Aztecs in Mexico harvested algae from lakes as a food source.

1400 AD:

While distillation of a variety of spirits from fermented grain was widespread, Egypt and Persia largely gave up brewing as a result of the influence of Islam. Fermented breads and cereals still maintained their hold in the African diet.

1665 AD:

Robert Hooke observed the cellular structure of cork. But it wasn't until almost 200 years later that scientists, armed with better microscopes, realized that all of us are divided into very small compartments.

1668 AD:

Francesco Redi (1626) used an experiment to compare two competing ideas that seek to explain why maggots arise on rotting meat. He observed that meat covered to exclude flies did not develop maggots, while similar uncovered meat did. This was among the first uses of a controlled experiment.

1660-1675 AD:

Marcello Malpighi (1628-1694) in this period used a microscope to study blood circulation in capillaries, described the nervous system as bundles of fibres

connected to the brain by the spinal cord, detailed the anatomy of the silkworm, described the development of the chick in its egg, and published work on plant anatomy.

1673 AD:

Anton van Leeuwenhoek (1632-1723), a Dutch merchant and civic administrator who ground glass lenses as a hobby used his microscopes to make discoveries in microbiology. He was the first scientist to describe protozoa and bacteria and to recognize that such microorganisms might play a role in fermentation.

(ii) 1700-1900: The Miracle of Life and Death Appears Smaller and Smaller

The empirical method and the industrial revolution brought monumental changes to farming and industry, while the biological sciences were inspired by the work of Darwin and Pasteur. The microbial nature of many diseases was established.

1701:

Giacomo Pylarini in Constantinople practiced "inoculation"--intentionally giving children smallpox to prevent a serious case later in life. Inoculation will compete with "vaccination"--an alternative method that uses cowpox rather than smallpox as the protecting treatment--for a century.

1724:

Cross-fertilization in corn was discovered.

1748:

Turbevill Needham heated various soups or "infusions" all of which eventually teem with life; he concluded "there is a vegetative force in every microscopical point of matter..." in support of the idea of spontaneous generation.

1750-1850:

Farmers in Europe increased their cultivation of leguminous crops and began rotating crops to increase yield and land use.

1798:

Edward Jenner published his book comparing vaccination (intentionally infecting humans with cowpox to induce resistance to smallpox) to inoculation (intentionally

infecting humans with a putatively mild strain of smallpox to induce resistance to severe strain of the disease). He derived his ideas from observing that people who had been exposed to cowpox were not vulnerable to smallpox. (Vaccine comes from the Latin word vaccinus "from cows.")

1799:

Lazaro Spallanzani described ingeniously crafted experiments using "hermetically sealed" flasks heated in boiling water to test the possibility of using heat to kill all the microbes in an "infusion" (liquid growth medium).

1809:

Nicolas Appert devised a technique using heat to sterilize food.

1816:

Recognizing the importance of agricultural diversity, the Tariff Act excluded foreign plants and trees from U.S. import duties.

1839:

Congress invested a huge amount of foreign exchange for the Congressional Seed Distribution Program, administered by the U.S. Patent Office, to increase the amount of free seeds.

1850's:

Horse drawn harrows, seed drills, corn planters, horse hoes, 2-row cultivators, hay mowers, and in the U.S. Industrially processed animal feed and inorganic fertilizer were first introduced.

1859:

Charles Darwin (1809 - 1882) hypothesized that animal populations adapt their forms over time to best exploit the environment, a process he referred to as "natural selection." He theorized that only the creatures best suited to their environment survive to reproduce. Darwin also inferred the process of adaptive radiation, wherein populations spread out into the environment to exploit specialized resources.

1863:

Louis Pasteur invented the process of pasteurization, heating wine sufficiently to inactivate microbes (that would otherwise turn the "vin" to "vin aigre" or "sour wine") while at the same time not ruining the flavor of the wine.

Anton de Bary proved that a fungus causes potato blight. A challenge for

scientists during this period was to discern whether a microbe was the cause of, or the result of, a disease.

1864:

Pasteur theorized that decayed organisms are found as small-organized 'corpuscles' or 'germs' in the air.

1868-1871:

Davaine used heat treatment to cure a plant of bacterial infection.

W. Flemming discovered mitosis.

Ernst Hoppe-Seyler discovered invertase, an enzyme that cuts the disaccharide sucrose into glucose and fructose. The enzyme is still widely used today in making sweeteners.

1873-1876:

Robert Koch investigated anthrax and developed techniques to view, grow, and stain organisms. He then photographed them, aided by Gram, Cohn, and Weigart.

1878-1879:

Joseph Lister described the "most probable number" technique, the first method for the isolation of pure cultures of bacteria, an important step in understanding infectious diseases.

Ralph Waldo Emerson suggested that weeds were actually plants "whose virtues have not yet been discovered".

1880:

Studying fowl cholera, Pasteur published his work on "attenuated" or weakened strains of organisms that could not cause disease but protected against severe forms of the same disease.

1881:

Robert Koch described bacterial colonies growing on potato slices, on gelatin medium, and on agar medium. Nutrient agar became a standard tool for obtaining pure cultures and for identifying genetic mutants. This was considered by T.D. Brock to be the single most important discovery in the rise of microbiology.

Pasteur used attenuation to develop vaccines against the bacterial pathogens of fowl cholera and anthrax; this was a founding moment in immunology and opened

new areas in the field of preventive medicine.

1882:

Robert Koch, using guinea pigs as an alternative host, described the bacterium that causes tuberculosis in humans. Koch became the first to uncover the cause of a human microbial disease. He established that specific organisms cause specific diseases.

Ilya Metchnikoff observed phagocytes surrounding microorganisms in starfish larvae. Later, he developed a cell theory to explain the action of vaccines.

1884-1887:

Robert Koch stated his "postulates" for testing whether a microbe is the causal agent of a disease.

Pasteur developed a rabies vaccine.

Christian Gram described the differential staining technique for bacteria known as the Gram stain. The first human trials of Pasteur's rabies vaccine took place. J.C. Arthur demonstrated that pear fire blight is a bacterial disease.

R.J. Petri described the circular glass plates with overlapping glass lids for growing microbes on nutrient agar. Petri plates are still standard tools of the microbiologist.

The Pasteur Institute was opened in Paris.

1892-1895:

Ivanovsky reported that the causal agent of the tobacco mosaic disease is transmissible and can pass through filters that trap the smallest bacteria. Such agents are later called "filterable viruses" or just "viruses."

Koch, Petri, Loffler, Yersin, and Erlich identified a host of human disease-causing organisms.

Emil von Behring developed the first antitoxin, for diptheria.

Winogradski demonstrated nitrogen fixation in the absence of oxygen by Clostridia bacteria.

1896:

Wilhelm Kolle, a German bacteriologist, developed cholera and typhoid vaccines.

1897:

Eduard Buchner demonstrated that fermentation could occur with an extract of yeast in the absence of intact yeast cells. This was a founding moment in biochemistry and enzymology.

Friedrich Loeffler and P. Frosch reported that the pathogen of the foot-and-mouth disease of cattle was so small that it passes through filters that trap the smallest bacteria; such pathogens came to be known as "filterable viruses." Ronald Ross discovered Plasmodium; the protozoan that causes malaria, in the Anopheles mosquito and showed the mosquito transmits the disease agent from one person to another.

1899:

Beijerinck's research on tobacco mosaic disease confirmed the work of Ivanovsky. Beijerinck proposed that the virus becomes incorporated into the protoplasm of the host plant.

(iii) The Modern Era

1900:

Walter Reed established that mosquitoes transmit yellow fever, the first time a human disease was shown to be caused by a virus.

1933:

Thomas Hunt Morgan was awarded with Nobel award for his discoveries concerning the role-played by the chromosome in heredity.

1953:

Double helix structure of DNA was first described by Watson and Crick.

1973:

Cohen and Boyer developed genetic engineering techniques to "cut and paste" DNA and to amplify the new DNA in bacteria.

1977:

The first human protein (somatostatin i.e. growth hormone) was produced in a bacterium (E. coli).

1978:

Arber, Nathan and Smith made the discovery of restriction enzymes and their application to problems of molecular genetics.

1980:

Paul Berg was awarded Nobel Prize for his fundamental studies of the biochemistry of nucleic acids, with particular regard to recombinant-DNA.

Walter Gilbert and Frederick Sanger contributed in the determination of base sequences in nucleic acids.

1982:

The first recombinant protein (human insulin) appeared in the market.

1983:

Polymerase chain reaction (PCR) technique conceived.

1990:

Human Genome Project (HGP), an international effort to sequence the human genome was launched.

1995:

The first genome sequence of an organism i.e., Haemophilus influenzae was determined.

1996:

A collaboration of scientists reported sequencing of the complete genome of a complex organism, Saccharomyces cerevisiae, otherwise known as baker's yeast. T-cell researchers determined the three-dimensional structure of these critical components of the immune system.

1997:

Researchers at Scotland's Roslin Institute cloned a sheep named Dolly from the cell of an adult ewe.

Artificial human chromosomes created for the first time.

Follistim, a recombinant follicle-stimulating hormone, approved for treatment of infertility.

A group of Oregon researchers claimed to have cloned two Rhesus monkeys.

Clock, the first gene providing the circadian rhythm of mammalian life identified.

Using a bit of DNA and some commonplace biological laboratory techniques, researchers have now engineered the first DNA computer "hardware" ever: logic made of DNA. A new DNA technique combines PCR, DNA chips, and computer programming providing a new tool in the search for disease-causing genes.

1998:

University of Hawaii scientists cloned three generations of mice from nuclei of adult ovarian cumulus cells.

Two research teams succeed in growing embryonic stem cells, the long sought grail of molecular biology.

Scientists at Japan's Kinki University clone eight identical calves using cells taken from a single adult cow.

Favorable results with a new antibody therapy against breast cancer, HER2neu (Herceptin), herald a new era of treatment based on molecular targeting of tumor cells.

1999:

A new technique based on unique individual antibody profiles offers an alternative to current DNA fingerprinting methods. The method is simple to use and has attracted considerable attention from law enforcement. A new medical diagnostic test will for the first time allow quick identification of BSE/CJD a rare but devastating form of neurologic disease transmitted from cattle to humans.

2000:

Human Genome Project was completed.

2001:

Modified foods with genes derived from no allergenic sources were evolved. Scientists are also trying to completely remove food allergens through biotechnology. Plant based vaccines along with those secreted by animal cells were worked out.

2002:

A primary focus of the renewed registration of Bt cotton (toxin gene of Bacillus thuringenesis has been introduced in cotton). For Bt cotton, to reduce the possibility

of insects developing resistance to Bt, the amended registration requires that some acres be set aside where non-Bt cotton will be grown to serve as a "refuge."

Genetically modified (GM) seed with better production and environmental friendly effects are the current issue.

Biotechnology at the End of 20th Century

The end of the nineteenth century was a milestone of biology. Microorganisms were discovered, Mendel's work on genetics was accomplished, and institutes for investigating fermentation and other microbial processes were established by Koch, Pasteur, and Lister.

Biotechnology at the beginning of the twentieth century began to bring industry and agriculture together. During World War I, fermentation processes were developed that produced acetone from starch and paint solvents for the rapidly growing automobile industry. Work in the 1930s was geared toward using surplus agricultural products to supply industry instead of imports or petrochemicals. The advent of World War II brought the manufacture of penicillin. The biotechnical focus moved to pharmaceuticals. The "cold war" years were dominated by work with microorganisms in preparation for biological warfare as well as antibiotics and fermentation processes.

Biotechnology is currently being used in many areas including agriculture, bioremediation, food processing, and energy production. DNA fingerprinting is becoming a common practice in forensics. Similar techniques were used recently to identify the bones of the last Czar of Russia and several members of his family. Production of insulin and other medicines is accomplished through cloning of vectors that now carry the chosen gene. Immunoassays are used not only in medicine for drug level and pregnancy testing, but also by farmers to aid in detection of unsafe levels of pesticides, herbicides and toxins on crops and in animal products. These assays also provide rapid field tests for industrial chemicals in ground water, sediment, and soil. In agriculture, genetic engineering is being used to produce plants that are resistant to insects and diseases. Modern biotechnology began when recombinant human insulin was first marketed in the United States in 1982. The effort leading up to this landmark event began in the early 1970's when researchers developed protocols to construct vectors, by cutting out and pasting pieces of DNA together to create a DNA (recombinant DNA), that could be inserted into the bacterium, *Escherichia coli* (transformation). If one of the pieces of DNA included a

gene, which produced an enzyme that broke down a particular antibiotic, the bacterium would be resistant to that antibiotic and could grow in a medium containing it. To the piece of DNA that conferred resistance of Escherichia coli to a particular antibiotic was added the human gene for the making of insulin. If this recombinant DNA containing the human insulin gene was used to transform Escherichia coli, and the bacteria were plated on an agar plate containing the antibiotic, the bacteria that grew contained not only the antibiotic resistant gene but also the insulin gene. Additional new pieces of DNA were then added to promote the expression of the human insulin gene so that this new recombinant DNA (expression vector) could be used to transform Escherichia coli. Thus, large quantities of human insulin messenger RNA were formed, which in turn were translated into large quantities of the human insulin protein.

The next step in the development of modern methods of protein production, now called as process development. During process development the best growth conditions are identified that produce desired protein, as efficiently as possible. This best process is scaled-up to produce the quantities of human protein that are needed for pre-clinical and clinical trials and for manufacture. Process development also includes the development of media, buffers, reagents, solutions, and assays and the choice of tools, such as bioreactors and liquid chromatography equipment, for the growth of recombinant cells (upstream processing), for the isolation and purification of the recombinant protein (downstream processing), and for tests to insure that both the upstream and downstream processes are proceeding in a predictable manner (quality control).

The protein characteristics and purity must conform to certain conditions determined during process development and tested at the manufacturing facility during process validation. The quality control division of the manufacturing facility monitors upstream and downstream processing. Quality control also handles environmental monitoring during production of the protein. Towards the end of process development, a master cell bank is laid down. The master cell bank is sized to last as long as the manufacture of the product will take place. Ordinarily the master cell bank is a large quantity of vials each containing 1ml of media within which there are about 1,000,000 recombinant cells (1,000,000 cells/ml) which are stored frozen in liquid nitrogen.

Since the manufacture of human insulin using recombinant Escherichia coli began in 1982, many other proteins (for human and veterinary therapeutics, vaccines and diagnostics) are being manufactured. Today, the Federal Drug Agency, USA (FDA) has approved for marketing 24 human therapeutic or vaccine proteins made by modern biotechnology methods (Table 1.1).

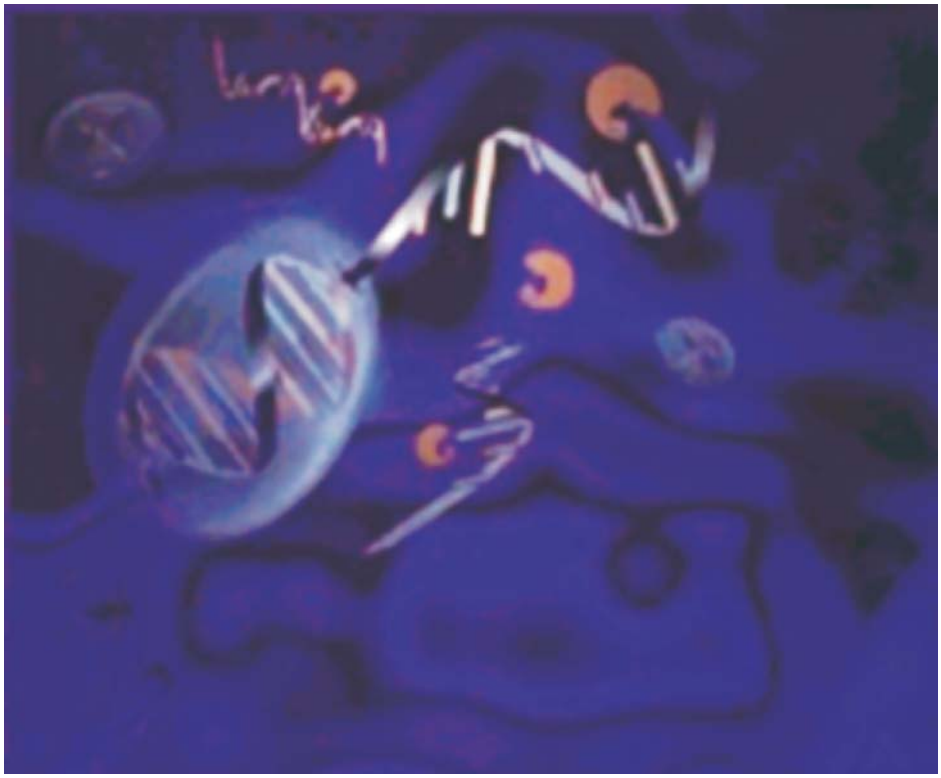
Table 1.1: List and Date of Approval of Vaccine Proteins by the FDA.

Product	Year
Actimmune	1990
Activase (this is human, recombinant tPA)	1990
Alferon N	1989
Betaseron	1993
Cerezyme	1994
Engerix-B	1989
Epogen	1993
Procrit	1993
Humatrope	1987
Humulin	1987
Intron A	1986-1992
KoGENate	1993
Leukine	1991
Neupogen	1994
Nutropin	1994
OncoScint	1992
Orthoclone	1993
Proleukin	1992
Protropin	1985
Pulmozyme	1993
Recombinate	1992
Recombivax hb	1986

There are more than 200 other human therapeutic and vaccine proteins in clinical trials. Products are being tested to target the following diseases: cancer, AIDS, heart disease, multiple sclerosis, rheumatoid arthritis and viral diseases.

Chapter 2

Fundamentals of Biotechnology



Chapter 2

FUNDAMENTALS OF BIOTECHNOLOGY

Cell: Unit of Life

The cell is the basic unit of life. There are millions of different types of cells. There are cells that are organisms onto themselves, such as microscopic amoeba and bacteria cells. The cell is the smallest unit of life in our bodies. All the cells in the body have unique functions and features. These have a 'skin', called the plasma membrane, protecting it from the outside environment. The cell membrane regulates the movement of water, nutrients and wastes into and out of the cell. Inside of the cell membrane are the working parts of the cell. At the center of the cell is the cell nucleus. The cell nucleus contains the cell's DNA, the genetic code that coordinates protein synthesis. In addition to the nucleus, there are many organelles inside of the cell small structures that help in carrying out the day-to-day operations of the cell (Figure 2.1).

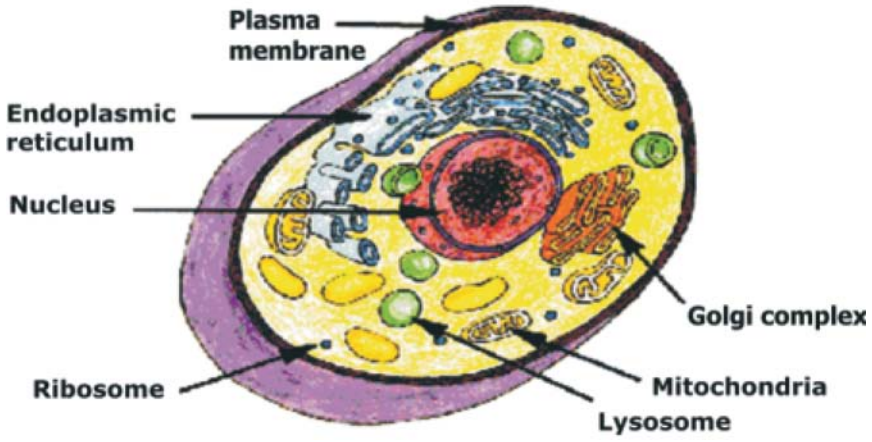


Figure 2.1: A Generalized Cell

Life is based on the universal principle of the specific interaction of molecules with each other. Molecules interact as pairs, one of which is called the ligand and the other the receptor. These ligand/receptor interactions involve associations of atoms

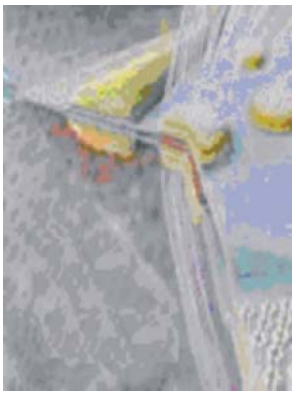
that attract one another so as to stick or bind the molecules together through a variety of weak bonds. The specificity is determined by the shape of the interactive regions on the ligand/receptor pairs that are similar to interactions between a lock and its key or the pieces of a puzzle. Imagine a lock/key system that when the key is inserted into the lock it strongly sticks in place so it is difficult to remove. This principle is applied to every biological phenomenon.

All cellular life has the following characteristics in common (Figure 2.2).

- Has a cell membrane that separates the chaos outside a cell from the high degree of organization within the cell. A cell without a cell membrane is not a cell.
- Contains DNA as its genetic material. All cells contain several varieties of RNA molecules and proteins; most of the latter are enzymes.
- Composed of the same basic chemicals: carbohydrates, proteins, nucleic acids, minerals, fats and vitamins.
- Regulate the flow of nutrients and wastes that enter and leave the cell.
- Reproduce and are the result of reproduction.
- Require a supply of energy.
- Are highly regulated by elaborate sensing systems (chemical "noses") that allow them to be aware of every reaction that is occurring within them and many of the environmental conditions around them; this information is continually processed to make metabolic decisions. For example: many flowers close up at night and open during the day.

The above criteria are the minimal requirements of life. Two general cell types have evolved. These are called Prokaryotic and Eukaryotic cells. Current data supports the theory that prokaryotic cells represent the initial or primitive cell type on earth and that eukaryotic cell types evolved from them.

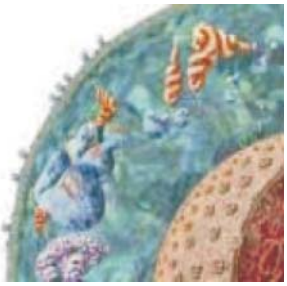
Prokaryotic cells are less complex than eukaryotic cells at several levels. Eukaryotic cells are structurally and biochemically more complex and are considered to represent a later stage of evolution. There is strong data to support the idea that Eukaryotic cells evolved from aggregates of Prokaryotic cells that became interdependent and eventually merged or fused into a single larger cell.



Cell junctions

Generalized plant structure

Plasmodesmata



Protein synthesis

Plant cell wall

Chloroplast

Figure 2.2: Important Cell Components

Prokaryotic Cell Structure-Escherichia coli (E. coli)

These are the main structures of a prokaryotic cell (Figure 2.3):

- **Ribosomes:** A small organelle constructed in the nucleoid, consisting of two subunits and functions as the site of protein synthesis in the cytoplasm.
- **Mesosome:** The infolding of a cytoplasmic membrane. Involved in the separation of chromosomes in reproduction.

Cell Surface Membrane: First, if not only, layer of every living cell, prokaryotic or eukaryotic, that acts as a selective barrier. It lets certain substances leave and enter the cell. Regulates the cell's chemical composition.

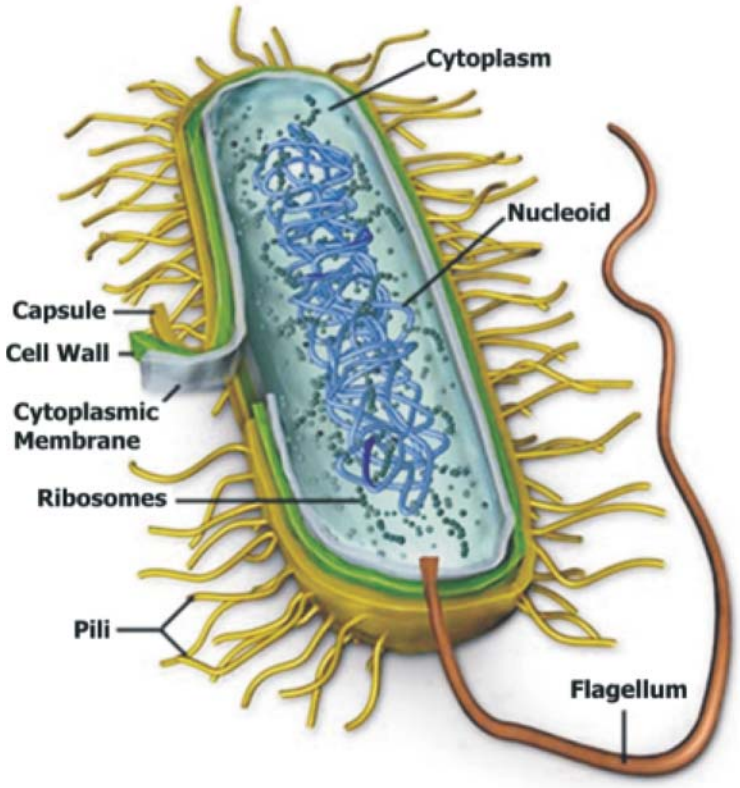


Figure 2.3: Bacterial Cell Structure

- Cell Wall: A protective layer external to the plasma membrane and internal to the slime capsule in prokaryotes. Also found in eukaryotes such as plants, fungi, and some protists. Protects and supports the organism.
- Slime Capsule: Secretion by prokaryotes of sticky substances that form another protective layer outside of the cell wall. Enable the organisms to adhere to their substrate and provide additional protection, including an increased resistance of pathogenic bacteria to host defenses.
- Flagellum: A long cellular appendage specialized for locomotion. Formed from a core of nine outer doublet microtubules and two inner single microtubules, ensheathed in an extension of plasma membrane. Helps to move the cell by whipping the appendage back and forth.

- ***Plasmid:*** Plasmids are circular, double-stranded DNA molecules capable of replication within living cells. Although not essential for the survival of their host, they may encode a wide variety of genes that increase survival in adverse environmental conditions.
- ***Naked Nucleic Acid:*** These are the DNA strands usually found in the nucleus of eukaryotes but in prokaryotes they are in the nucleoid. Naked Nucleic Acids serve as the genes (chromosomes) that control the cell.

Eukaryotic Cell (Plant)

Eukaryotic cell has following components (Table 2.1 & Figure 2.4).

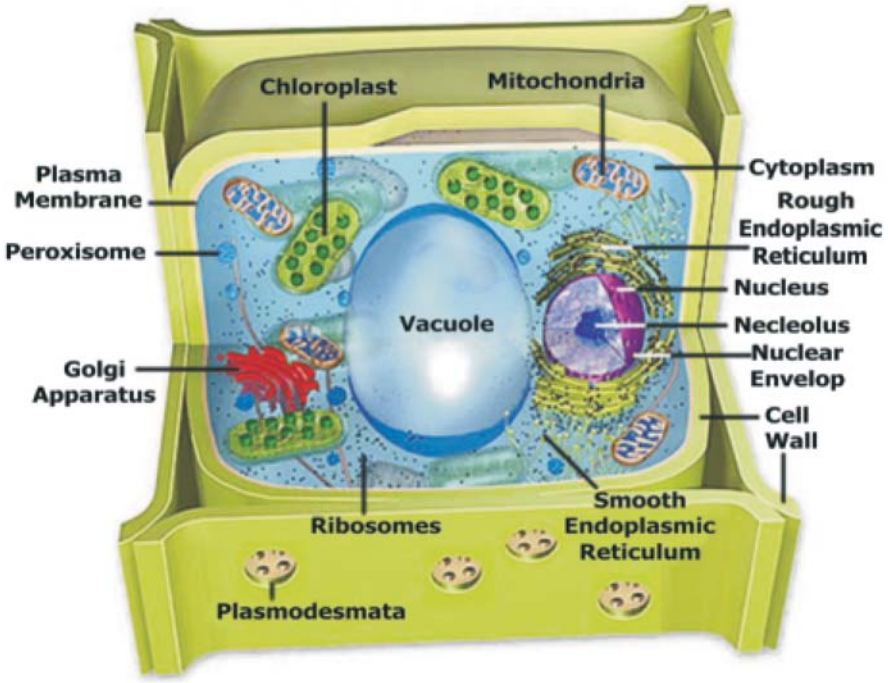


Figure 2.4: Eukaryotic Cell (Plant)

- ***Cell Wall:*** Prokaryotic cells and plant cells both have a rigid cell wall made up of polysaccharides. The cell wall provides and maintains the shape of these cells and serves as a protective barrier.

Cell Membrane: Every cell is enclosed in a membrane. The membrane is a double layer of lipids (lipid bilayer) but is made quite complex by the presence of numerous proteins that are important to cell activity. These proteins include receptors, pores, and enzymes. The membrane is responsible for the controlled entry and exit of ions like sodium (Na) potassium (K), calcium (Ca++).

Vacuole: A vacuole is a membrane-bound sac that plays roles in intracellular digestion and the release of cellular waste products. In animal cells, vacuoles are generally small. Vacuoles tend to be large in plant cells and play a role in turgor pressure. When a plant is well watered, water collects in cell vacuoles producing rigidity in the plant. Without sufficient water, pressure in the vacuole is reduced and the plant wilts.

The Nucleus and Nucleolus: The nucleus is the most obvious organelle in any eukaryotic cell. It is a membrane-bound organelle and is surrounded by a double membrane. It communicates with the surrounding cytosol via numerous nuclear pores (Figure 2.5).

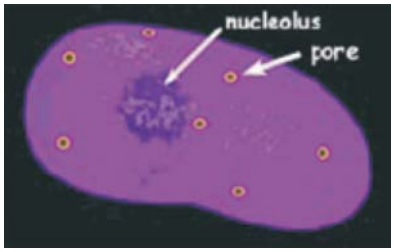


Figure 2.5: Nucleus

Within the nucleus is the DNA, which is responsible for providing the cell with its unique characteristics. The DNA is similar in every cell of the body, but depending upon the specific cell type, some genes may be turned on or off that's why a liver cell is different from a muscle cell, and a muscle cell is different from a fat cell.

Chloroplast: Chloroplasts are specialized organelles found in all higher plant cells (Figure 2.6). These organelles contain the chlorophyll, hence give the green color. They have a double outer membrane. Within the stroma are other membrane structures the thylakoids and grana (singular=granum) where photosynthesis takes place.

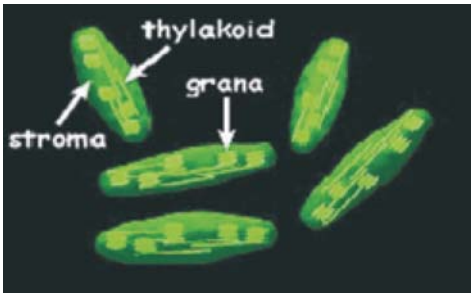


Figure 2.6: Chloroplasts

Mitochondria: Mitochondria provide the energy a cell needs to move, divide, produce secretory products, contract, in short, they are the power centers of the cell. They are about the size of bacteria but may have different shapes depending on the cell type. Mitochondria (singular, mitochondrion) are oblong shaped organelles found in the cytoplasm of all eukaryotic cells (Figure 2.7). They occur in varying numbers, depending on the cell and its function.

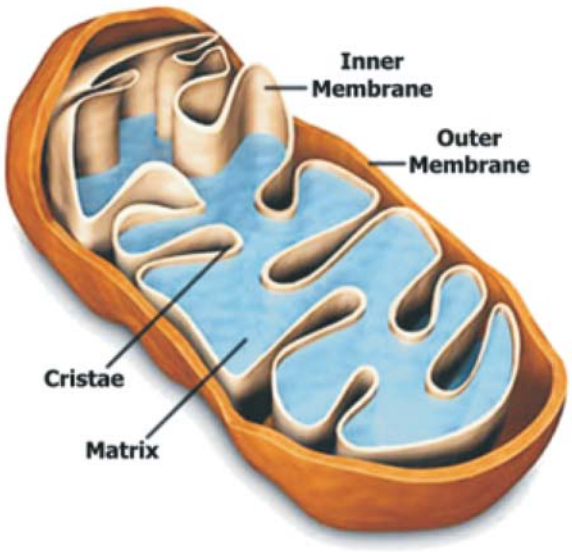


Figure 2.7: Mitochondrion

Golgi Apparatus: The Golgi apparatus is a membrane-bound structure with a single membrane. It is actually a stack of membrane-bound vesicles that are important in

packaging macromolecules for transport elsewhere in the cell. Numerous smaller vesicles containing those packaged macromolecules surround the stack of larger vesicles. The enzymatic or hormonal contents of lysosomes, peroxisomes and secretory vesicles are packaged in membrane-bound vesicles at the periphery of the Golgi apparatus.

Table 2.1: Some of the Structures Found in a Eukaryotic Cell and Their Known Functions.

Structure	Some of proposed functions
Cell Membrane	Regulation of passage of materials into and out of the cell
Cell Wall	Protection and maintenance of cell shape
Nucleus	Contains most of the hereditary material (DNA) of the cell
Nucleolus	Involved in the synthesis of ribosomes which then travel to the cytoplasm
Endoplasmic reticulum	Synthesis and intracellular storage and transport of materials such as carbohydrates, proteins and steroids
Golgi Body (Dictyosome)	Synthesis and secretion (often to the outside of the cell) of materials such as carbohydrates and proteins
Mitochondrion	Derives chemical energy (in form of ATP) from orgainc materials for use by rest of cell (respiration)
Chloroplast	Synthesis of orgainc materials using light as an energy source (photosynthesis)
Microbody	Various functions, depending on type, including fat breakdown and the packaging of "suicidal" hydrolytic enzymes (e.g. lysosomes)
Microtubules	Maintenance of cell shape (where there is no cell wall), formation of "tracks" along which organelles can run, and formation of the spindle during cell division
Centriole	A group of microtubules which provide the base on which a flagellum can develop
Flagellum (Cilium)	Provides motility, primarily for single-celled organisms.
Ribosome	Site of protein synthesis
Vacuole	Storage of substances (plants), site of digestion of particulate food.

Eukaryotic vs. Prokaryotic Cells

Eukaryotic cells have a higher degree of organization than do Prokaryotic cells, in that they contain many organelles or structures separated from the other cytoplasm

components by a membrane, whereas prokaryotic cells contain no organelles. The major eukaryotic organelles are:

- The Nucleus, which contains the DNA and the enzymes for DNA synthesis and the process of transcription. The DNA in eukaryotic cells is packaged in a highly organized way. It consists of a basic unit called a nucleosome (Figure 2.8); a beadlike structure with 146 base pairs of DNA wrapped around eight histone molecules. The nucleosomes are linked to one another by a segment of DNA approximately 60 DNA base pairs long.

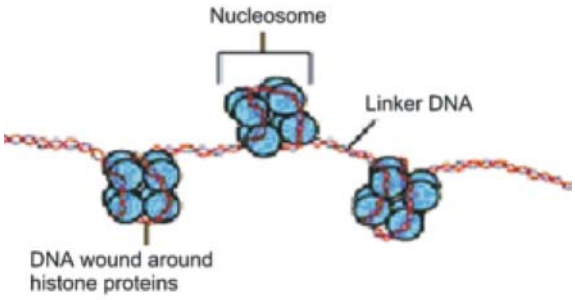


Figure 2.8: Nucleosome

- Chloroplasts, which allow plants to trap sunlight as energy and to carry out photosynthesis. It is photosynthesis, the process of using the trapped sunlight to fix carbon dioxide in the form of sugar that makes human life possible on this planet since plants ultimately provided the food for most Eukaryotes.
- Golgi Apparatus is a structure in eukaryotic cells that secretes substances.
- Mitochondria are the energy producing structures of Eukaryotic cells and supply the cells with ATP. The mitochondria contain some DNA and some protein synthesizing machinery that resembles similar Prokaryotic machinery. The human mitochondria are inherited entirely from the mother (the egg) and thus changes (mutations) in mitochondrial DNA are traced through the mother only. A detailed animal cell model can be studied (Figure 2.9).
- Ribosomes, which are the structures upon which proteins are made. All cells contain these, but they are different in prokaryotes and eukaryotes.

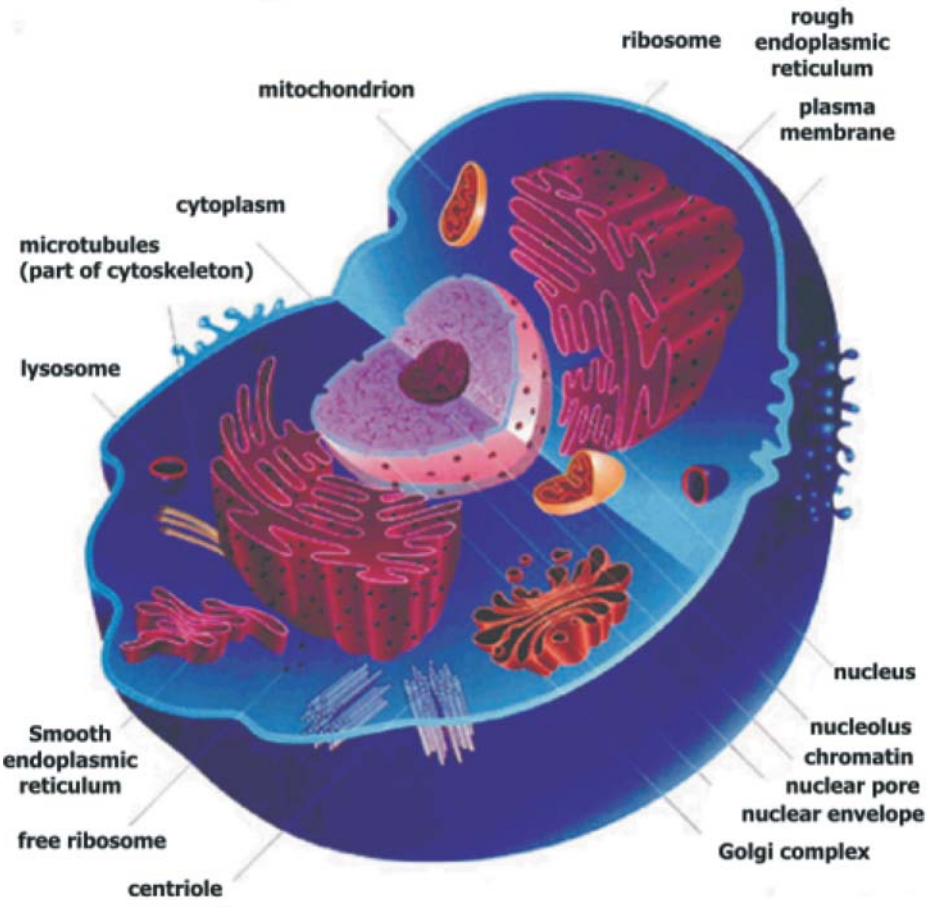


Figure 2.9: Eukaryotic Cell (Animal)

- Photosynthetic cells contain forms of the light-trapping pigment, chlorophyll. Almost all life on earth is dependent upon chlorophyll. There are many photosynthetic bacteria (prokaryotes), but many of them have a mechanism of photosynthesis that is different from that in eukaryotic photosynthetic cells.
- Motility Structures: Not all cells are motile, but both groups have cells that are. Some cells have flagella that are long thin structures that rotate so as to propel the cell; others crawl across solid surfaces and others have stiff fiber-like structures that move like oars. The bacterial flagella are composed of

repeating protein subunits that form a stiff organ. The base of the bacterial flagella acts like a motor and contains a flexible "joint". The motor can change direction when necessary, but it only moves forward when the flagella turns in a counterclockwise direction for most bacteria. The eukaryotic flagella are very complex and are composed of several filaments.

Biomolecules

Life as a word is difficult to define because the living state is not a clear-cut condition. Rather, it is a continuum upon which many objects exist. There are some fundamental properties that constitute the living condition. Certain objects possess all of these properties and are obviously alive. We call these objects organisms. Other items in our universe possess only some of these properties and are considered to a greater or lesser extent non-living. Biomolecules like proteins, nucleic acids, enzymes etc are essential part of life.

Proteins

General Function: Proteins and peptides (small proteins) are essential to the cell. They serve two major functions in the cell. Some proteins are enzymes that catalyze most biological reactions in a living organism. Other proteins perform a structural role for the cell - either in the cell wall, the cell membrane or in the cytoplasm. In this section, we will look at the basic structure that all proteins have in common.

Primary Structure of Proteins: Proteins are polymers of amino acids. Amino acids are primary amines that contain an alpha carbon that is connected to an amino group (NH2), a carboxyl group (COOH), and a variable side group (R) (Figure 2.10). The side group gives each amino acid its distinctive properties and helps to dictate the folding of the protein.

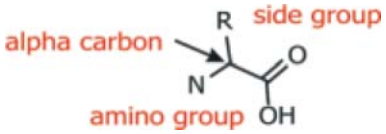


Figure 2.10: A General Amino Acid

Linking an amino group to a caroboxyl group on another amino acid creates polymers of amino acids. This is termed a peptide bond (Figure 2.11).

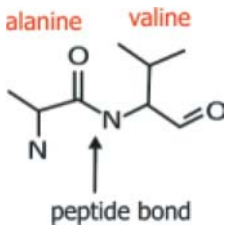


Figure 2.11: A Peptide Bond

There are 20 common amino acids found in proteins and these amino acids can be classified into 3 groups; polar, non-polar and charged. Polar and charged amino acids will most often be found on the surface of a protein, interacting with the surrounding water, while the non-polar (or hydrophobic) amino acids will bury themselves in the interior. The number and position of these types of amino acids in protein can greatly influence its function. Peptides and proteins are formed when a ribosome and the rest of the translation machinery link 10-10,000 amino acids together in a long polymer. This long chain is termed the primary sequence. The properties of the protein are determined, for the most part, by this primary sequence. In many cases an alteration of any amino acid in the sequence will result in a loss of function for the protein (a mutation). Genetic diseases in humans are often caused by changes in important proteins that cause illness. Sickle cell anemia is caused by a single amino acid change from glutamic acid to valine at position 6 of the hemoglobin (the oxygen carrying protein found in humans and other mammals). Below is the primary sequence of hemoglobin (Figure 2.12).

NH_2—Val His Leu Thr Pro Glu Glu Lys Ser Ala Val Thr Ala

Leu Trp Gly Lys Val Asn Val Asp Glu Val Gly Gly Glu--

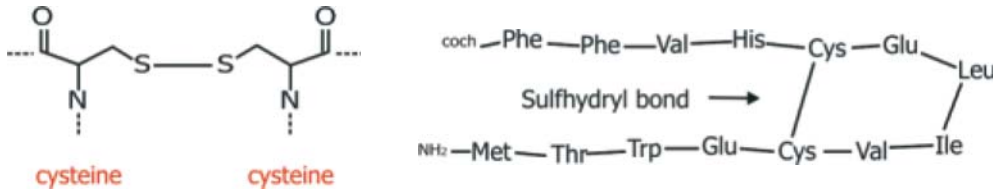
Figure 2.12: Hemoglobin amino acid sequence: Only the first 26 amino acids are shown.

Secondary Structure

- Basic attractive forces: During and after synthesis, the primary sequence will associate in a fashion that leads to the most stable, comfortable structure for

the protein. How a protein folds is largely dictated by the primary sequence of amino acids? Each amino acid in the sequence will associate with other amino acids to conserve the most energy. Hydrogen bonds, hydrophobic interactions, ionic interactions, and sulfhydryl linkages stabilize this structure.

- *Sulfhydryl linkages:* These are covalent bonds between cysteine groups. Cysteine is a unique amino acid in that, it has a sulfur group available for binding to other groups. Often in proteins, adjacent sulfhydryl groups on cysteines will form a covalent link in a protein (Figure 2.13) and are often crucial for the mature protein to perform its function.



The chemical structure of a sulhydryl bond A sulfhydryl bond in a peptide

Figure 2.13: Two Views of Sulfhydryl Linkages

Common Secondary Structures: Proteins often have stretches of amino acids that associate into two common structures. These are the alpha helix and the beta (pleated) sheet. Formation of these structures is driven by favorable hydrogen bonding and hydrophobic interactions between nearby amino acids in the protein.

The alpha helix resembles a ribbon of amino acids wrapped around a tube to form a staircase like structure. This structure is very stable, yet flexible and is often seen in parts of a protein that may need to bend or move. In the beta sheet, two planes of amino acids will form, lining up in such a fashion so that hydrogen bonds can form between facing amino acids in each sheet. The beta pleated sheet or beta sheet is different than the alpha helix in that far distant amino acids in the protein can come together to form this structure. Also, the structure tends to be rigid and less flexible. Following is the diagram of enzyme lysozyme showing alpha helix and beta-pleated sheet (Figure 2.14).

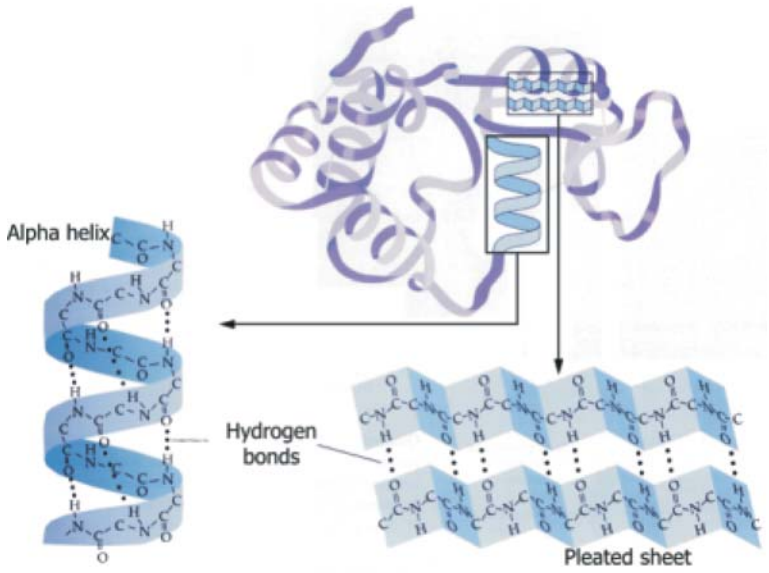
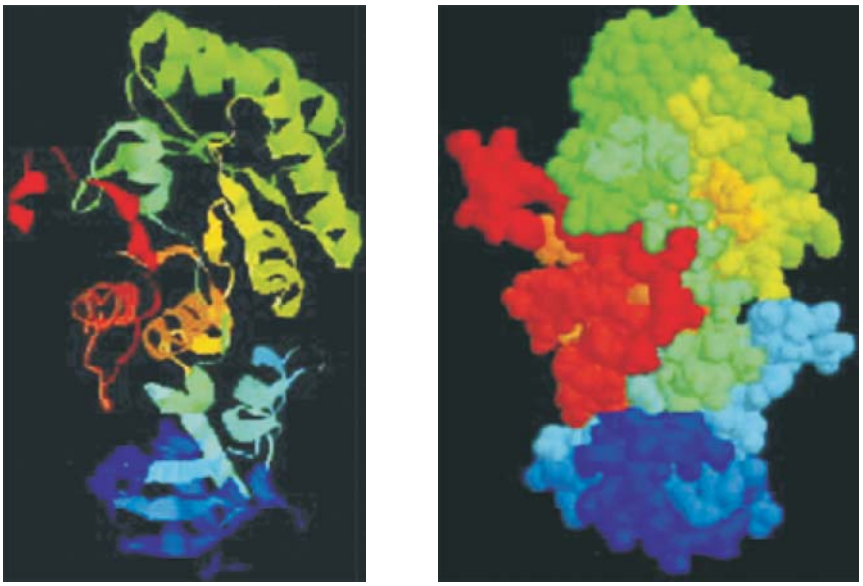


Figure 2.14: The secondary structure of protein. Two types of secondary structure, alpha helix and pleated sheet can both be found in the protein Lysozyme. Both patterns depend on hydrogen bonding along the polypeptide chain. The R groups of amino acid are omitted in these diagrams.

Tertiary Structure: During and after synthesis, a protein folds into alpha helices and beta sheets. These areas of secondary structure are connected by bridging sequences that will cause the protein to fold in specific ways. At the completion of this process, the protein takes on its final shape. The mature stable structure of a single peptide sequence is termed its tertiary structure. Hydrogen bonds, hydrophobic interactions, ionic interactions, and disulphide linkages stabilize this structure.

Following is the tertiary structure of ribulose bisphosphate carboxylase/oxygenase (RUBISCO), one of the most important enzymes on this planet (Figure 2.15). Life would not exist without it. This enzyme takes energy, obtained most often from the sun, and uses it to convert carbon dioxide into carbohydrate. It is found in many photosynthetic organisms and is probably the most abundant protein on the earth.



Ribbon Space filling

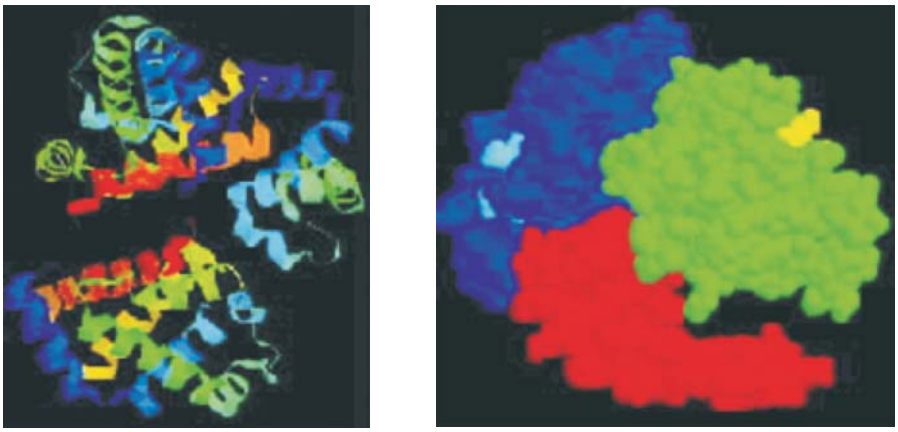
Figure 2.15: RUBISCO

Quaternary Structure: Many enzymes and structures are actually complexes of several polypeptides. The arrangement of these polypeptides is termed the quaternary structure of a protein. Proteins complexes can contain several copies of an identical protein or they may consist of any number of polypeptides in various ratios.

Hemoglobin (the oxygen carrier in blood) is an example of a protein containing identical subunits (Figure 2.16). Notice the haeme groups associated with each polypeptide (the light blue group that is surrounded by the dark blue protein). These haeme groups contain iron and the iron is the atom that actually binds the oxygen. Haeme iron has an even greater affinity for carbon monoxide. If all your blood binds carbon monoxide, you literally suffocate, and this is why it is such a deadly poison.

Function of Proteins: One of the proteins called ribosomes are the protein synthesizing factories of the cell. They translate the information present in mRNA into protein sequences. Ribosomes also give the cytoplasm its granular look in the electron micrograph (EM). Often they aggregate to form structures known as "polysome" in the mRNA. Ribosomes sit down on mRNA at two sites. The A site,

where the new amino acid is accepted and the P site, where the growing polypeptide is held. The prokaryotes and eukaryotes have different size ribosomes and have different proteins. Eukaryotic ribosomes may be free in the cytoplasm or bound to an internal membrane structure known as the endoplasmic reticulum.



Ribbon Space filling

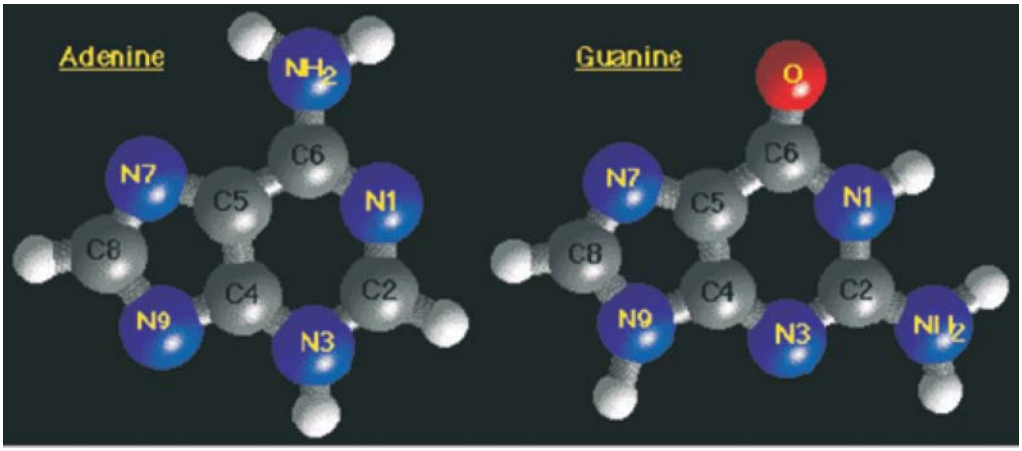
Figure 2.16: Hemoglobin: Only 3 of the Four Subunits are Visible.

Nucleic Acids

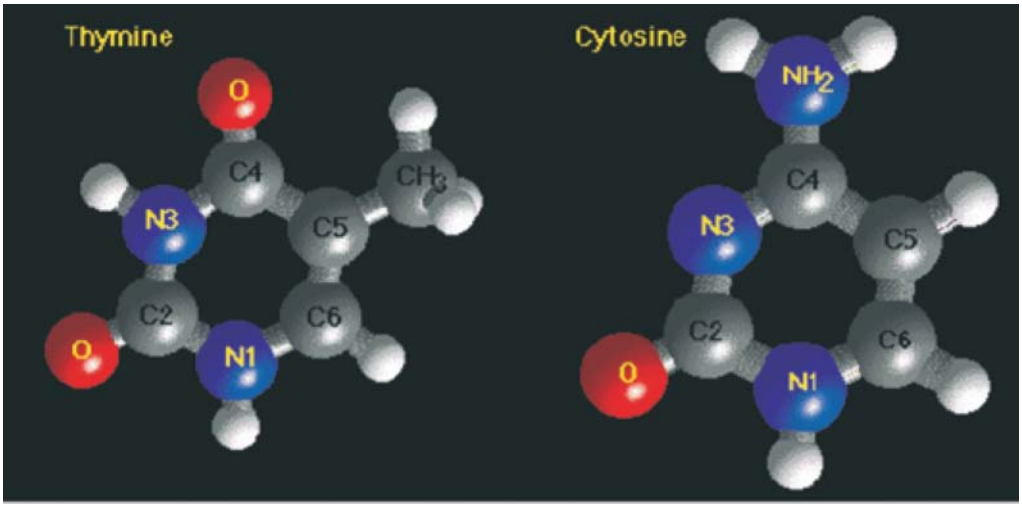
Nucleic acids, Ribonucleic acid (RNA) and Deoxyribonucleic acid (DNA), serve as storage units for the hereditary information. Deoxyribonucleic acid (DNA) can be thought of as a large cookbook with recipes for making every protein in the cell. Ribonucleic acid (RNA) helps the ribosome translate the information in DNA into protein.

Chemistry

The building blocks: Despite their importance in cellular function, nucleic acid structure is surprisingly simple. RNA and DNA are long polymers of only 4 nucleotides, adenine, guanine, cytosine and thymine (or uracil for RNA) (Figure 2.17 a & b).



a. Purines



b. Pyrimidines

Figure 2.17: The Structure of Nucleotides

The nucleotide structure can be broken down into 2 parts. The sugar-phosphate backbone and the base. All nucleotides share the sugar-phosphate backbone. Linking the monomer units using oxygen on the phosphate, and a hydroxyl group on the sugar forms nucleotide polymers (Figure 2.18).

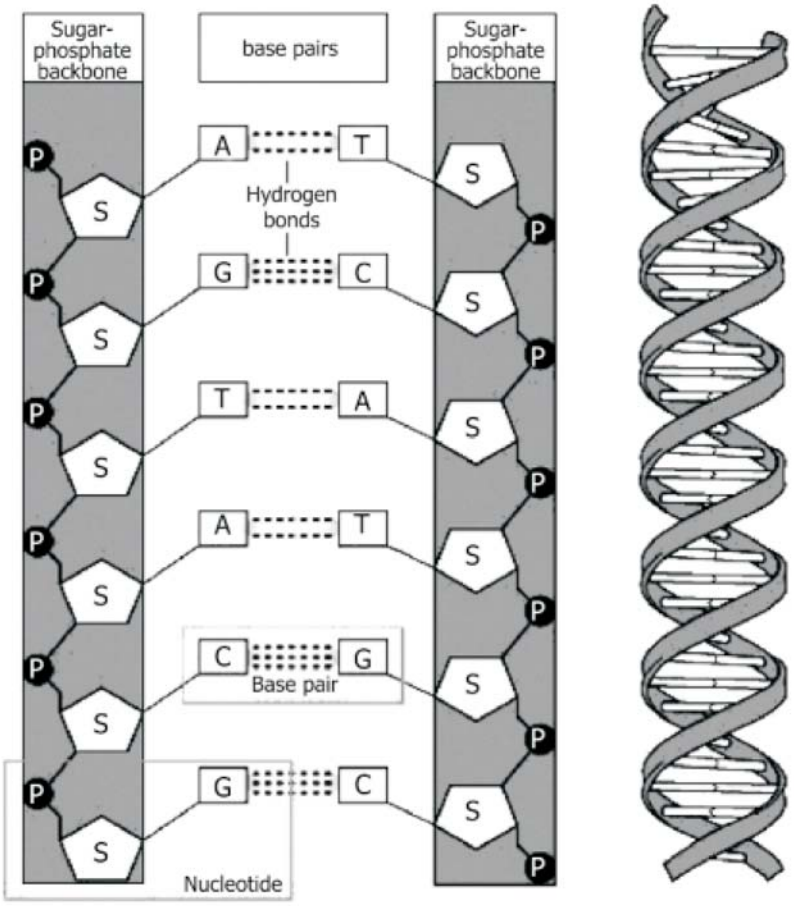
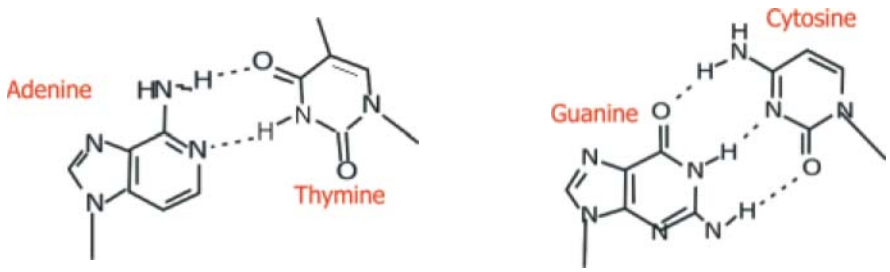


Figure 2.18: A Schematic Representation of the Bonding of one DNA Strand to Another

Adenine, Thymine, Guanine and Cytosine are capable of being linked together to form a long chain. The 3'-hydroxyl group on the ribose unit reacts with the 5'-phosphate group on its neighbor to form a chain. The base on each nucleotide is different, but they still show similarities. Adenine (A) and guanine (G) are purines; notice the two ring structure, with the differences in the molecules coming in the groups attached to the ring. Likewise, cytosine (C) and thymine (T) and uracil (U) are pyrimidines and share a similar structure, but differ in their side groups.

Base Pairing: If two strands of nucleic acid are adjacent to one another, the bases along the polymer can interact with complementary bases in the other strand. As

shown in Figure 2.19, adenine is capable of forming hydrogen bonds with thymine and cytosine can base pair with guanine. Adenine forms two hydrogen bonds with thymine, cytosine forms 3 with guanine. The G to C pair is 33% stronger than the A to T pair due to the extra hydrogen bond.



A and T (or U) base pair G and C base pair

Figure 2.19: Base Pairing Between Nucleic Acids

All the life is based on a living unit called DNA (deoxyribonucleic acid). It is considered that DNA is genetic material and life is based upon functioning and form of this material in an organism. Therefore, it is necessary to study DNA in detail as we step towards biotechnology basics.

Structure of DNA

The structure of DNA is illustrated by a right-handed double helix, with about 10 nucleotide pairs per helical turn (Figure 2.20). Each spiral strand, composed of a sugar phosphate backbone and attached bases, is connected to a complementary strand by non- covalent bonding called hydrogen bonding between paired bases, adenine (A) with thymine (T) and guanine (G) with cytosine (C). Adenine and thymine are connected by two hydrogen bonds (non-covalent) while guanine and cytosine are connected by three. James Watson and Francis Crick first described this structure in 1953 (Figure 2.21).

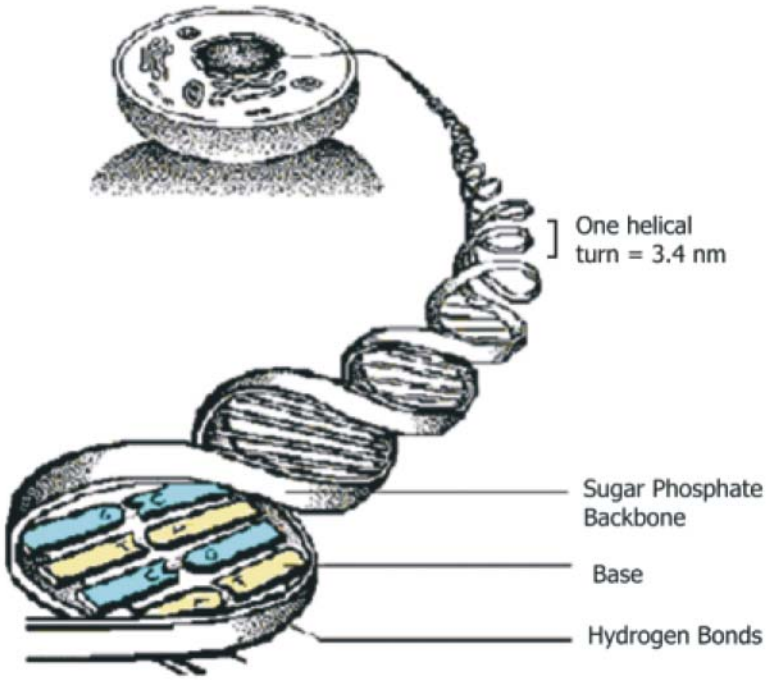


Figure 2.20: Illustration of the Double Helical Structure of the DNA Molecule

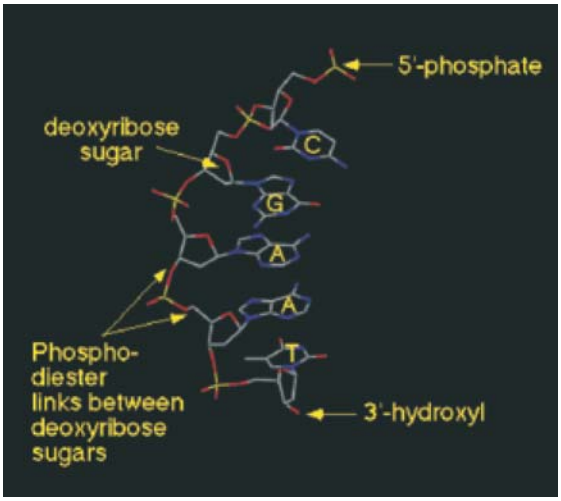


Figure 2.21: The Single Strand of the DNA is Shown Along with Details of how the Bases, Sugars and Phosphates Connect to Form the Structure of the Molecule

To explain the phenomenon of heredity, biological information must be accurately copied and transmitted from each cell to all of its progeny. Three ways for DNA molecules to replicate may be considered, each obeying the rules of complementary base pairing (Figure 2.22).

- **Conservative replication** would leave intact the original DNA molecule and generate a completely new molecule.
- **Dispersive replication** would produce two DNA molecules with sections of both old and new DNA interspersed along each strand.
- **Semi conservative replication** would produce molecules with both old and new DNA, but each molecule would be composed of one old strand and one new one. The replication is semi conservative. Each strand acts as a template for the synthesis of a new DNA molecule by the sequential addition of complementary base pairs, thereby generating a new DNA strand that is the complementary sequence to the parental DNA. Each daughter DNA molecule ends up with one of the original strands and one newly synthesized strand.

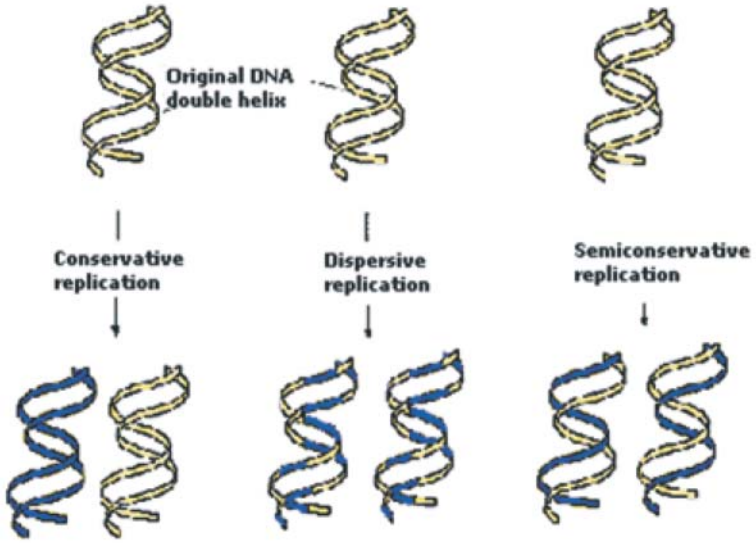


Figure 2.22: Three possible models in which DNA can replicate are illustrated. The two original strands of DNA are shown in yellow (light) newly synthesized DNA is blue (dark).

Structure of RNA

There are three different RNA species. All of them are single stranded nucleic acids.

Ribosomal RNA (rRNA): Ribosomal RNA is part of the ribosome structure and assists in the catalytic role of the ribosome. During rapid growth, the cell has to synthesize large amounts of rRNA and there are several copies of the rRNA genes on the chromosome. These are essential components of an important part of the protein synthesis machinery: the ribosomes. In addition to rRNA, there are some 70 different proteins in a ribosome. There are hundreds of copies of rRNA genes per genome, thus making the production of lots of rRNA possible. There are four different rRNAs, each with a different size. Each ribosome contains one molecule of each of the four rRNA types. In prokaryotes, ribosomes bind to the mRNA close to the translation start site. This ribosome-binding site is referred to as the Shine-Dalgarno sequence or as the ribosome recognition element. In eukaryotes, ribosomes bind at the 5' end of the mRNA and scan down the mRNA until they encounter a suitable start codon.

Transfer RNA (tRNA) carries amino acids to the ribosomes, to enable the ribosomes to put this amino acid on the protein that is being synthesized as an elongating chain of amino acid residues, using the information on the mRNA to "know" which amino acid should be put on next. For each kind of amino acid, there is a specific tRNA that will recognize the amino acid and transport it to the protein that is being synthesized, and tag it on to the protein once the information on the mRNA calls for it.

All tRNA's have the same general shape resembling a clover leaf. Parts of the molecule fold back in characteristic loops, which are held in shape by nucleotide pairing between different areas of the molecule (Figure 2.23). There are two parts of the tRNA that are of particular importance: the aminoacyl attachment site and the anticodon. The aminoacyl attachment site is the site at which the amino acid is attached to the tRNA molecule. Each type of tRNA specifically binds only one type of amino acid. The anticodon (three bases) of the tRNA base pairs with the appropriate mRNA codon at the mRNA-ribosome complex. This temporarily binds the tRNA to the mRNA, allowing the amino acid carried by the tRNA to be incorporated into the polypeptide in its proper place. Thus, the sequence of the codon (three bases) in the mRNA dictates the amino acid to be put in the protein at a specific site. The "dictionary" of codons coding for amino acids is called the genetic code.

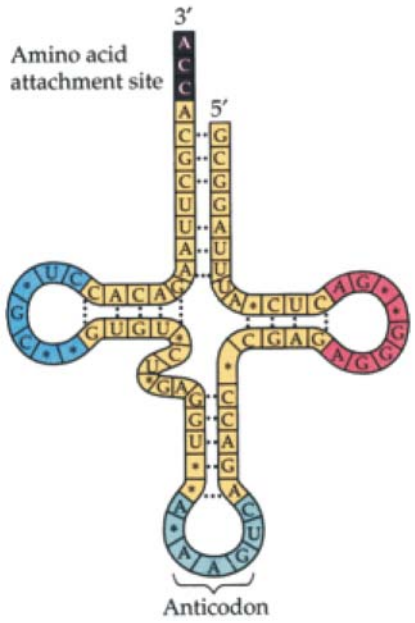


Figure 2.23: Model of Transfer RNA

Messenger RNA (mRNA): Messenger RNA directs the incorporation of amino acids into proteins. It can be thought of as a "photocopy" of DNA that the ribosome works from (Figure 2.24). In eukaryotes, generally the transcripts need to be processed before they can serve as a blueprint for a protein while in prokaryotes, transcripts don't need to be processed.

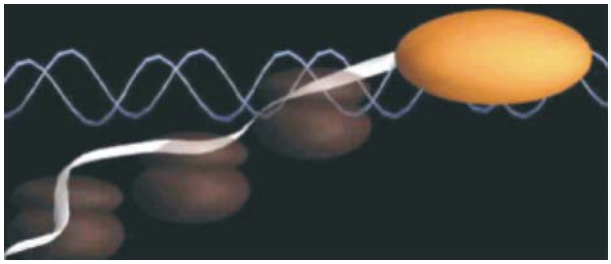


Figure 2.24: mRNA at Work in the Cell

Central Dogma of Molecular Biology

Transcription of DNA to RNA to Protein: This dogma forms the backbone of molecular biology and is represented by four major stages (Figure 2.25).

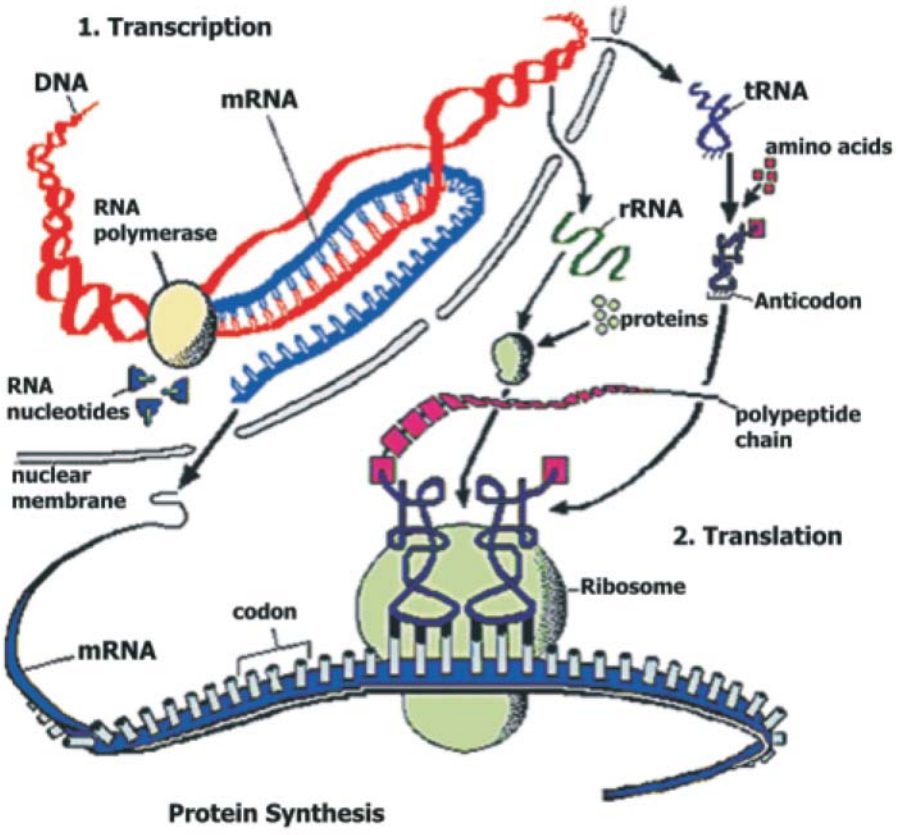


Figure 2.25: Transcription of DNA to RNA to Protein

1. ***Replication:*** The DNA replicates its information in a process that involves many enzymes. When DNA replicates, many different proteins work together to accomplish the following steps:

- The two parent strands unwound with the help of DNA helicases.

- Single stranded DNA binding proteins attach to the unwound strands, preventing them from winding back together (Figure 2.26). The strands are held in position that binding of DNA polymerase becomes easy, which catalyzes the elongation of the leading and lagging strands. DNA polymerase also checks the accuracy of its own work.

- While the DNA polymerase on the leading strand can operate in a continuous fashion, RNA primer is needed repeatedly on the lagging strand to facilitate synthesis of Okazaki fragments. DNA primase, which is one of several polypeptides bound together in a group called primosomes, helps to build the primer.

- Finally, each new Okazaki fragment is attached to the completed portion of the lagging strand in a reaction catalyzed by DNA ligase.

2. ***Transcription:*** The DNA codes for the production of messenger RNA (mRNA).

3. In eukaryotic cells, the mRNA is processed (essentially by splicing) and migrates from the nucleus to the cytoplasm.

4. Messenger RNA carries coded information to ribosomes. The ribosomes "read" this information and use it for protein synthesis. This process is called translation. Proteins do not code for the production of protein, RNA or DNA. They are involved in almost all biological activities, structural or enzymatic.

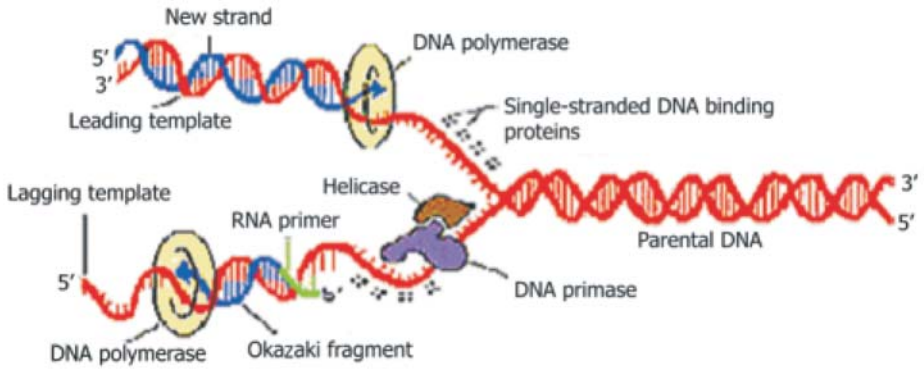


Figure 2.26: The Major Types of Proteins, Which Must Work Together During the Replication of DNA Showing their Positions

DNA Replication

It may be worth recapitulating that DNA comes as double stranded right handed helix with three isoforms, two with right handed turns (A and B conformation) with a major and minor groove and one left handed one (Z-DNA) with only one groove (Figure 2.27).

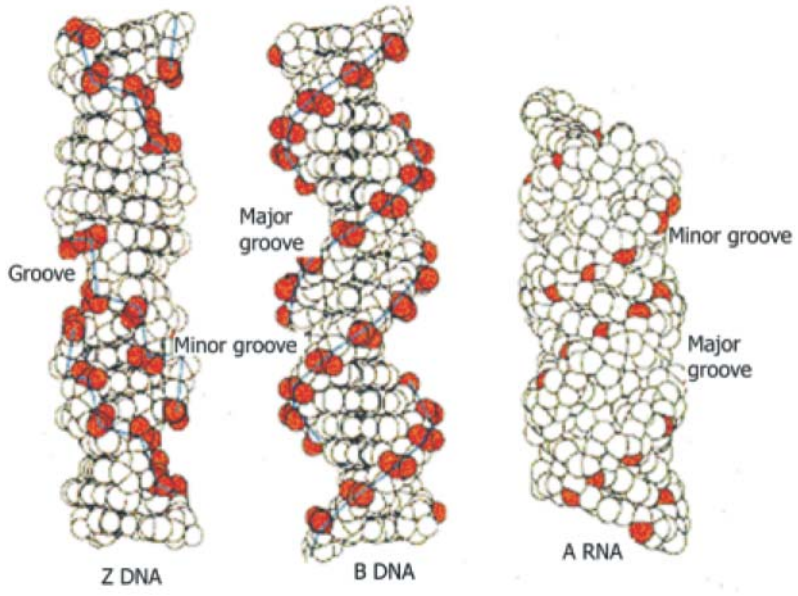


Figure 2.27: Topology of DNA and Chromosomal Structure

In order to accommodate the immense length of eukaryotic DNA inside the minute size of the cell, the DNA strand is wound up into so called superstructures. The first level of such a superstructure in eukaryotes is the nucleosome in which the DNA is wound in two full turns as left-handed super helix around a protein disc consisting of histone proteins forming the so called histone octamer. These nucleosomes are separated by short linker DNA regions stabilized by histone H1 giving the structure some resemblance to "beads on a string". The next higher structural order is the 30 nm chromatin structure, which is clearly visible under the electron microscope. This structure arises by a further super coiling of the string of nucleosomes around a fixed axis with about 6-7 nucleosomes per turn. The next more complex structural feature is called the 100 nm chromosomal domain in which 20-100 kbp of DNA are held

together by so called matrix proteins; this structure is visible under the light microscope in lampbrush chromosomes of Chironomus tentans oocytes for example.

Further (but transient) condensation of chromosomes occurs during cell division in late G2 phase and during mitosis (metaphase chromosomes) in which the DNA assumes a clear cytological structure which can be visualized for example in karyotyping.

a) ***Initiation:*** Begins at a specific site in the chromosome called "origin of replication" or ORI. DnaA initiator subunits bind to repeated sequences at ORI. DNA replication in bacteria is performed by a DNA-dependent DNA polymerase. As in eukaryotes, DNA replication is semi-conservative. Bacteria have three DNA polymerases (I, II, III). DNA polymerase I has three enzymatic functions: polymerizing activity, 3'-5' exonuclease activity (proofreading function) and a 5'-3' exonuclease activity (removal of RNA primer). Replication proceeds bidirectionally (Figure 2.28).

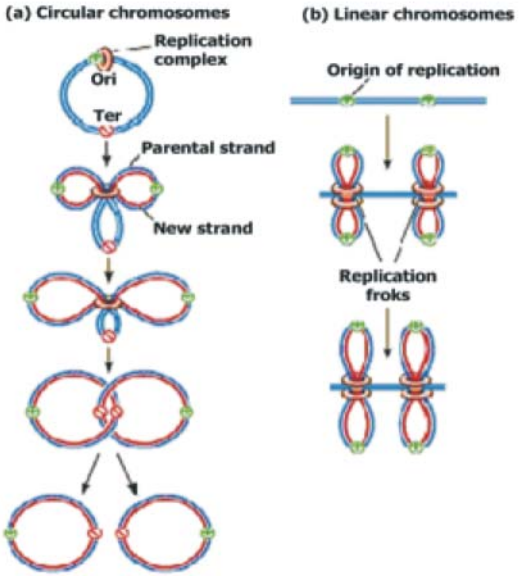


Figure 2.28: Replication of the Bacterial Circular Chromosome in Comparison with Linear One

b) ***Elongation:*** Proceeds at a constant rate irrespective of growth conditions. Once replication begins, it continues until the end and does not require additional protein synthesis.

c) ***Termination:*** The two-replication forks meet at the terminus (terC). The terC region is bound by a termination protein (tus) and is flanked by DNA sequences of opposite polarity that block progression of the replication forks.

Chromosome Partitioning: Concatenated sister chromosomes are decatenated by DNA gyrase and DNA topoisomerase. Chromosome partitioning is probably an active process involving unknown factors that aid in the movement of the nucleoid to daughter cells.

Cytokinesis: Nucleoid partitioning and Cytokinesis are coupled. The cell envelope (cell wall and cytoplasmic membrane) invaginates and eventually separates two cells by a directional shift in the growth of the peptidoglycan layer and occurs at a predetermined position by the ingrowth of a septum.

Proof that DNA is Genetic Material

Griffith: Griffith added dead, virulent pneumonia bacteria to a mouse, it lived; but if he added dead virulent bacteria to live non-virulent bacteria some mice died. He termed the material that changed the non-virulent bacteria to virulent the transforming principle.

Hershey and Chase The "Blender Experiment": Hershey and Chase knew that the DNA - has P but no S, Protein has S but not P. They also knew that something from T2 phage entered E. coli cells and directed the bacteria to produce more phage. They assumed that the genetic material was the material that entered the cell. So they set out to determine the chemical nature of the material. In separate experiments they infected E. coli cells with 35S or 32P labeled phage. By analyzing the products they determined that 32P entered the cell. Thus DNA must be the genetic material (Figure 2.29).

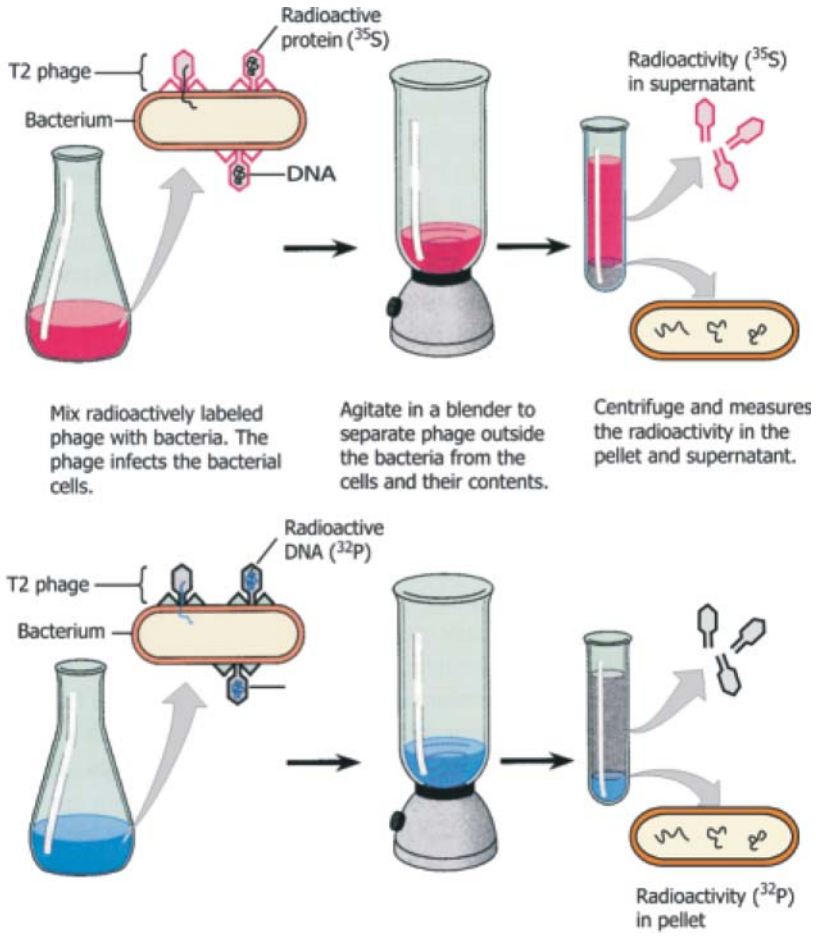


Figure 2.29: In Hershey Chase experiment they demonstrated that it was DNA, not protein, that functioned as the phages genetic material. Viral proteins, labeled with radioactive sulfur, remained outside the host cell during infection. In contrast, viral DNA, labeled with radioactive phosphorus, entered the bacterial cell.

Avery, MacLeod and McCarty: Avery, Macleod and McCarty used biochemical purification of cellular fractions to determine that DNA and not RNA or protein was the transforming principle. Transformation is the alteration of phenotype by the addition of foreign DNA.

Genetic Code

The Genetic Code and Language:

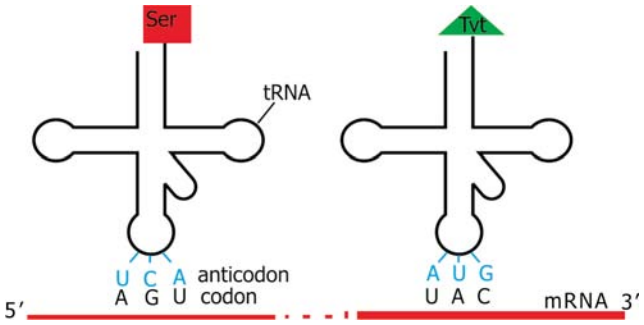
Introduction: Genetic information is like a language. We use letters of the alphabet to make words and then join these words together to make sentences, paragraphs and books.

In the Case of DNA:

- Alphabet is only 4 letters long
- Each letter represents a chemical compound called a base or nucleotide
- These 4 letters are used to form the genetic words called codons
- Unlike a normal language all genetic words are only three letters long
- These words combine together to form sentences called genes
- At the end of each sentence is a special word or full stop called a stop codon
- All the sentences join together to form a book that contains all the genetic information called genome.

DNA transfers information to mRNA in the form of a code defined by a sequence of nucleotides bases. During protein synthesis, ribosomes move along the mRNA molecule and "read" its sequence three nucleotides at a time (codon) from the 5' end to the 3' end. Each amino acid is specified by the mRNA's codon, and then pairs with a sequence of three complementary nucleotides carried by a particular tRNA (anticodon).

Since RNA is constructed from four types of nucleotides, there are 64 possible triplet sequences or codons (444). Three of these possible codons specify the termination of the polypeptide chain. They are called "stop codons". That leaves 61 codons to specify only 20 different amino acids. Therefore, most of the amino acids are represented by more than one codon. The genetic code is said to be degenerate (Figure 2.30).



2nd base in codon

1st base in codon	U	C	A	G	3rd base in codon
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	Stop	Stop	A
	Leu	Ser	Stop	Trp	G
C	Leu	Pro	His	Aug	U
	Leu	Pro	His	Aug	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Ser	Ser	U
	Ile	Thr	Ser	Ser	C
	Ile	Thr	Arg	Aug	A
	Met	Thr	Arg	Aug	G
G	Val	Ala	Gly	Gly	U
	Val	Ala	Gly	Gly	C
	Val	Ala	Gly	Gly	A
	Val	Ala	Gly	Gly	G

Figure 2.30: Amino Acids Specified by Each Codon Sequence on mRNA

Ala: Alanine	Cys: Cysteine	Asp: Aspartic acid	Glu: Glutamic acid
Phe: Phenylalanine	Gly: Glycine	His: Histidine	Ile: Isoleucine
Lys: Lysine	Leu: Leucine	Met: Methionine	Asn: Asparagine
Pro: Proline	Gln: Glutamine	Arg: Arginine	Ser: Serine
Thr: Threonine	Val: Valine	Trp: Tryptophane	Tyr: Tyrosisne

A = adenine G = guanine C = cytosine T = thymine U = uracil

An Explanation of the Genetic Code: DNA is a two-stranded molecule. Each strand is a polynucleotide composed of A (adenosine), T (thymidine), C (cytidine), and G (guanosine) residues polymerized by "dehydration" synthesis in linear chains with

specific sequences. Each strand has polarity, such that the 5'-hydroxyl (or 5'-phospho) group of the first nucleotide begins the strand and the 3'-hydroxyl group of the final nucleotide ends the strand; accordingly, we say that this strand runs 5' to 3' ("Five prime to three prime"). It is also essential to know that the two strands of DNA run antiparallel such that one strand runs 5' to 3' while the other one runs 3' to 5'. At each nucleotide residue along the double-stranded DNA molecule, the nucleotides are complementary. That is, A forms two hydrogen bonds with T; C forms three hydrogen bonds with G. In most cases the two-stranded, antiparallel, complementary DNA molecule folds to form a helical structure, which resembles a spiral staircase. This is the reason why DNA has been referred to as the "Double Helix".

One strand of DNA holds the information that codes for various genes; this strand is often called the template strand or antisense strand (containing anticodons). The other, and complementary, strand is called the coding strand or sense strand (containing codons). Since mRNA is made from the template strand, it has the same information as the coding strand. The table in figure 2.32 refers to triplet nucleotide codons along the sequence of the coding or sense strand of DNA as it runs 5' -> 3'; the code for the mRNA would be identical but for the fact that RNA contains U (uridine) rather than T.

An example of two complementary strands of DNA would be:

(5'->3')	ATGGAATTCTCGCTC	(Coding, sense strand)
(3'<- 5')	TACCTTAAGAGCGAG	(Template, antisense strand)
(5'->3')	AUGGAAUUCUCGCUC	(mRNA made from Template strand)

Since amino acid residues of proteins are specified as triplet codons, the protein sequence made from the above example would be Met-Glu-Phe-Ser-Leu... (MEFSL...).

Practically, codons are "decoded" by transfer RNAs (tRNA), which interact with a ribosome-bound messenger RNA (mRNA) containing the coding sequence. There are 64 different tRNAs, each of which has an anticodon loop (used to recognize codons in the mRNA). 61 of these have a bound amino acyl residue; the appropriate "charged" tRNA binds to the respective next codon in the mRNA and the ribosome catalyzes the transfer of the amino acid from the tRNA to the growing (nascent) protein/polypeptide chain. The remaining 3 codons are used for "punctuation"; that is, they signal the termination (the end) of the growing polypeptide chain.

Lastly, the Genetic Code in the given table has also been called "The Universal Genetic Code". It is known as "universal", because all known organisms use it as a code for DNA, mRNA, and tRNA. The universality of the genetic code encompases animals (including humans), plants, fungi, archaea, bacteria, and viruses. However, all rules have their exceptions, and such is the case with the genetic code; small variations in the code exist in mitochondria and certain microbes.

Transcription and Translation

Because the nucleoid does not have a membrane surrounding it, as soon as mRNA is transcribed from the DNA, ribosomes attach and begin translating the mRNA into protein. Thus, transcription and translation are "coupled" in prokaryotes.

Transcription

1. Binding of the promoter by the RNA polymerase holoenzyme. The DNA helix is unwound for approximately 10 bp of DNA.

2. Formation of the first phosphodiester bond resulting in a RNA dinucleotide that is used as a primer for transcription elongation (almost always begins with a A or G). The sigma factor dissociates from the complex as transcription proceeds in a 5' to 3' direction using a 3' to 5' DNA template (Figure 2.31).

3. Termination of transcription commonly occurs at specific palindromic sequences capable of forming hairpin structures. Type I terminators form GC rich stem-loop structures followed by a T-rich sequence (Figure 2.32a). Type II terminators require an additional protein factor called Rho, which causes RNA polymerase to terminate transcription when it pauses at specific sequences (Figure 2.32b).

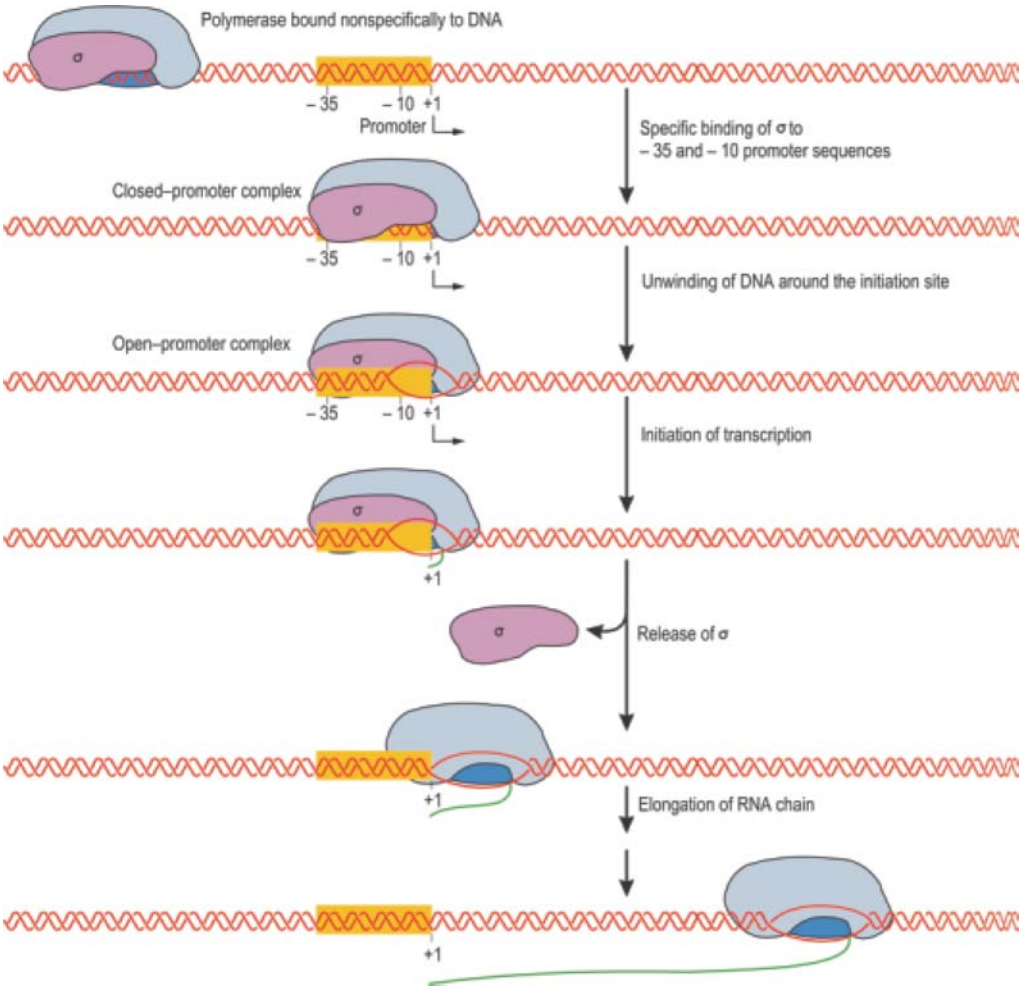
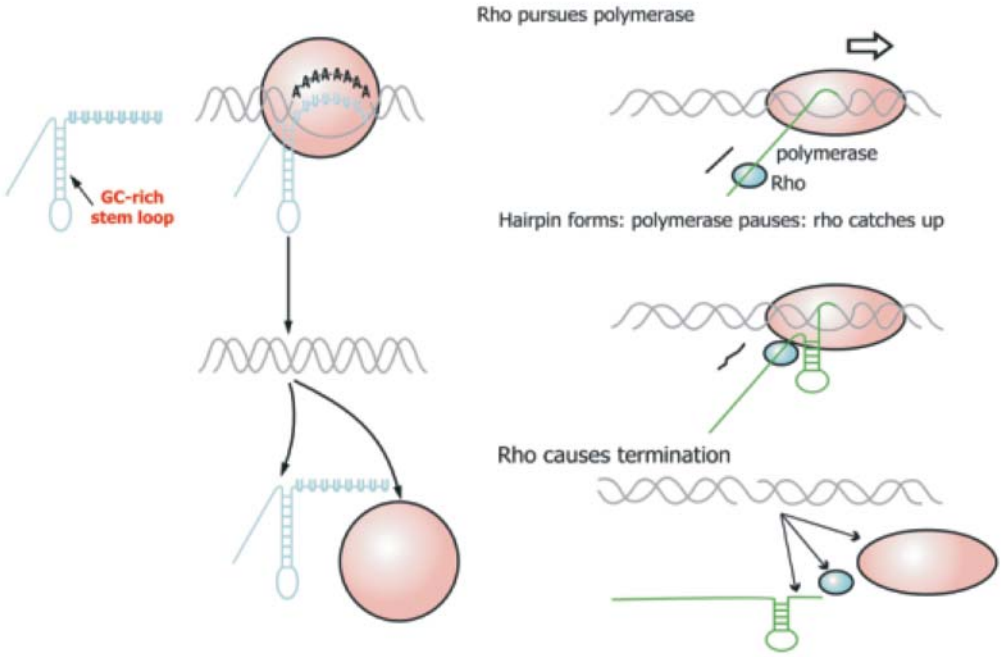


Figure 2.31: Transcription by E. coli RNA Polymerase



(a) Rho independent termination **(b)** Rho dependent termination

Figure 2.32: Termination

Archaea

a. Like bacteria, Archaea have a single RNA polymerase, but it is more closely related to eukaryotic RNA polymerase II than bacterial RNA polymerase. The RNA polymerase of Archaea is composed of 8-10 subunits.

b. Binding of RNA polymerase occurs at a sequence similar to the TATA box of eukaryotes, but which also has some similarities to the Pribnow box (Figure 2.33).

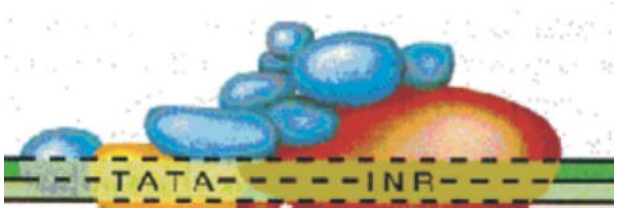


Figure 2.33: Archaea RNA Polymerase

c. Transcription termination is not well understood, but occurs in some cases after inverted repeats followed by AT rich regions, similar to termination signals in Bacteria.

General Mechanisms of Transcriptional Control in Prokaryotes

Bacteria:

a. RNA polymerase in bacteria (such as E. coli) is composed of a core RNA polymerase composed of 4 polypeptides (alpha2, beta, beta') and accessory factors called sigma subunits. The sigma subunits enable RNA polymerase to recognize and bind to promoter regions.

b. Bacteria have a number of sigma factors that allow the organism to differentially regulate genes. Sigma factors are DNA binding-specific and so provide specificity to RNA polymerase that allow it to bind to specific promoter sequences. The major vegetative sigma factor in E. coli is sigma 70. Different sigma factors allow the cells to deal with stress (heat shock) and developmental decisions including formation of flagella and sporulation by differentially regulating different sets of genes.

c. At positions -35 and -10 are specific sequences (Pribnow box) that allow regulation by sigma factors/RNA holoenzyme in E. coli (Figure 2.34).

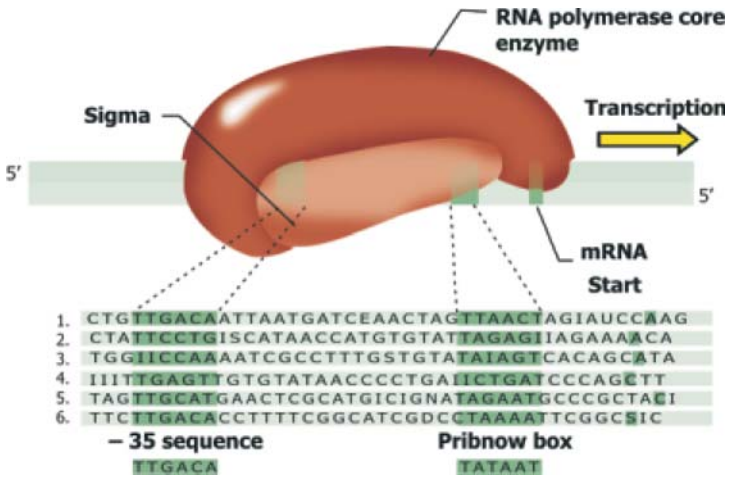


Figure 2.34: Transcriptional Control

General Mechanisms of Translation in Prokaryotes

a. Transcription and translation in both bacteria and Archaea are coupled. During transcription, the mRNA is bound by multiple ribosomes (polysomes) and is translated successively.

b. The ribosomes of prokaryotes are smaller than eukaryotic ribosomes and have a sedimentation coefficient of 70S. Ribosomes are composed of two major subunits (50S and 30S). In E. coli, the 30S subunit is composed of the 16S rRNA and 20 different proteins. The 50S subunit is composed of the 5S rRNA, the 23S rRNA and approximately 30 proteins. The 30S ribosome binds to specific sequences in the mRNA (ribosome binding sites) and subsequently recruits the 50S subunit (Figure 2.35).

c. The initiation of translation is the rate-limiting step and can be regulated (Figure 2.36). Initiation of translation in bacteria occurs at the first AUG in the mRNA from the 5' end and begins with a tRNA carrying a specialized amino acid, N-formyl-methionine. In Archaea, like eukaryotes, translation begins at the first AUG in the mRNA, but the tRNA carries methionine.

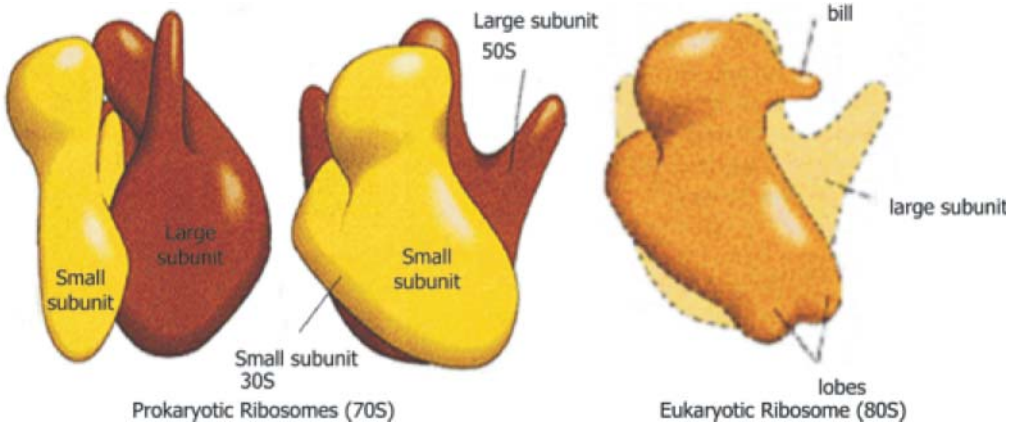
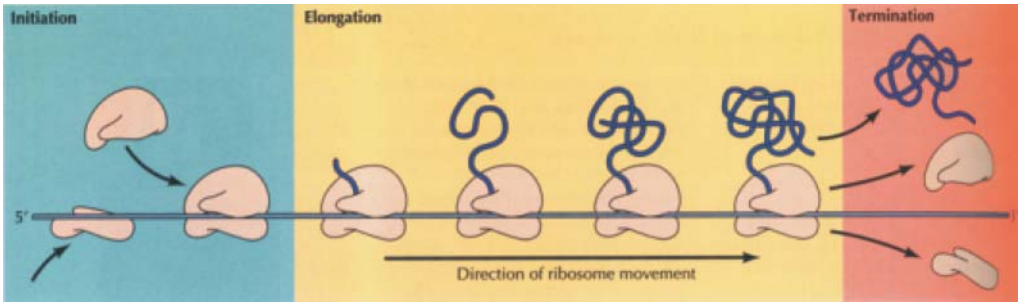


Figure 2.35: Ribosomes

d. Secondary ribosomal binding sites or start codons within the polycistronic mRNA can be buried in stem-loop structures, making these sequences inaccessible to ribosomes for binding and therefore can affect translation. In some operons, stop

codons and start codons can overlap, ensuring the coordinate translation of genes within an operon and stoichiometric amounts of proteins.



Ribosome binds mRNA at start codon

Polypeptide chain elongates by successively adding amino acids

When a stop codon is encountered, polypeptide is released and ribosome dissociates

Figure 2.36: Overview of Translation

Gene Control in Prokaryotes

In bacteria, genes are clustered into operons, gene clusters that encode the proteins necessary to perform coordinated function, such as biosynthesis of a given amino acid. RNA that is transcribed from prokaryotic operons is polycistronic a term implying that multiple proteins are encoded in a single transcript. In bacteria, control of the rate of transcriptional initiation is the predominant site for control of gene expression. As with the majority of prokaryotic genes, initiation is controlled by two DNA sequence elements that are approximately 35 bases and 10 bases, respectively, upstream of the site of transcriptional initiation and as such are identified as the -35 and -10 positions. These 2 sequence elements are termed promoter sequences, because they promote recognition of transcriptional start sites by RNA polymerase. The consensus sequence for the -35 position is TTGACA, and for the -10 position, TATAAT. (The -10 position is also known as the Pribnow-box.) These promoter sequences are recognized and contacted by RNA polymerase.

The activity of RNA polymerase at a given promoter is in turn regulated by interaction with accessory proteins, which affect its ability to recognize start sites.

These regulatory proteins can act both positively (activators) and negatively (repressors). The accessibility of promoter regions of prokaryotic DNA is in many cases regulated by the interaction of proteins with sequences termed operators. The operator region is adjacent to the promoter elements in most operons and in most cases the sequences of the operator bind a repressor protein. However, there are several operons in E. coli that contain overlapping sequence elements, one that binds a repressor and one that binds an activator.

Prokaryotic genes that encode the proteins necessary to perform coordinated function are clustered into operons. Two major modes of transcriptional regulation function in bacteria (E. coli) to control the expression of operons. Both mechanisms involve repressor proteins. A classic example of a catabolite-regulated operon is the lac operon, responsible for obtaining energy from b-galactosides such as lactose (Figure 2.37).

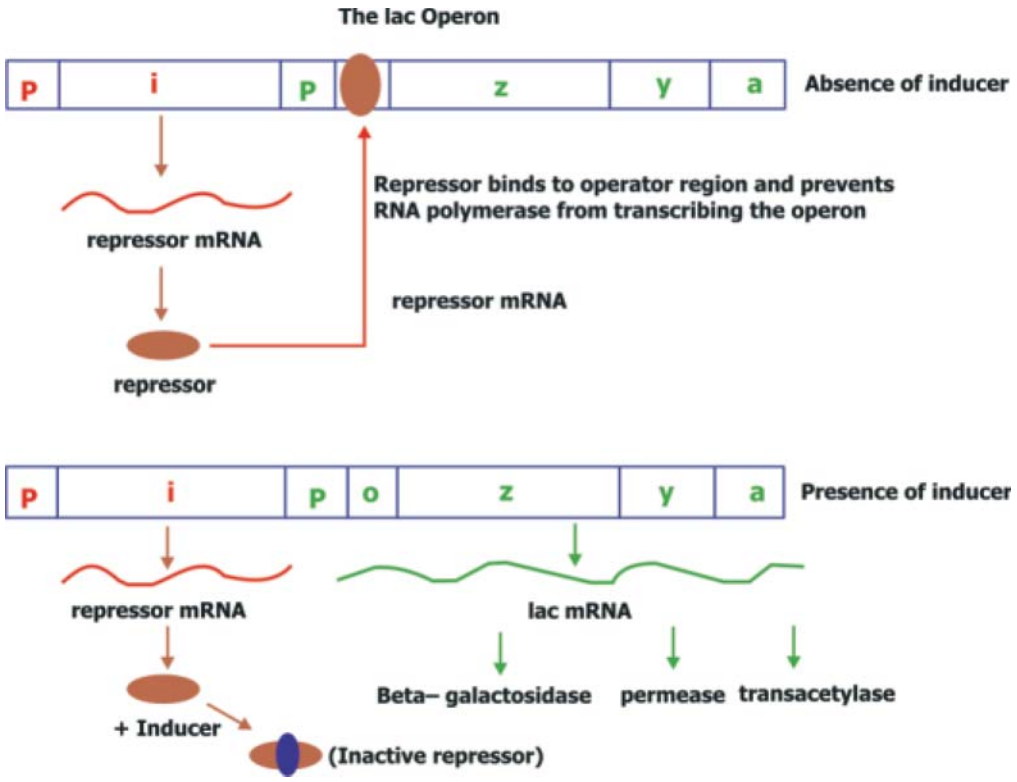


Figure 2.37: Lac Operon Showing Mechanism of Gene Regulation

Gene Control in Eukaryotes

In eukaryotic cells, the ability to express biologically active proteins comes under regulation at several points:

1. ***Chromatin Structure:*** The physical structure of the DNA, as it exists compacted into chromatin, can affect the ability of transcriptional regulatory proteins (termed transcription factors) and RNA polymerases to find access to specific genes and to activate transcription from them. The presence of the histones and CpG methylation most affect accessibility of the chromatin to RNA polymerases and transcription factors.

2. ***Transcriptional Initiation:*** This is the most important mode for control of eukaryotic gene expression. Specific factors that exert control include the strength of promoter elements within the DNA sequences of a given gene, the presence or absence of enhancer sequences (which enhance the activity of RNA polymerase at a given promoter by binding specific transcription factors), and the interaction between multiple activator proteins and inhibitor proteins.

3. ***Transcript Processing and Modification:*** Eukaryotic mRNAs must be capped and polyadenylated, and the introns must be accurately removed. Several genes have been identified that undergo tissue-specific patterns of alternative splicing, which generate biologically different proteins from the same gene. Cap-dependent initiation is the major translation initiation pathway in eukaryotes (Figure 2.38):

- The eukaryotic mRNAs are monocistronic, capped at the 5' end and polyadenylated at the 3' end
- Ribosomes dissociate into 40S and 60S subunits
- 40S subunits locate the initiator AUG codon by scanning the mRNA from the cap structure in the 3' direction for the first AUG codon
- at the AUG codon the 60S ribosomal subunit joins the 40S initiation complex to form an 80S ribosome competent for translation elongation:

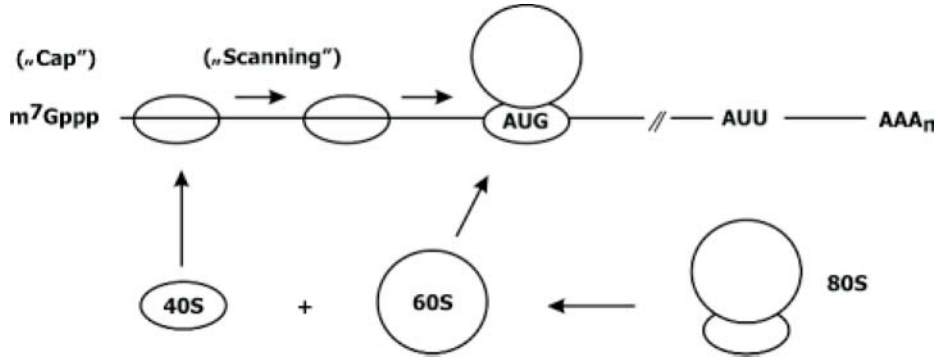


Figure 2.38: Principle of Cap-Dependent Translation Initiation. AUU, stop codon; AAAn, poly(A) tract

4. ***RNA Transport:*** A fully processed mRNA must leave the nucleus in order to be translated into protein.

5. ***Transcript Stability:*** Unlike prokaryotic mRNAs, whose half-lives are all in the range of 1--5 minutes, eukaryotic mRNAs can vary greatly in their stability. Certain unstable transcripts have sequences (predominately, but not exclusively, in the 3'-non-translated regions) that are signals for rapid degradation.

6. ***Translational Initiation:*** Since many mRNAs have multiple methionine codons, the ability of ribosomes to recognize and initiate synthesis from the correct AUG codon can affect the expression of a gene product. Several examples have emerged demonstrating that some eukaryotic proteins initiate at non-AUG codons. This phenomenon has been known to occur in E. coli for quite some time, but only recently has it been observed in eukaryotic mRNAs.

7. ***Post-Translational Modification:*** Common modifications include glycosylation, acetylation, fatty acylation, disulfide bond formations, etc.

8. ***Protein Transport:*** In order for proteins to be biologically active following translation and processing, they must be transported to their site of action.

9. ***Control of Protein Stability:*** Many proteins are rapidly degraded, whereas others are highly stable. Specific amino acid sequences in some proteins have been shown to bring about rapid degradation.

Control of Eukaryotic Transcription Initiation

Transcription of the different classes of RNAs in eukaryotes is carried out by three different polymerases. RNA pol I synthesize the rRNAs, except for the 5*S* species. RNA pol II synthesizes the mRNAs and some small nuclear RNAs (snRNAs) involved in RNA splicing. RNA pol III synthesizes the 5*S* rRNA and the tRNAs. The vast majority of eukaryotic RNAs are subjected to post-transcriptional processing.

The most complex controls observed in eukaryotic genes are those that regulate the expression of RNA pol II-transcribed genes, the mRNA genes. Almost all eukaryotic mRNA genes contain a basic structure consisting of coding exons and non-coding introns and basal promoters of two types and any number of different transcriptional regulatory domains (Figure 2.39). The basal promoter elements are termed CCAAT-boxes (pronounced cat) and TATA-boxes because of their sequence motifs. The TATA-box resides 20 to 30 bases upstream of the transcriptional start site and is similar in sequence to the prokaryotic Pribnow-box (consensus $TATA^{T}/_{A}A^{T}/_{A}$, where $^{T}/_{A}$ indicates that either base may be found at that position).

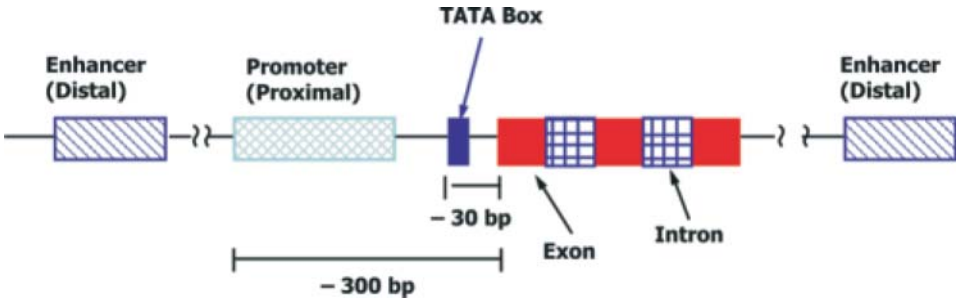


Figure 2.39: Typical Structure of a Eukaryotic mRNA Gene

Eukaryotic transcripts must be processed before they can be translated. Here is a diagram outlining the steps involved in the production of a protein in eukaryotic cells (Figure 2.40).

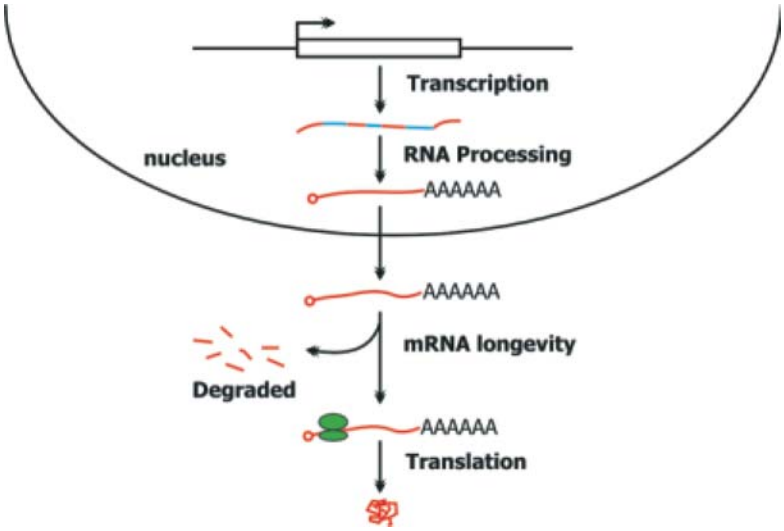


Figure 2.40: Pathway for Protein Production

Regulation can occur at any point in this pathway; specifically, it occurs at the levels of transcription, RNA processing, mRNA lifetime (longevity), and translation. Each of these types of regulation will be considered in turn.

Cell Cycle

Bacterial growth and reproduction require a balanced process of growth, chromosome replication and cell division. The integration of the processes is required so that cells do not divide before they are big enough to do so, and that each cell contains a correctly partitioned nucleoid after cell division. Cell division by either mitosis or meiosis is only one phase in the life of a cell. Cells are not always dividing. The majority of their life is spent carrying out their life function in a phase known as Interphase.

Generally the cycle is composed of two phases, a division phase denoted as "M" (mitosis or meiosis) and Interphase. Interphase is itself composed of three phases denoted as G1, M and G2 (Figure 2.41). The G1 stage, or first stage is the period after cytokinesis but before DNA synthesis. It is a time of cell growth, high metabolic activity, and synthesis of enzymes needed for DNA replication. The S stage, or synthesis stage, is the period where DNA synthesis takes place. The single long strand of nucleic acids making up one chromatid or one half the double stranded structure of DNA is precisely copied to yield two identical molecules called sister

chromatids. They are held together at the centromere, which consists of DNA that was not replicated along with the rest of the molecule. The G2, or second gap stage, begins as soon as all the chromosomes are replicated. Here normal metabolic activity and growth occur in addition to the synthesis of proteins needed for cell division stage.

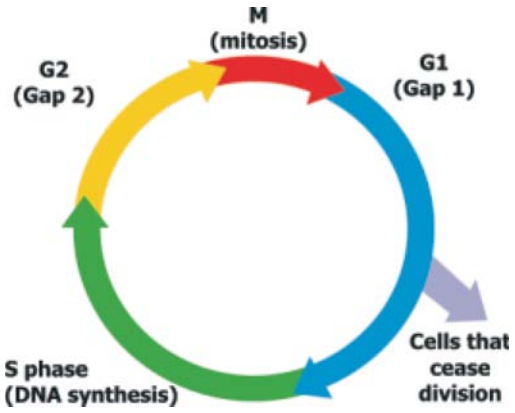


Figure 2.41: Cell Cycle

Cell Growth and Development

Most cells divide by a process called binary fission where a cell divides in half forming two daughter cells, which are smaller duplicates of each other (Figure 2.42). A few cells reproduce by budding, where the daughter cell grows out of the parent and gradually increases in size.

All cells have their genes arranged in linear chains called chromosomes. Eukaryotic cells contain 2 (or more) copies of each gene (with some exceptions) carried in duplicate chromosomes. During cell division the chromosomes of eukaryotic cells undergo an organized process of chromosome replication that is visible under the light microscope. This process, called mitosis insures that each daughter cell receives a complete copy of the parental genome. Prokaryotic cells usually contain only a single chromosome and, while its process of replication etc. is also highly organized, it is not visible under the light microscopes.

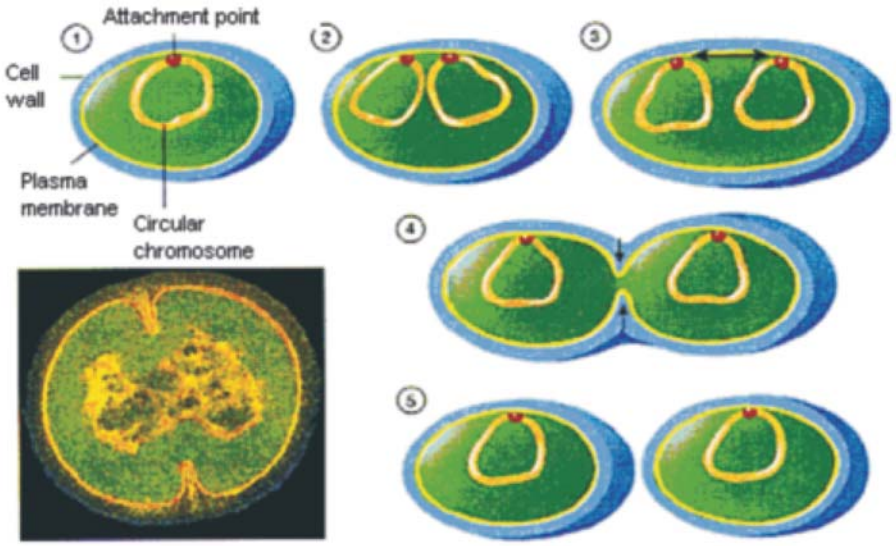


Figure 2.42: Binary Fission in Bacteria

Mitosis

Mitosis is the mechanism that allows the nuclei of cells to split and provide each daughter cell with a complete set of chromosomes during cellular division. This, coupled with cytokinesis (division of the cytoplasm), occurs in all multicellular plants and animals to permit growth of the organism.

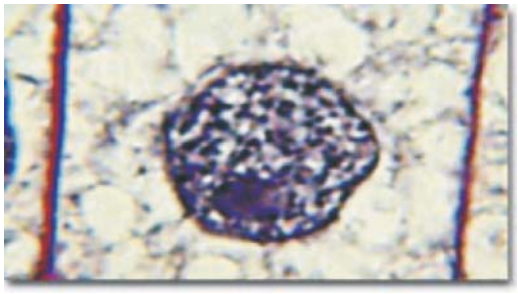


Figure 2.43a: Resting Stage

Resting Cell: A normal resting cell exists in a state called interphase in which the chromatin is undifferentiated in the heavily stained nucleus, as illustrated above

(Figure 2.43a). Before the cell enters the mitosis phase, it first undergoes a synthesis or S phase where each chromosome is duplicated and consists of two sister chromatids joined together by a specific DNA sequence known as a centromere. These are crucial to segregation of the daughter chromatids during mitosis. The first phase of mitosis is known as the prophase, where the nuclear chromatin starts to become organized and condenses into thick strands that eventually become chromosomes.

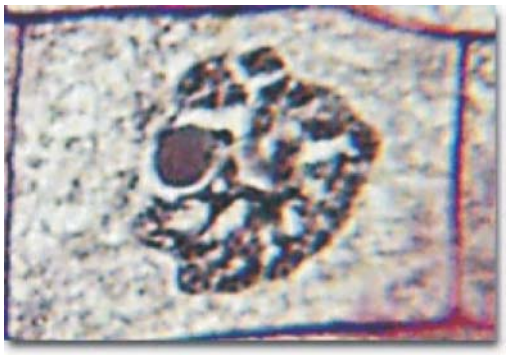


Figure 2.43b: Early Prophase

Early Prophase: During prophase, the cytoskeleton (composed of cytoplasmic microtubules) begins to disassemble and the main component of the mitotic apparatus, the mitotic spindle begins to form outside the nucleus at opposite ends of the cell. The photomicrograph above depicts the initial chromosome condensation at the beginning of prophase (early prophase) when the nucleolus is still intact (Figure 2.43b).

Late Prophase or Prometaphase: This phase begins with the disruption of the nuclear envelope, which is broken down into small membrane vesicles that closely resemble the endoplasmic reticulum and tend to remain visible around the mitotic spindle. During this period the chromosomes continue to condense and gradually shorten and thicken until they have completely formed the units that will undergo mitosis. The nucleolus also disappears during this period (Figure 2.43c).

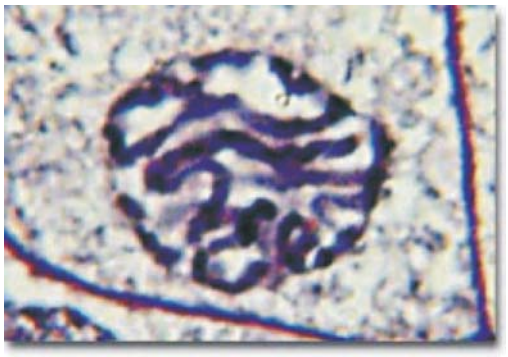


Figure 2.43c: Late Prophase

The mitotic spindle microtubules are now free to enter the nuclear region, and formation of specialized protein complexes called kinetochores begins on each centromere. These complexes become attached to some of the spindle microtubules, which are then termed kinetochore microtubules. Other microtubules in the spindle (not attached to centromeres) are termed polar microtubules and these help form and maintain the spindle structure along with astral microtubules, which remain outside the spindle.

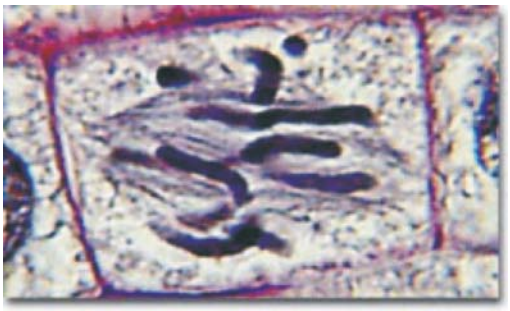


Figure 2.43d: Metaphase

The next identifiable phase is called metaphase where the chromosomes, attached to the kinetochore microtubules, begin to align in one plane (the metaphase plate) halfway between the spindle poles. The kinetochore microtubules exert tension on the chromosomes and the entire spindle-chromosome complex is now ready for the next event. The photomicrograph depicts onion root tip cell chromosomes in

metaphase, ready for separation (Figure 2.43d). The kinetochore and polar microtubules re clearly visible and radiate out the ends of the cell leaving the chromosomes in the middle of the complex.

Metaphase: This sets the stage for chromosome separation in the next stage of mitosis: anaphase. Almost immediately after the metaphase chromosomes are aligned at the metaphase plate, the two halves of each chromosome are pulled apart by the spindle apparatus and migrate to the opposite spindle poles. The kinetochore microtubules shorten as the chromosomes are pulled toward the poles, while the polar microtubules elongate to assist in the separation. The photomicrograph below illustrates the early stage of anaphase where the chromosomes are just becoming completely separated. The microtubules are clearly visible in this complex.

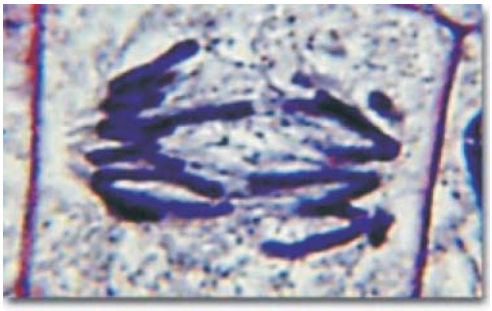


Figure 2.43e: Early Anaphase

Early Anaphase: Anaphase typically is a rapid process that lasts only a few minutes. When the chromosomes have completely migrated to the spindle poles, the kinetochore microtubules begin to disappear, although the polar microtubules continue to elongate (Figure 2.43e). This is the junction between late anaphase and early telophase, the last stage in chromosome division. The photomicrograph below shows the positioning of the chromosomes in late anaphase (Figure 2.43f). The polar microtubules are a clearly formed network and the synthesis of a new cell membrane has been initiated in the cytoplasm between the two spindle poles.

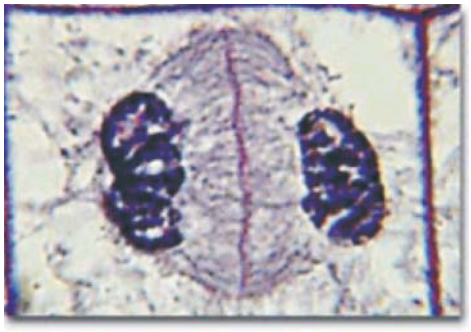


Figure 2.43f: Late Anaphase

Late Anaphase: In telophase, the daughter chromosomes arrive at the spindle poles and are eventually redistributed into chromatin. The process of cytokinesis, where the cytoplasm is divided by cleavage, also starts sometime in late anaphase and continues through telophase. After complete separation of the chromosomes and their extrusion to the spindle poles, the nuclear membrane begins to reform around each group of chromosomes at the opposite ends of the cell. The nucleoli also reappear in what will eventually become the two new cell nuclei. The photomicrograph below captures a cell in late telophase where the new membrane is beginning to divide the cell but the nuclei have not completely reformed and cytokinesis has not yet finished (Figure 2.43g).

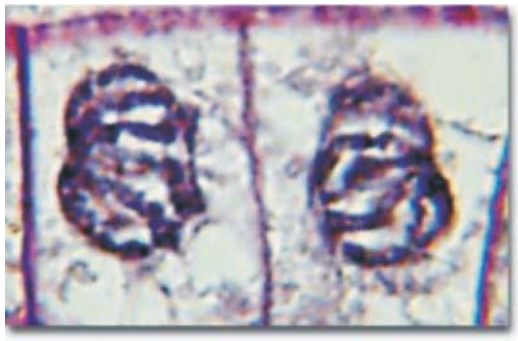


Figure 2.43g: Telophase

Telophase: When telophase is complete and the new cell membrane (or wall in the case of the onion root tips) is being formed, the nuclei have almost matured to the pre-

mitotic state. The final steps are completion of the total formation of a membrane between each of the new daughter cells to yield two separate new cells. The photomicrograph below illustrates two newly formed cells that have just completed the process of mitosis (Figure 2.43h).

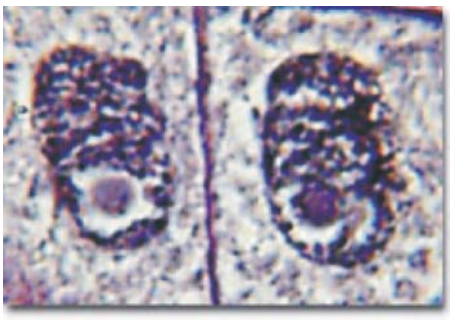
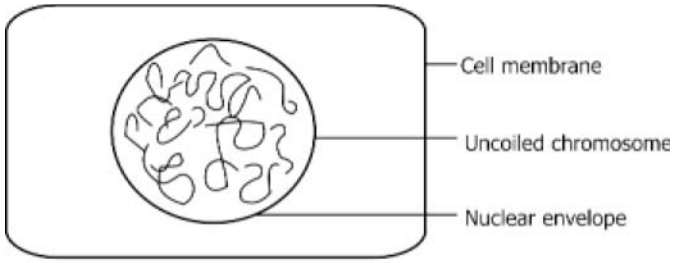


Figure 2.43h: Daughter Cells

Meiosis

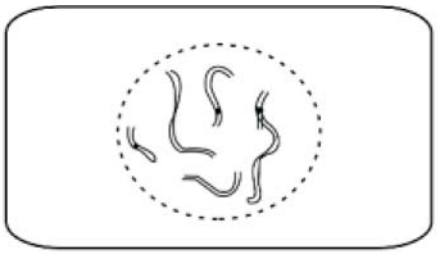
Meiosis starts with interphase in which replication occurs in metabolically active cell. In early Prophase I, chromosomes become bigger and thick while nuclear envelop begins to disappear. In Metaphase I, homologous pairs of chromosomes begin to arrange on equatorial plate. These pairs of chromosome begin to separate in Anaphase I towards opposite poles. In Telophase I, the separation of chromosomes completes and cell division starts.

In Interphase, chromosomes uncoil and nuclear envelope reappears, resulting in two haploid nuclei. First cell division is completed and two haploid cells are produced. In Prophase II, chromosomes become aligned on equatorial plane and spindle fibers again formed. Chromatids are separated in Metaphase II and Anaphase II. In Telophase II, cell division starts and second cell division is completed in Interphase again resulting in four haploid cells (Figures 2.44a-k).



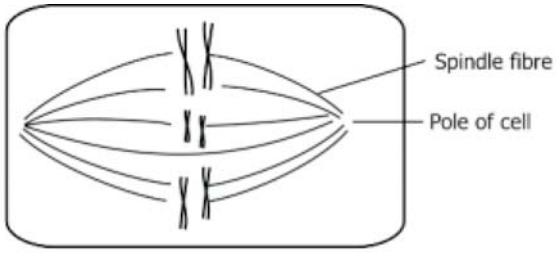
1. Metabolically active cell with a diploid nucleus (2n-6)
2. DNA replication occurs

Figure 2.44a: Interphase



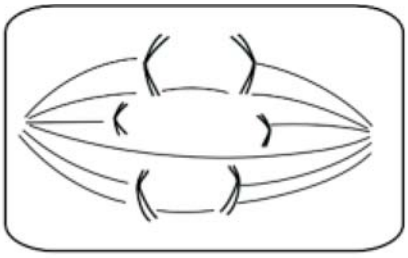
1. Chromosomes become shorter, thicker and more easily stained
2. Nuclear envelope begins to disintegrate

Figure 2.44b: Prophase I



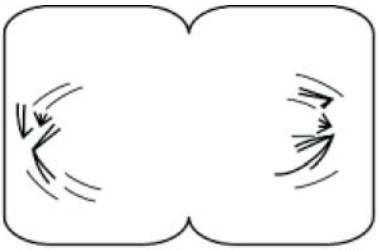
1. Pair of homologous chromosomes migrate to the equatorial plane of cell

Figure 2.44c: Metaphase I



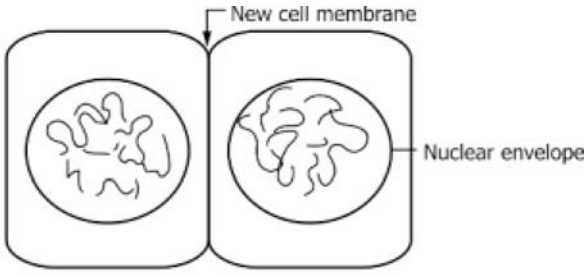
1. Homologous chromosomes (each consisting of two chromatids held together by a centromere move to opposite poles

Figure 2.44d: Anaphase I



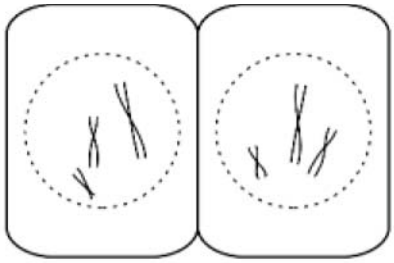
1. Movement of chromosomes to poles is complete
2. The Spindle disappears
3. Cell division starts (cytokinesis)

Figure 2.44e: Telophase I



1. The chromosomes uncoil and resume the appearance
2. The nuclear envelope reappears, resulting in two haploid nuclei
3. First cell division is completed, resulting in two haploid cells

Figure 2.44f: Interphase



1. Chromosomes become aligned on the equatorial plane
2. Fibres of spindle reformed.

Figure 2.44g: Prophase II

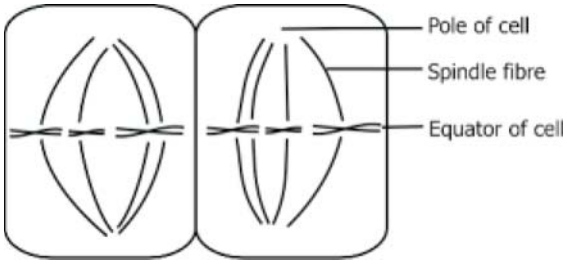
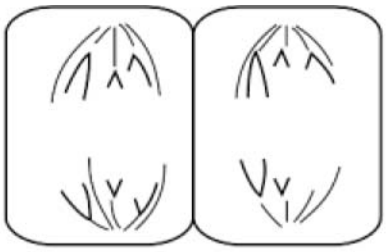


Figure 2.44h: Metaphase II



1. Centromeres divide, separating the chromatids which move to opposite poles as chromosomes

Figure 2.44i: Anaphase II

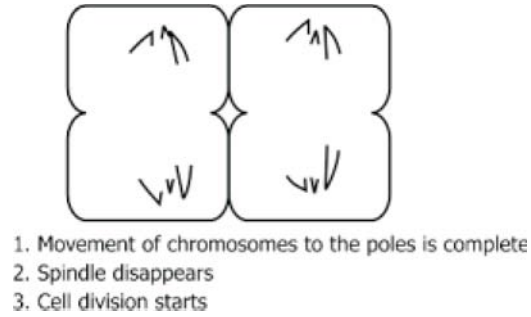


Figure 2.44j: Telophase II

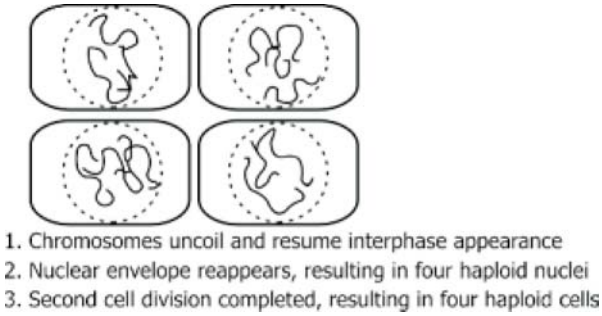


Figure 2.44k: Interphase

Eukaryotic cells range in size between 2 and 100 micro meters (=10-6 meters) and are usually much larger than prokaryotic cells, which run between 0.5 and 2 micrometers. However, a large prokaryotic cell that is 600 micrometers long has recently been discovered.

Genome: Organization and Functions

Genome

The DNA code that comprises the complete genetic composition of an organism. In other words, the genome of an organism is its set of chromosomes, containing all of its genes and associated DNA.

The Prokaryotic Genome

Prokaryotes are divided into two major taxonomic groups, the eubacteria and the archaea or archaebacteria. The eubacteria are the more commonly known form, but since the recognition of the Archaea they have been found in thousands of environments so we can't say which group is the most common on the planet. However, there are no reports of any pathogenic Archaea. The Archaea are generally found in environmental extremes (as far as we're concerned) in that they exist in hot springs at 100oC, in the bottom of the ocean at ~110oC and in water the density of gold, in saturated salt ponds and other environments that would seem to be inamicable to life. They can metabolize unusual substances for energy like methane, sulfur, and hydrogen gas. Their origin and relationship to eubacteria and eukaryotes is currently being hotly debated among microbiologists. However, some of their biochemical characteristics resemble eukaryotes more than they do eubacteria.

All evolution and adaptation is directed towards propagation i.e. DNA replication and cell division. Development of adaptation to environmental stress must be coordinated with processes affecting replication and cell division, conservation of mechanisms throughout the living world argues for their importance e.g. rRNA genes, mechanism of replication, cell division

Genome Size: 600 kb for *Chlamydomonas,* 13, 000 kb for *Calothrix, Candida* (yeast), 4,500 kb for *E. coli.* Not long ago it was thought that all prokaryotic genomes (both bacteria and Archae) were much smaller than eukaryotic genomes. However, the application of new techniques for constructing physical maps and whole genome sequencing has demonstrated that there is tremendous diversity in the size and organization of prokaryotic genomes. The following figure shows some examples of genome sizes of bacteria and Archae (Figure 2.45). The size of bacteria chromosomes ranges from 0.58 Mbp to 10 Mbp, and the size of Archae chromosomes range from 0.5 Mbp to 5.8 Mbp. For comparison, Eukaryotic chromosomes range from 2.9 Mbp (Microsporidia) to over 4,000 Mbp.

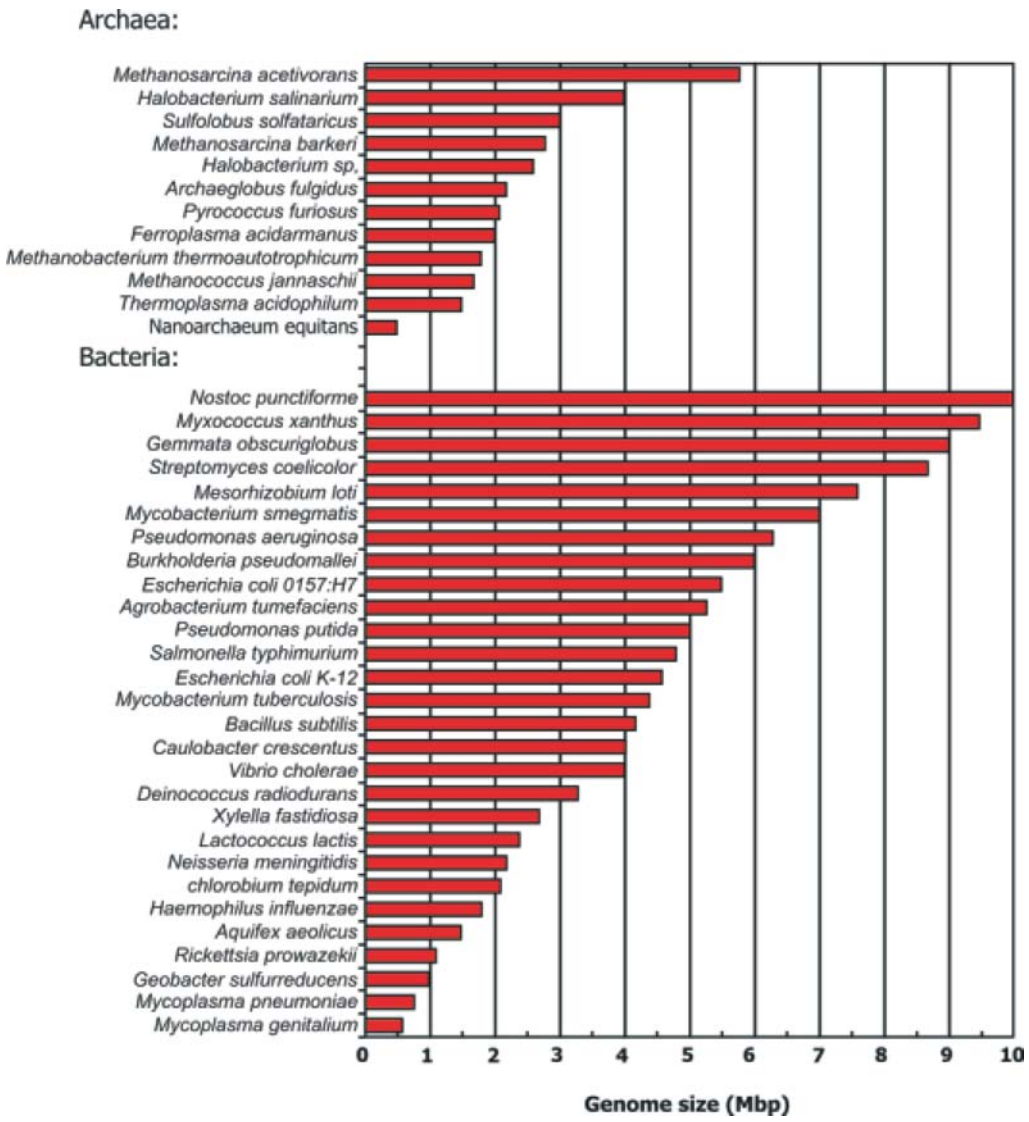


Figure 2.45: Genome Size

The smallest genomes identified thus far are in the *Mycoplasma*. For example, the complete *M. genitalium* genome is only 0.58 Mbp (580 Kbp) and is predicted to encode 480 proteins. These organisms are intracellular pathogens that rely upon their host for many products. In contrast, free-living bacteria must dedicate many genes toward the synthesis or acquisition of these products. The smallest free-living bacteria have a genome size of about 1 Mbp.

The Eukaryotic Genomes

Initially it is important to obtain an idea of the relative sizes of various eukaryotic genomes and to put them into perspective with bacterial and viral genomes. In principle the rule holds that an increase in structural complexity of an organism goes hand in hand with a more complex genome (since it takes obviously more DNA to encode more proteins); but there are some exceptions to this rule such as the extraordinary large sizes of some amphibian and plant genomes (Figure 2.46).

The genomes of the structurally simplest unicellular or filamentous eukaryotes such as the fungi *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* are around 12 and 18 Mbp respectively i.e. only about four times the size of the average bacterial genome. Multicellular eukaryotes with differentiated cells (tissues) again contain more DNA in their somatic/germ line cells: Nematodes *(Caenorhabditis elegans)* and small dicot plants (such as *Arabidopsis thaliana*) around 100 Mbp; insects (dipters) have genomes nearly twice as large again (165 Mega base pairs for *Drosophila melanogaster*) but then there is a large jump in genome size when one looks at the animal phylum of the vertebrates.

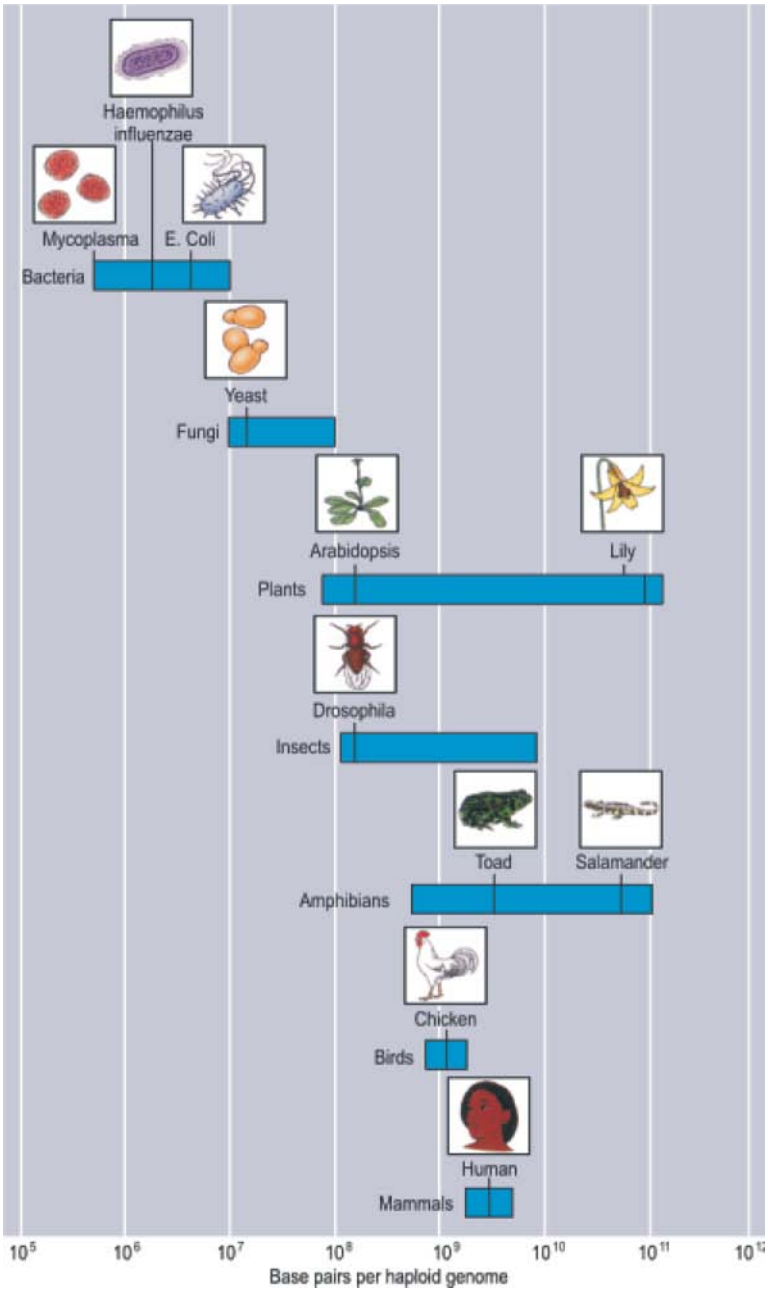


Figure 2.46: The Range of Sizes of the Genomes of Representative Groups of Organisms is Shown on a Logarithmic Scale.

Genome Organization

a. Operon

As discussed earlier many transcriptional units encode more than one gene, which is termed an operon (Figure 2.47). Genes with related functions are often located together in an operon. An operon is a group of genes that has a single promoter site (site where RNA polymerase binds and transcribes mRNA) and is transcribed as a single polycistronic mRNA molecule. Translation of genes within the polycistronic mRNA begins at the first AUG and proceeds to transcription termination signals. Ribosomes may or may not detach from the mRNA between cistrons. A basic operon is composed of a promoter, transcriptional start point, the leader sequence, ribosome binding site and transcription termination signal.

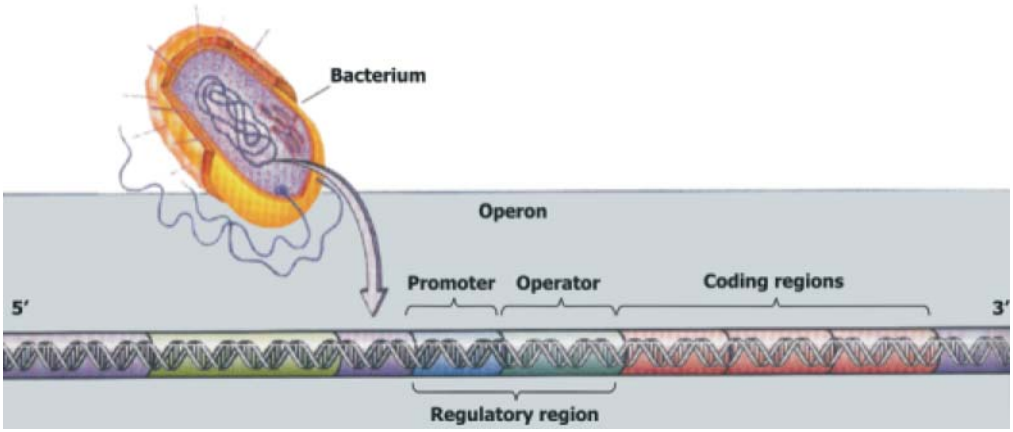


Figure 2.47: Structure of an Operon

The promoter region and upstream regulatory region may be bound by DNA binding proteins that may function either as transcriptional repressors (thus interfering with binding and transcriptional capabilities of RNA polymerase) or as activators (facilitating the binding or transcriptional activity of RNA polymerase). tsp=transcriptional start point. The operon has two cistrons. Ribosomes attach at the ribosome-binding site (rbs) just before the start codon (AUG) on the mRNA. Transcription termination occurs at the end of the operon and is facilitated by the formation of a hairpin loop structure in the mRNA.

b. Regulon

A regulon is a set of operons that may be scattered across the chromosome but that are all controlled by the same regulatory protein. These operons encode proteins within a common pathway or phenomenon. An example is genes involved in glycerol catabolism. Many of the genes involved in regulons and modulons are being assessed by gene microarray technology (A technology used to assess the impact of many genes at once by measuring the amount of mRNA bound to each site, on the array).

Comparative Analysis of Genomes

The full genomic sequence of approximately 35 bacterial and archeal species has been determined and 40 more are near completion. Comparative genomic analysis has become a powerful tool to decipher the function of genes encoded by various genomes. The smallest free-living prokaryotic genome that has been sequenced to date is that of *Mycoplasma genitalium* which encodes approximately 450 genes. The largest prokaryotic genome is estimated to be approximately 10 Mbp. Sequencing technologies has progressed to the point that the DNA sequence of an entire bacterial genome can be determined in just a few days. Following is a list of prokaryotic genomes that have been or are currently being sequenced (Table 2.2).

Table 2.2: Important Features of Prokaryotic Organisms and Genome size.

Prokaryotic organism	Important features	Size of genome (Mbp)
Haemophilus influenzae	Human pathogen	1.83
Helicobacter pylori	Human pathogen, peptic ulcers	1.7
Mycoplasma genitalium	Smallest genome	0.58
Staphylococcus aureus	Human pathogen, wound infections	2.8
Bacillus subtilis	Model genetic system	4.2
Escherichia coli	Model genetic system	4.7
Methanobacterium	Marine Archaeal species, methanotrophic	1.7
Mycobacterium leprae	Human pathogen, leprosy	2.4
Mycobacterium tuberculosis	Human pathogen, tuberculosis	4.4

Chapter 3

Genetic Engineering

Chapter 3

GENETIC ENGINEERING

Introduction

Genetic engineering is the technique of removing, modifying or adding genes to a DNA molecule in order to change the information it contains. By changing this information, genetic engineering changes the type or amount of proteins an organism is capable of producing. Genetic engineering is used in the production of drugs, human gene therapy, and the development of improved plants. For example, an “insect protection” gene (Bt) has been inserted into several crops like corn, cotton, and potatoes to give farmers new tools for integrated pest management. Bt corn is resistant to European corn borer. This inherent resistance thus reduces a farmer's pesticide use for controlling European corn borer, and in turn requires fewer chemicals and potentially provides higher yielding an example of Agricultural Biotechnology.

Natural Selection

Natural selection is nature's own form of genetic engineering. The fit organisms survive through natural selection. The rate of evolution of new species through natural selection is incredibly slow, but methods have been discovered by which nature has optimized the process. The entire genome (all the genes) of higher animals and plants are broken up into functional components known as exons and separated by regions called introns. Special genes known as transposable elements serve to mix and match functional components of genes in an effort to maximize the likelihood of creating better genes and organisms. There is some evidence that bacteria, one of the simplest organisms, had introns and exons in some past era, but lost them in favor of efficiency and other means of acquiring new DNA.

Artificial Selection

Selective breeding or "artificial selection” is man's most basic effort at genetic engineering by creating selective pressures. Many conventional farm animals, domesticated dogs and cats were likely created ages ago by selectively breeding animals together with desired traits. Gregor Mendel helped to establish the rules of

genetics through his work on selectively breeding plants in the 1800's. Selective Breeding has worked well for engineering animals and plants, but it can take whole human lifetimes to bring about small changes in a species.

Through unnatural selection certain attributes and characteristics can be enhanced by selectively killing all organisms that do not have the desired traits. Parents could produce a large number of fertilized eggs through in vitro fertilization. Each could be grown for a while in vitro and then be tested for desired traits. Only an egg with all the traits desired by the parents would then be implanted in the mother. There are obvious drawbacks, not the least of which is the large number of fertilized eggs that are not selected. This option is not a viable alternative for many couples for religious reasons.

Another drawback is that selecting for a very large number of traits is close to impossible. Each gene desired at least doubles the number of fertilized eggs required. Certain traits are the result of many genes acting in concert, which could inflate egg requirements very quickly. Last of all, fertilized eggs must have one copy of each gene from each parent. Even with an infinite number of eggs a bad gene cannot be totally eliminated if one parent has two copies of that gene.

Genetic Manipulations

Genetic Manipulations are becoming common as a means of genetic engineering. There are many methods of introducing new genetic material into a cell or organism, or altering the existing material. Radiation and mutagenic compounds are able to wreak havoc on DNA. Special viruses have been altered and put to use, which can introduce new genetic material to an organism. Transposable elements, nature's own gene shuffling tools, have been put to use moving genes around in cells and organisms. Gene Targeting is a way of replacing a specific gene with another within a cell.

These kinds of genetic manipulations are great for research with animals. Gene targeting seems to be the most precise way of altering known genes. Gene therapies often try to replace or repair defective genes in tissues where the genes are in use. This technique does not usually alter the "germ line", that is the reproductive cells, so even if it corrects a problem, new borns can still inherit the problem.

Gene Therapy on the reproductive cells, or better yet, on a fertilized egg could be used to introduce whatever genes are desired into an organism, even a human, when they are still a single cell. With cloning technology, not even a fertilized egg is needed, just a cell that will grow in cell culture. This is where genetic engineering stands right now. It is technically possible to repair and/or replace any known gene, but it is not very efficient and requires a large number of cells, of which only a few will be properly repaired. The other limitation is the number of known genes.

True genetic engineering is the creation of whole new genes and proteins, or even new organisms. By understanding the genetic code and random or specific proteins can be created quite readily, but creating new proteins precisely for a given purpose for example, to strongly catalyze a particular chemical reaction is difficult. Research into the structure and folding of proteins may yield some answers. Mixing and matching of the components of known proteins and organisms may yet be mastered, but that is a large step beyond even the manipulation of known genes.

The major reason that lies behind the advance of genetic engineering is the universal presence of DNA (deoxyribonucleic acid) in the cells of all living organisms (Figure 3.1).

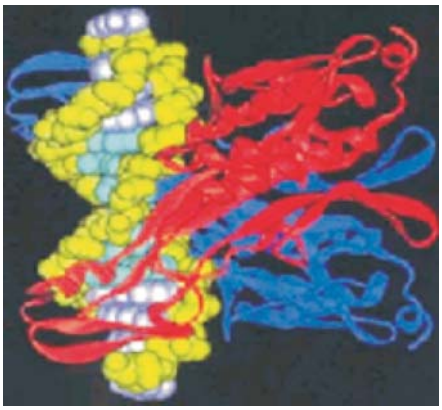


Figure 3.1: DNA Construction

This molecule stores the organism's genetic information and orchestrates the metabolic processes of life. Genetic information is specified by the sequence of four chemical bases (adenine, cytosine, guanine, and thiamine) along the length of the

DNA molecule. Genes are discrete segments of DNA that encode the information necessary for assembly of a specific protein. The proteins then function as enzymes to catalyze biochemical reactions, or as structural or storage units of a cell, to contribute to expression of a trait. The general sequence of events by which the information encoded in DNA is expressed in the form of proteins via an mRNA intermediary is shown in the diagram below.

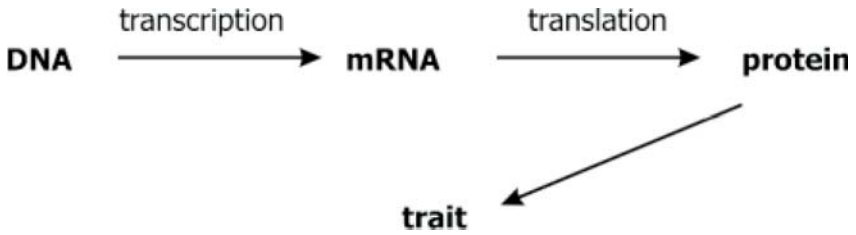


Figure 3.2: Genetic Information Flow

The transcription and translation processes, as discussed in previous chapter, are controlled by a complex set of regulatory mechanisms, so that a particular protein is produced only when and where it is needed. Even species that are very different have similar mechanisms for converting the information in DNA into proteins; thus, a DNA segment from bacteria can be interpreted and translated into a functional protein when inserted into a plant.

Among the most important tools in the genetic engineer's tool kit are enzymes that perform specific functions on DNA. Restriction enzyme (EcoR1), which recognizes and cuts the DNA at a specific region of the DNA, is one of the most important enzymes. Other enzymes known as ligases join the ends of two DNA fragments. These and other enzymes (DNA modifying enzymes) enable the manipulation and amplification of DNA, essential components in joining the DNA of two unrelated organisms.

Steps Involved in Gene Manipulations

There are many diverse and complex techniques involved in gene manipulation but the basic principles are reasonably simple (Figure 3.3).

1) Generation of DNA fragments using enzymes that recognize and cut DNA molecules at specific DNA sequences.

2) Splicing of these sequences to other DNA molecules that serve as Vectors. The vector can replicate autonomously and these facilitate the manipulation and identification of the newly created recombinant DNA molecule.

3) Transfer of vector carrying inserted DNA segment into a cell, which it is to be replicated, a process called Transformation of the cell.

4) Selection of those cells that carry the desired recombinant DNA molecule and their replication as clones.

In addition the cloned DNA segment can be recovered from the host cell, purified and amplified.

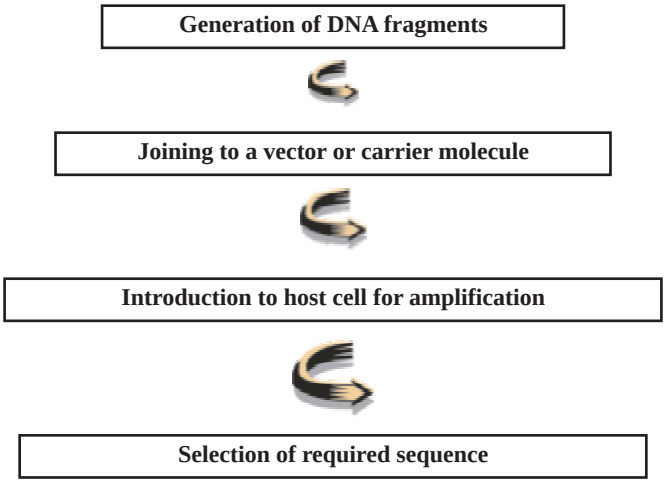


Figure 3.3: Gene cloning protocol

1. The Production of DNA Fragments

In genetic engineering, the immediate goal of an experiment is usually to insert a particular fragment of chromosomal DNA into a plasmid or a viral DNA molecule. This is accomplished by techniques for breaking molecules at specific sites and for isolating particular DNA fragments. Deoxyribonucleic acid fragments are usually obtained by treating DNA samples with an important sub-class of endonucleases called Restriction Endonucleases.

i) Restriction Enzymes: Restriction enzymes are DNA-cutting enzymes found in bacteria (and harvested from them for use). Because they cut within the molecule, they are often called restriction endonucleases and molecular scissors. A restriction enzyme recognizes and cuts DNA only at a particular sequence of nucleotides. These restriction sites are generally 4-6 base pairs long and that sites, which differ for restriction endonuclease, are palindromes i.e., they exhibit two-fold rational symmetry. For example, the bacterium *Hemophilus aegypticus* produces an enzyme named HaeIII that cuts DNA wherever it encounters the sequence:

5'GGCC3'
3'CCGG5'

The cut is made between the adjacent G and C. This particular sequence occurs at 11 places in the circular DNA molecule of the virus phiX174. Thus treatment of this DNA with the enzyme produces 11 fragments, each with a precise length and nucleotide sequence. These fragments can be separated from one another and the sequence of each determined.

ii) Sticky and Blunt ends: HaeIII and AluI cut straight across the double helix producing "**blunt**" ends. However, many restriction enzymes cut in an offset fashion. The ends of the cut have an overhanging piece of single-stranded DNA. These are called "sticky ends" because they are able to form base pairs with any DNA molecule that contains the complementary sticky end. Any other source of DNA treated with the same enzyme will produce such molecules. Mixed together, these molecules can join with each other by the base pairing between their sticky ends. The union can be made permanent by another enzyme, DNA ligase that forms covalent bonds along the backbone of each strand. The result is a molecule of recombinant DNA (rDNA).

***Restriction Endonucleases have been Divided into Two General Types: I & II* (Figure 3.4):**

Type I restriction endonucleases catalyze both the methylation of the host DNA and the cleavage of unmethylated DNA. Type II restriction endonucleases are simpler in that they only cleave double stranded DNA at or near a specific unmethylated recognition sequence; separate enzymes, specific methyltranferases known as “restriction methylases” catalyze methylation at the same recognition sequence to protect host DNA. Well over 200 types, type II and I restriction nucleases have been characterized.

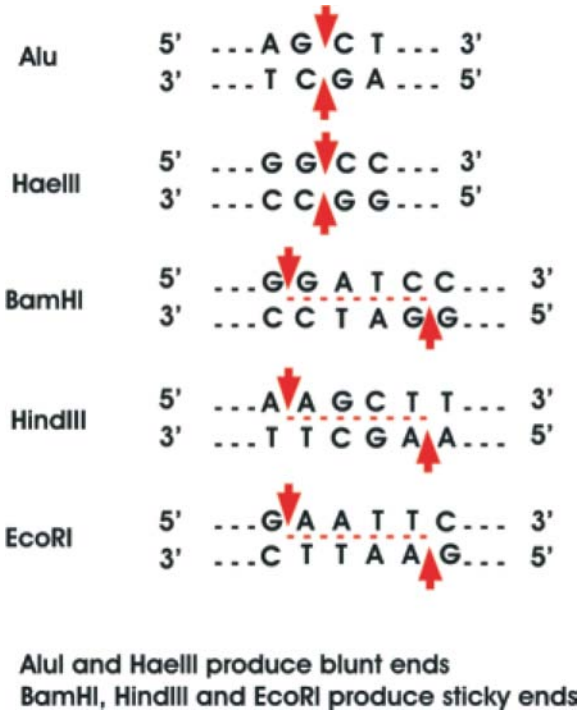


Figure 3.4: Restriction Enzymes

2. Joining to a Vector or Carrier Molecule

In genetic engineering, a particular DNA segment of interest is joined to a small but essentially complete DNA molecule that is able to replicate a vector to form recombinant DNA molecule. This is placed in a cell in which the molecule can replicate. When a stable transformant has been isolated, the genes or DNA sequences linked to the vector are said to be cloned or recombinant DNA.

Vector: A DNA molecule that is capable of replication in a host organism, and can act as a carrier molecule for the construction of recombinant DNA" or a vector is a DNA molecule into which a DNA fragment can be cloned.

The useful vector has following properties.

- Vector should be fairly small DNA molecule to facilitate isolation and handling.

- The vector DNA can be introduced into a host cell.
- The vector contains a replication origin and so can replicate inside the host cell.
- Cells containing the vector can usually be selected by a straightforward assay, most conventionally by allowing the growth of the host cell on a solid selective medium.

At present the most commonly used vectors are plasmids and derivatives of bacteriophage ë and M13. One of the most extensively used plasmid is pBR 32. This plasmid has all the features of a good vector, such as low molecular weight, antibiotic resistant gene, an origin of replication and several single cut restriction endonuclease restriction sites.

A piece of target DNA can be inserted into a plasmid if both the circular plasmid and the target DNA have been cleaved by the same restriction nuclease in such a way as to create sticky ends. The newly created recombinant molecule is stabilized with the DNA ligase enzyme, which repairs nicks in the backbone of the DNA molecule (Figure 3.5).

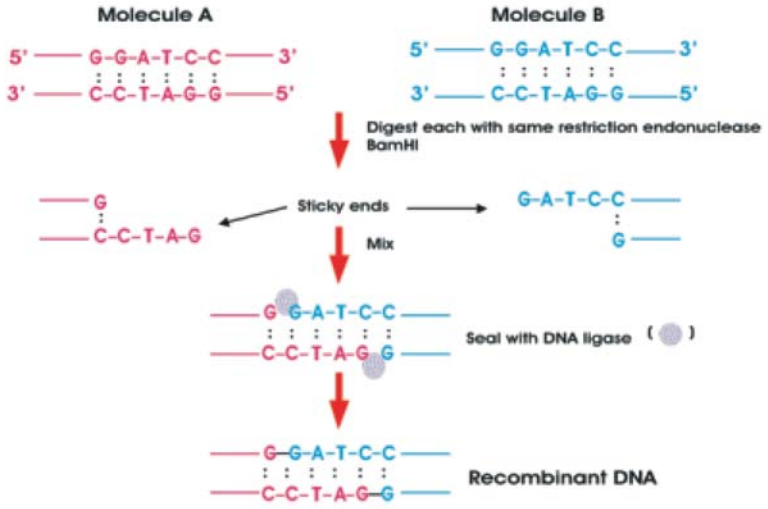


Figure 3.5: DNA Recombination

The Splicing of DNA

Three general splicing methods can be used to form recombinant DNA molecules;

1) Because a particular restriction enzyme produces fragments with identical sticky ends, with out regard for the source of DNA, fragments from the different organisms can be joined e.g., the restriction enzyme Eco RI is used to digest DNA from any organism of interest and to cleave a bacterial plasmid that contains only one Eco RI restriction site. The donor DNA is digested into many fragments. When the donor fragments and the linearized plasmids are mixed, recombinant molecules can form by base pairing between the complementary single stranded ends. At this point the DNA is treated with DNA ligase to seal the joints and the fragments become permanently joined in a combinations that may never have existed before. The ability to join a donor DNA fragment of interest to a vector is the basis of the recombinant DNA technology. DNA ligase is an important cellular enzyme, as its function is to repair broken phosphodiester bonds that may occur at random or as a consequence of DNA replication or recombination *in vivo*. In genetic engineering, it is used to seal discontinuities in the sugar phosphate chains that arise when recombinant DNA is made by joining DNA molecules from different sources. It can therefore be thought of as molecular glue, which is used to stick pieces of DNA together.

2) This method is based on the capacity of T4 DNA ligase to join together blunt ended fragments. Thus a fragment of foreign DNA may be linked to a synthetic oligonucleotide of prescribed sequence. If these linker molecules incorporate recognition sites for a specific restriction enzyme, the foreign DNA can be inserted into a plasmid vector, although the foreign DNA did not originally carry any of the enzyme restriction sites present in the plasmid.

3) The availability of terminal transferase enzyme that can add sequences of the same nucleotide (homopolymer) to the 3' end of the DNA chain provides a third splicing method. In this technique one type of DNA carrying exposed 3' hydroxyl end; produced either by exonucleases or restriction enzymes is incubated wit terminal transferase and a pool of only one type of nucleotides, e.g., dGTP. At the same time DNA from another source is given similar treatment but with a pool of complementary nucleotides, e.g., dCTP.

Complementary homopolymer with 3' “tails” are thus generated in two types of DNA permitting them to anneal. Ligation can then follow between the two strands to form the recombinant DNA molecule (Figure 3.6).

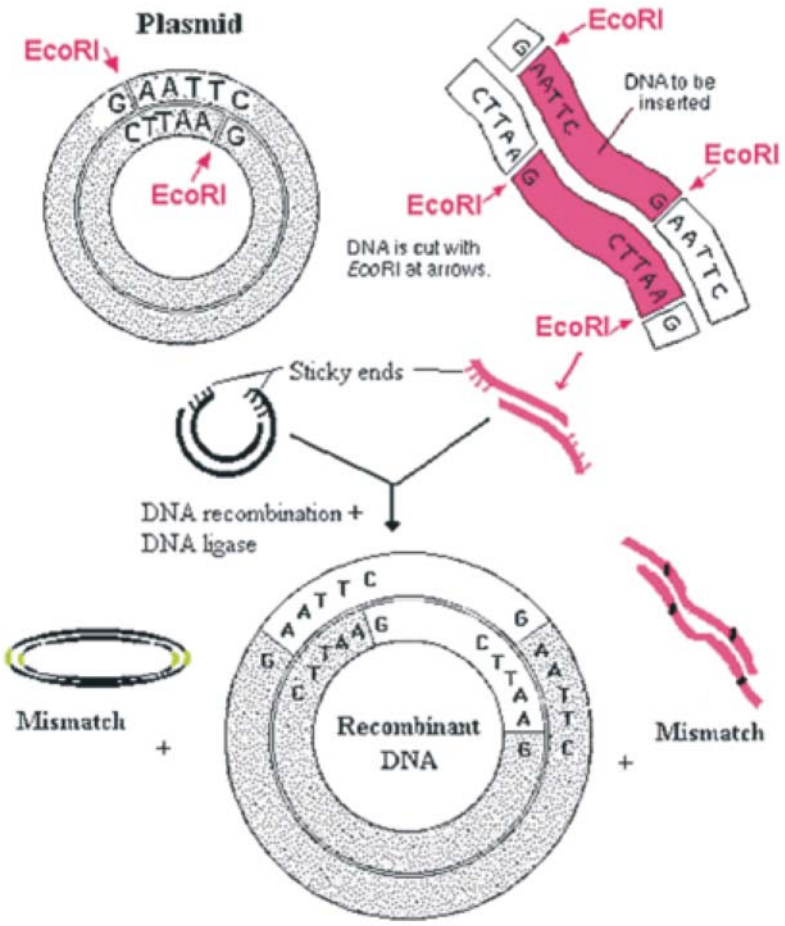


Figure 3.6: DNA Interaction With Plasmid

3. Getting DNA into the Cell or Introduction of DNA into the Host Cell.

After the formation of recombinant DNA molecules, the next step is getting them into the host cell for propagation. The methods available depend upon the type of host/vector system and range from very simple procedures too much more complicated and esoteric ones. Some of the methods available for getting recombinant DNA into the host cells are as follows:

Transformation and Transfection: The techniques of transformation and transfection represent the simplest methods available for getting recombinant DNA into cells. In the context of cloning in *E. coli* cells, transformation refers to the uptake of plasmid DNA and transfection refers to the uptake of phage DNA. Fredrick Griffith first demonstrated transformation in bacteria in 1928, in his famous "transforming principle" experiment that paved the way for the discoveries that eventually showed that genes were made of DNA. However, not all bacteria can be transformed easily, and it was not until the early 1970s that the transformation was demonstrated in *E. coli*, the main stay of gene manipulation technology. To affect the transformation of *E. coli*, the cells need to be made competent. This is achieved by soaking the cells in an ice-cold solution of Calcium chloride, which induces competence in a way that is still not fully understood. Transformation of competent cells is carried out by mixing the plasmid DNA with the cells, incubating on ice for 20-30 minutes, and then giving a brief heat shock (2 minutes at 42°C is often used), which appears to enable the DNA to enter the cells (Figure 3.7). The transformed cells

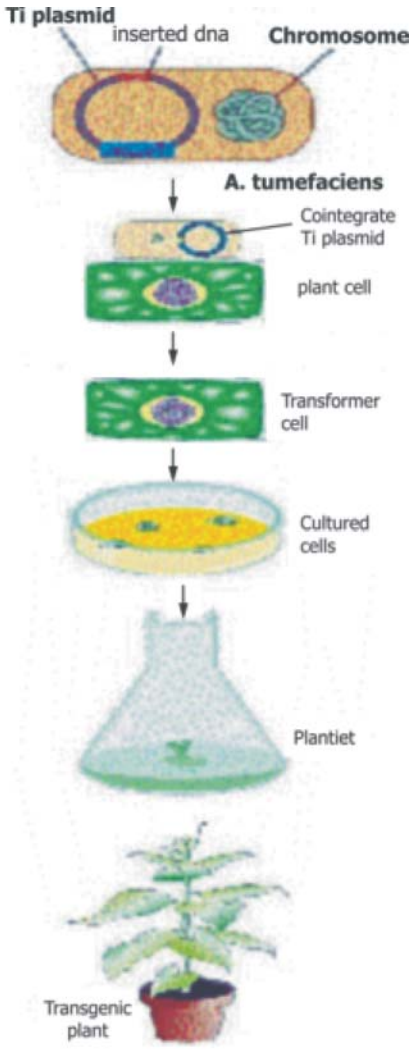


Figure 3.7: Transformation

are usually incubated in nutrient broth at 37°C for 60-90 minutes to enable the plasmid to become established and permit phenotypic expression of their trait.

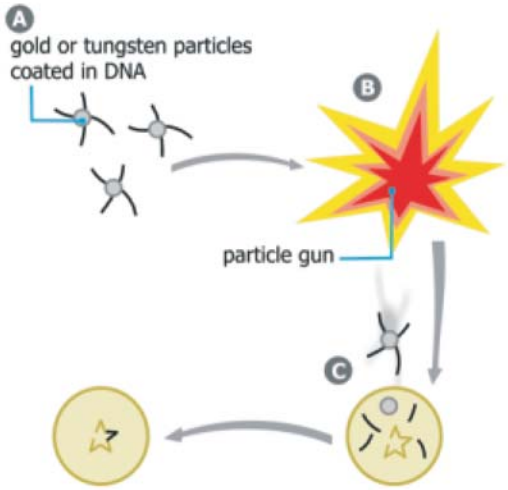
Alternative DNA Delivery Methods: The methods available for introducing DNA into the bacterial cells are not easily transferred to other cell types. The phage specific system is not available for other systems, and transformation by normal methods may prove impossible or too inefficient to be a realistic option. However, there are some alternative methods for introducing DNA into the cells. These are;

a. *Microinjection*

An alternative to transformation procedures is to introduce DNA into the cells by some sort of physical method. One way of doing this is to use a very fine needle and inject the DNA directly into the nucleus.

b. *Biolistic*

A recent development has proved extremely useful in transformation of plant cells. The technique, which is called biolistic DNA delivery, involves literally shooting DNA into the cell by a specific tool called 'Gene Gun'. The DNA is used to coat microscopic tungsten particles known as microprojectile, which are accelerated on a macroprojectile by firing a gunpowder change. At one end of the gun, there is a small aperture that stops the macroprojectile but allows the microprojectile carrying the DNA into the cell and in some cases, stable transformation will occur (Figure 3.8).



A: Particles coated in DNA
B: Particles fired at plant by "biolistic" gun
C: New DNA enters cell, new gene is built into chromosome

Figure 3.8: Transferring a Gene using Biolistic Particle Gun

c. *Electroporation:*

Most of the problems associated with getting DNA into the bacterial cells have involved plant cells. Animal cells are relatively flimsy and can be transformed easily. However, plant cells pose the problem of a rigid cell wall, which is a barrier to DNA uptake. This can be alleviated by the production of protoplasts, in which the cell wall is removed enzymatically. The protoplast can then be transformed using a technique such as electroporation. Where an electric pulse is used to create transient holes in the cell membranes, through which DNA can pass. The protoplasts can then be regenerated (Figure 3.9).

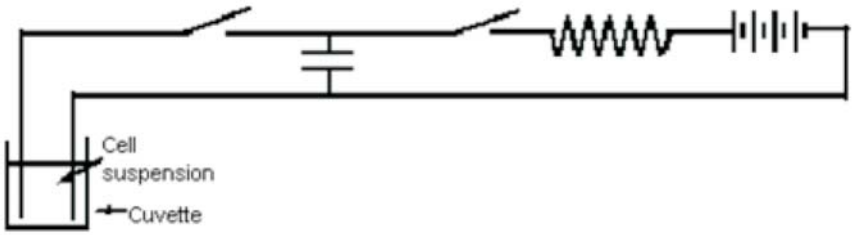


Figure 3.9: Electroporation Procedure

4. Selections and Screening of Recombinant DNA Molecules

When a vector is cleaved by a restriction enzyme and renatured in the presence of many different restriction fragments from a particular organism, many of molecules result, including a self-joined vector molecules containing one or more fragments and a molecule consisting only particular gene. There is a need of some means to ensure that the vector indeed possesses an inserted DNA fragment. So, the success of any cloning experiment depends on being able to identify the desired gene sequence among the many recombinants that may be produced. Before proceeding we should be able to distinguish between the two terms "Selection and Screening".

"Selection" is where some sort of pressure (e.g., the presence of an antibiotic) is applied during the growth of host cells containing recombinant DNA. The cells with the desired characteristics are therefore selected by their ability to survive. This approach ranges in sophistication from simple selection for the presence of a vector,

upto direct selection of cloned DNA fragments by complementation of defined mutation.

Genetic selection methods can be simple or complex depending on the characteristics of the vector/insert combination and one of the types of the host strain used. Such methods are extremely powerful and there is a wide variety of genetic selection and screening techniques available for many diverse applications. Some of these are described below;

Use of Antibiotics: One of the simplest genetic selection methods involves the use of antibiotics to select for the presence of vector molecules. For example, the plasmid pBR322 contains genes for ampicillin resistance (Ap^r) and tetracycline resistance (Tc^r).

Use of Chromogenic Substrates: The use of chromogenic substrates in genetic screening methods has been important aspects of the development of the technology. The most popular system uses the compound X-gal (5-bromo-4-choloro-3-indolyl-ß-D-galactopyranoside), which is a colorless substrate for the ß-galactosidase. On cleavage of X-gal, a blue colored precipitate is formed, thus the expression of the Lac Z (ß-galactosidase) gene can be detected easily.

"Screening" is a procedure by which a population of viable cells is subjected to some sort of analysis that enables the desired sequence to be identified.

Transforming Plants

Transformation is the heritable change in a cell or organism brought about by the uptake and establishment of introduced DNA. There are two main methods of transforming plant cells and tissues:

1. The "Gene Gun" method (also known as microprojectile bombardment or biolistics). This technique has been especially useful in transforming monocot species like corn and rice.
2. The *Agrobacterium* method. *Agrobacterium tumefaciens* is a remarkable species of soil-dwelling bacteria that has the ability to infect plant cells with a piece of its DNA (Figure 3.10).

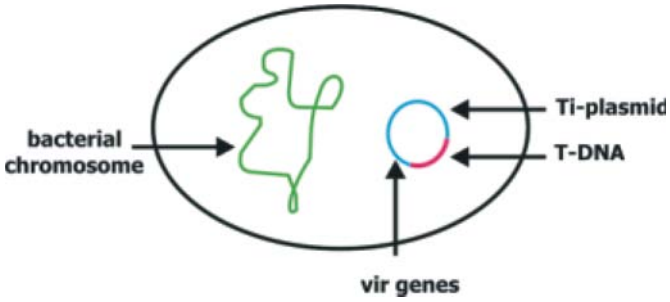


Figure 3.10: Agrobacterium Tumefaciens Cell

Transformation via *Agrobacterium* has been successfully practiced in dicots (broadleaf plants like soybeans and tomatoes) for many years, but only recently has it been effective in monocots (grasses and their relatives) (Figure 3.11). In general, the *Agrobacterium* method is considered preferable to the gene gun, because of the greater frequency of single-site insertions of the foreign DNA, making it easier to monitor.

Figure 3.11: Crown gall of raspberry caused by *Agrobacterium tumefaciens*

The DNA in an *A. tumefaciens* cell is contained in the bacterial chromosome as well as in another structure known as a Ti (tumor-inducing) plasmid. The Ti plasmid contains

- a stretch of DNA termed T-DNA (~20 kb long) that is transferred to the plant cell in the infection process.
- a series of *vir* (virulence) genes that direct the infection process.

Selection of Successfully Transformed Tissues

Following the gene insertion process, plant tissues are transferred to a selective medium containing an antibiotic or herbicide, depending on which selectable marker was used. Only plants expressing the selectable marker gene will survive and it is assumed that these plants will also possess the transgene of interest. Thus, subsequent steps in the process will only use these surviving plants.

Regeneration of Plants

To obtain whole plants from transgenic tissues such as immature embryos, they are grown under controlled environmental conditions in a series of media containing nutrients and hormones, a process known as tissue culture. Once whole plants are generated and produce seed, evaluation of the progeny begins. This regeneration step has been a stumbling block in producing transgenic plants in many species, but specific varieties of most crops can now be transformed and regenerated.

Plant Breeding and Testing

Intrinsic to the production of transgenic plants is an extensive evaluation process to verify whether the inserted gene has been stably incorporated without detrimental effects to other plant functions, product quality, or the intended agro ecosystem. Initial evaluation includes attention to:

- Activity of the introduced gene
- Stable inheritance of the gene
- Unintended effects on plant growth, yield, and quality

If a plant passes these tests, most likely it will not be used directly for crop production, but will be crossed with improved varieties of the crop. This is because only a few varieties of a given crop can be efficiently transformed, and these generally do not possess all the producer and consumer qualities required of modern cultivars. The initial cross to the improved variety must be followed by several cycles of repeated crosses to the improved parent, a process known as backcrossing. The goal is to recover as much of the improved parent's genome as possible, with the addition of the transgene from the transformed parent.

Genetic Engineering is NOT Breeding: The biotech industry and departments of agriculture claim that genetic engineering is a natural extension of traditional breeding. However, traditional agriculture methods, such as cross-pollination or selective breeding, are based on natural reproductive mechanisms. These traditional methods will cross only one kind of plant or animal with a similar species. Genetic engineering crosses the coded DNA barrier and utilizes very powerful (and unnatural) laboratory techniques for transferring genetic material directly between plants and animals. Using these techniques, genes from any plant, animal, virus, or other organism, including a human, can be inserted into any other organism. Using the traditional methods, one could never mate a tomato and a fish, or a canola plant with a human. Yet genetic engineers have done this. Many crops on the market (e.g. soybeans, canola oil, corn) are genetically engineered to be resistant to herbicides, allowing more herbicide use. The herbicides involved are meant to break down more quickly, but the actual rates are highly dependent on local conditions, and soil and water pollution do occur. Genetically engineered insect-resistant crops create different environmental problems. These crops include potatoes, cotton, maize, and corn. They are already on the market and contain their own pesticide (Bt toxin) genetically inserted into the plant to kill insects.

Cloning in Nature

Cloning has been going on in the natural world for thousands of years. A clone is simply one living thing made from another, leading to two organisms with the same set of genes. In that sense, identical twins are clones, because they have identical DNA. Sometimes, plants are self-pollinated, producing seeds and eventually more plants with the same genetic code.

Some forests are made entirely of trees originating from one single plant; the original tree spread its roots, which later sprouted new trees. When earthworms are cut in half, they regenerate the missing parts of their bodies, leading to two worms with the same set of genes. However, the ability to intentionally create a clone in the animal kingdom by working on the cellular level is a very recent development.

Early Progress

Hans Dreisch created the first cloned animals in the late 1800's. Dreich's original goal was not to create identical animals, but to prove that genetic material is not lost

during cell division. In 1902, another scientist, embryologist Hans Spemman, used a hair from his infant son as a knife to separate a 2-celled embryo of a salamander, which also grow externally. He later separated a single cell from a 16-celled embryo.

New Advances

There were no major advances in cloning until November of 1951, when a team of scientists in Philadelphia working at the lab of Robert Briggs cloned a frog embryo. They took the nucleus out of a frog embryo cell and used it to replace the nucleus of an unfertilized frog egg cell. Once the egg cell detected that it had a full set of chromosomes, it began to divide and grow (Figure 3.12). This was the first time that this process, called nuclear transplant, was ever used, and it continues to be used today, although the method has changed slightly.

First Cloned Mammal

A breakthrough came in 1986. Two teams, working independently but using nearly the same method, each on opposites side of the Atlantic, announced that they had cloned a mammal. Steen Willadsen in England, which cloned a sheep's embryo, led one team. Neal First in America, which cloned a cow's embryo, led the other team.

Dolly: Dolly, the scientific sensation of 1997, is the most famous sheep in the world. Ian Wilmut at the Roslin Institute in Scotland created a sheep that produced a certain chemical in its milk. One of Wilmut's colleagues, who had experience with cloning from early embryo cells, suggested that the reason so many cloning attempts failed was that the cells were in incompatible stages of life. In one stage, the cells are adding to the DNA, in another, they are proofreading it, and in another, splitting it. Wilmut's team learned that by starving the cells, they could be forced into what is called the G_0 phase, similar to cellular hibernation.

This advance increased the survival rate of the cloned cells; Megan and Morag, two lambs, were cloned from sheep embryos. More work was done, and on July 5, 1996, a lamb was born, cloned from a frozen mammary cell from another adult sheep. Wilmut, who names his animals very creatively, named her Dolly after Dolly Parton (Figure 3.13). The manipulation was done using microscopic needles, a method pioneered in human fertility treatments twenty years ago (Figure 3.14). A third, surrogate sheep, was implanted with the resulting embryo.

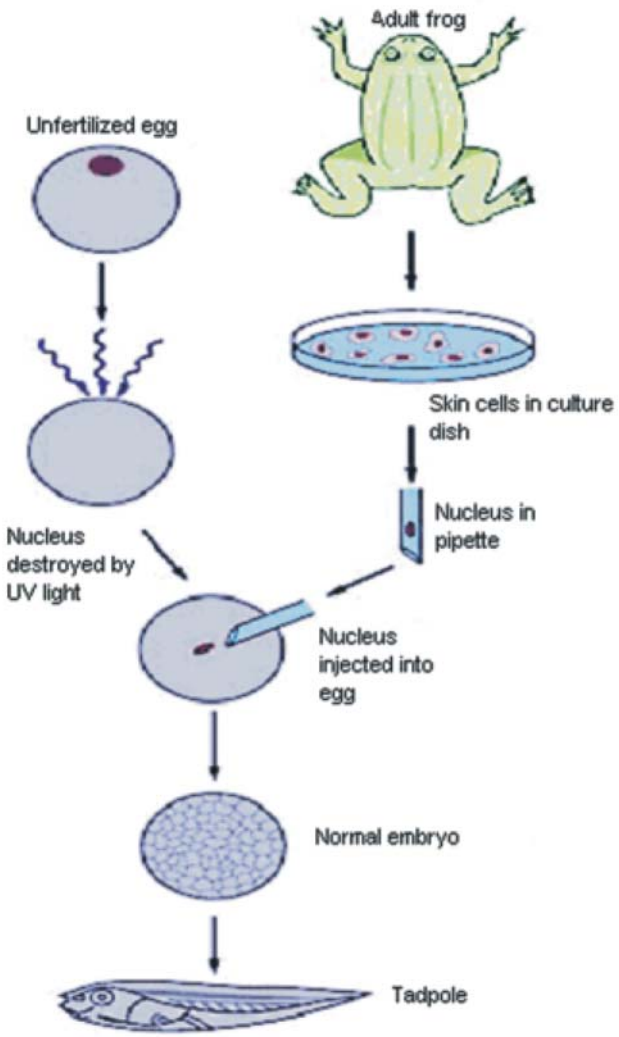


Figure 3.12: Cloning from Adult Vertebrate Cells

Figure 3.13: The young lamb named Dolly (left), with her surrogate mother, was created by cloning at the Roslin Institute.

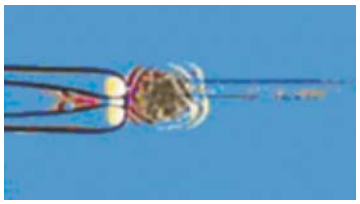


Figure 3.14: Removing the egg nucleus by microscopic needle

Herd of Mice: Honolulu Technique created Cumulina 'the cloned mouse' in 1997. She was cloned from cumulus cells (cells which surround developing egg cells) using traditional nuclear transfer. The nucleus was taken from the cumulus cell and implanted in an egg cell from another mouse. The new cell was then treated with a chemical to make it grow and divide. The scientists repeated the process for three generations, yielding over fifty mice that are virtually identical by the end of July, 1998. As cloning technology improves, more and more applications will be seen in everyday life.

Applications

Reliable cloning can be used to make farming more productive by replicating the best animals. It can make medical testing more accurate by providing test subjects that all react the same way to the same drug. It can allow mass production of genetically altered animals, plants, and bacteria. It may settle once and for all what part of personality is dependent on genetics and what part on environment. In short, it can be beneficial to almost every area of biological science.

DNA cloning is a technique that allows the wholesale production of a specific DNA sequence. DNA containing a gene of interest is inserted into the purified DNA genome of a self-replicating element, which can be a plasmid, a virus or in this case, a yeast artificial chromosome (YAC). A YAC can be considered as a functional artificial chromosome (self replicating element), since it includes three specific DNA sequences that enable it to propagate from one cell to its offspring:

- **TEL:** The telomere, which is located at each chromosome end, protects the linear DNA from degradation by nucleases.
- **CEN:** The centromere, which is the attachment site for mitotic spindle fibers, "pulls" one copy of each duplicated chromosome into each new daughter cell.
- **ORI:** Replication origin sequences, which are specific DNA sequences that allow the DNA replication machinery to assemble on the DNA and move at the replication forks.

It also contains few other specific sequences like:

- **A** and **B:** selectable markers that allow the easy isolation of yeast cells that have taken up the artificial chromosome.
- Recognition site for the two restriction enzymes EcoRI and BamHI.

While DNA cloning into plamsid allows the insertion of DNA fragment of about 10,000 nucleotide base pairs, DNA cloning into a YAC allows the insertion of DNA fragments up to 1,000,000 nucleotide base pairs.

Cloning Human Genomic DNA into a YAC: Genomic DNA is partially digested by the restriction enzyme EcoRI. Very large DNA fragments are obtained. The YAC is digested by the two restriction enzymes EcoRI and BamHI. Those two elements

recombine at the EcoRI sites and are covalently linked by the DNA ligase. A recombinant YAC vector, a yeast artificial chromosome with genomic DNA inserted, is produced. This vector can be used to infect yeast cells and generated an unlimited number of copies.

Monoclonal Antibody Production

Process by which large quantities of antibodies (targeted against a particular antigen X) can be produced. A mouse is immunized by injection of an antigen X to stimulate the production of antibodies targeted against X. The antibody forming cells are isolated from the mouse's spleen. Fusing single antibody-forming cells to tumor cells grown in culture produces monoclonal antibodies. The resulting cell is called a hybridoma (Figure 3.15).

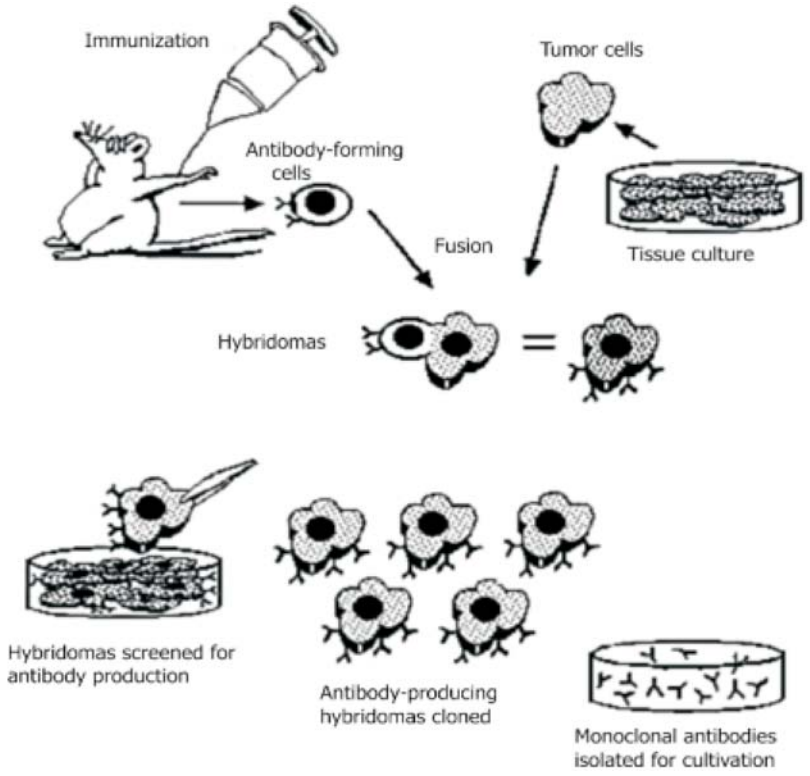


Figure 3.15: Antibody Production

Each hybridoma produces relatively large quantities of identical antibody molecules. By allowing the hybridoma to multiply in culture, it is possible to produce a population of cells, each of which produces identical antibody molecules. These antibodies are called "monoclonal antibodies" because they are produced by the identical offspring of a single, cloned antibody-producing cell.

Transgenic Plants

A transgenic crop plant contains a gene or genes that have been artificially inserted instead of the plant acquiring them through pollination. The inserted gene sequence (known as the transgene) may come from another unrelated plant, or from a completely different species:

Transgenic Bt corn, for example, which produces its own insecticide (Figure 3.16), contains a gene from a bacterium. Plants containing transgenes are often called genetically modified or GM crops although in reality all crops have been genetically modified from their original wild state by domestication, selection and controlled breeding over long periods of time.

Figure 3.16: Results of insect infestation on Bt (right) and non-Bt (left) cotton bolls

Future Transgenic Products

Corn Hybrids Resistant to Corn Rootworm: Corn rootworm (*Diabrotica* spp.) is a serious pest of corn. It damages roots of young corn seedlings, resulting in reduced growth and poor standability of the plant (Figure 3.17). This insect is responsible for the application of the largest amount of insecticide to cornfields What's more, to control this pest the insecticide must be applied directly to the soil, where it may leave residues or leach into the ground water. By replacing these chemical insecticides, corn rootworm resistant hybrids may provide major benefits to environmental quality.

Figure 3.17: Corn Rootworm Feeding on a Young Maize Root

Golden Rice: Millions of people in the world suffer from Vitamin A deficiency, which leads to vision impairment and increased susceptibility to diarrhea, respiratory diseases, and measles. Rice is a staple food in many countries, particularly in Asia, but does not contain Vitamin A or its immediate precursors. By inserting two genes from daffodil and one gene from a bacterial species into rice plants, Swiss researchers have produced rice capable of synthesizing beta-carotene, the precursor of Vitamin A. This rice variety is now being crossed into adapted varieties, with field tests possible in a year or two (Figure 3.18).

Figure 3.18: Developing Kernels of Rice

Tomato: Because tomatoes are one of the world's most popular vegetables, they have benefited from a long history of genetic improvement that continues in the transgenic age. Lycopene, a naturally occurring constituent of tomato, is a nutritional factor related to Vitamin A. Tomato varieties with transgenically enhanced lycopene content are under investigation (Figure 3.19). Another trait of interest is delayed ripening. Tomatoes that ripen slower can remain on the vine longer and develop improved flavor, compared to commercial varieties that are picked at the green stage.

Figure 3.19: Improved nutritional content and delayed ripening are transgenic traits of interest in tomatoes

Canola: Canola is a major oilseed crop. Transgenic research has focused on improving the nutritional quality of canola oil by enhancing the Vitamin E content or by modifying the balance of fatty acids.

Plant-based Vaccines: Food crops engineered to produce edible vaccines against infectious diseases would make vaccination more readily available to children around the world. Transgenic bananas containing inactivated viruses that cause cholera, hepatitis B, and diarrhea have been produced and are currently undergoing evaluation.

Sunflower: White mold (*Sclerotinia*) is a serious problem for sunflower producers in some areas. Resistance to this disease would expand the area in which sunflowers can be grown and might improve yields in areas of current cultivation. A commercially available cultivar is not expected before 2005. Resistance to the Argentina looper, an insect that eats sunflower leaves, is also being investigated

(Figure 3.20). Some research has been done on developing sunflowers that can tolerate being sprayed with the weed killer Roundup.

Figure 3.20: Sunflower

Coffee and Tea: Decaffeinated coffee is now made by treating coffee beans to remove the caffeine. Two scientists in Hawaii and Scotland have identified different genes that lead to the production of caffeine in coffee beans and tealeaves. If these genes can be "turned off" in some plants, coffee and tea trees could be developed that would produce naturally decaffeinated products with full flavor and aroma.

Grapes and Wine: Grape vines (*Vitis vinifera*) are susceptible to several diseases that reduce the amount and the quality of wine grapes and table grapes or even kill the vine. Genes that confer resistance to particular diseases would reduce the cost of battling diseases in the vineyard. Researchers at the University of Florida have patented a method for producing grape vines that carry a silkworm gene to provide protection from Pierce's disease, a fatal bacterial disease of grapes as shown in figure 3.21.

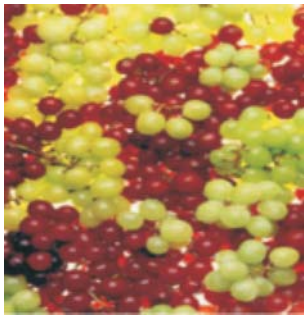


Figure 3.21: GM Grapes

Tobacco: Nicotine-free tobacco is now being grown for a projected introduction of nicotine-free cigarettes. Previous attempts to make low-nicotine products removed some of the flavor along with the nicotine. Genetically engineered nicotine-free tobacco doesn't synthesize nicotine in the leaf.

Trees: Forest trees such as poplar, aspen, and spruce have been transformed with various genes to provide resistance to insects, tolerance to herbicides, and higher

levels of the commercial product (Figure 3.22). For example, reducing the lignin content of a tree can make it easier to recover wood pulp.

Figure 3.22: Gymnosperm Plant

cDNA

Human genes composed of coding and non-coding sequences. The copy of the coding sequences is called cDNA. It can be obtained from the reverse transcription of messenger RNA. The transcription and translation of the insulin cDNA allows the production of a functional insulin molecule.

cDNA Library Construction

"Reverse transcription" is a mechanism whereby genetic information contained in mRNA is converted back into a double stranded DNA form (Figure 3.23). The enzyme responsible for this is an RNA dependent DNA polymerase called reverse transcriptase. Reverse transcriptases have traditionally been isolated from viruses whose genome are in an RNA form and must be converted to duplex DNA. These viruses typically carry a functional reverse transcriptase along with their mRNA genetic component when they infect cells. One of the most common commercially available reverse transcriptases is Moloney murine leukemia virus (MMLV). This RNA dependent DNA polymerase (as all polymerases) adds nucleotides to a nascent polynucleotide in the 5' to 3' direction. It does not contain any 3' to 5' exonuclease (proofreading) activity.

If we can introduce "nicks" into the RNA half of this DNA/RNA duplex then the situation would be very similar to that observed in "lagging strand" synthesis of prokaryotic genomic DNA. Nicks in the RNA half of the molecule can be introduced via the action of the enzyme RNAse H.

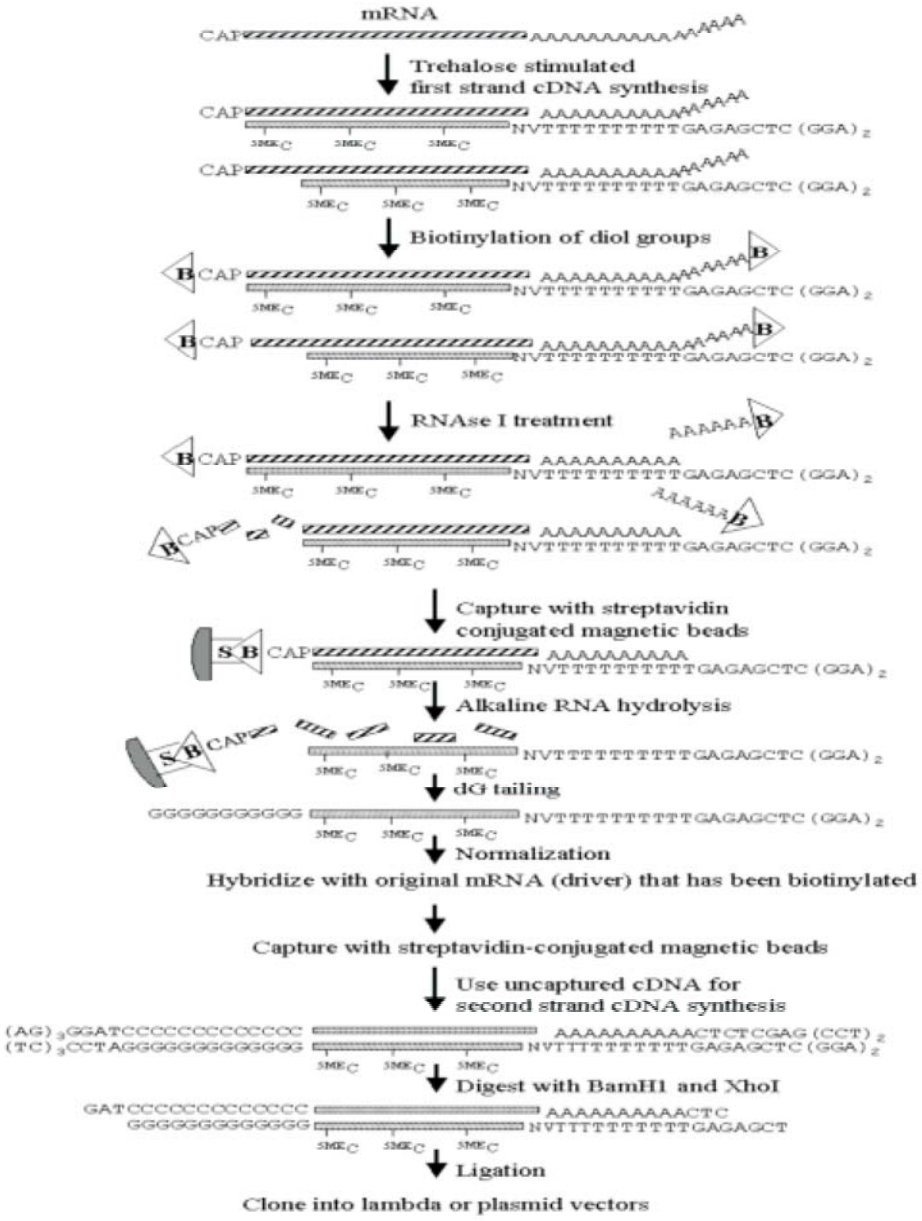


Figure 3.23: cDNA Library Construction

This enzyme exhibits endonucleolytic cleavage of the RNA moiety of RNA/DNA hybrids, as well as 5' to 3' and 3' to 5' exoribonuclease activity. In other words, it will nick the RNA and then proceed to digest back in both directions. These RNA fragments can now serve as primers for DNA synthesis by *E. coli* Pol I. This enzyme will also translate the "nicks" to effectively remove the RNA primers.

Synthesis of Double Stranded cDNA from mRNA: A short oligo(dT) chain is hybridized to the poly(A) tail of an mRNA strand. The oligo(dT) segment serves as a primer for the action of reverse transcriptase, which uses the mRNA as a template for the synthesis of complementary DNA strand. The resulting cDNA ends in a hairloop (Figure 3.24).

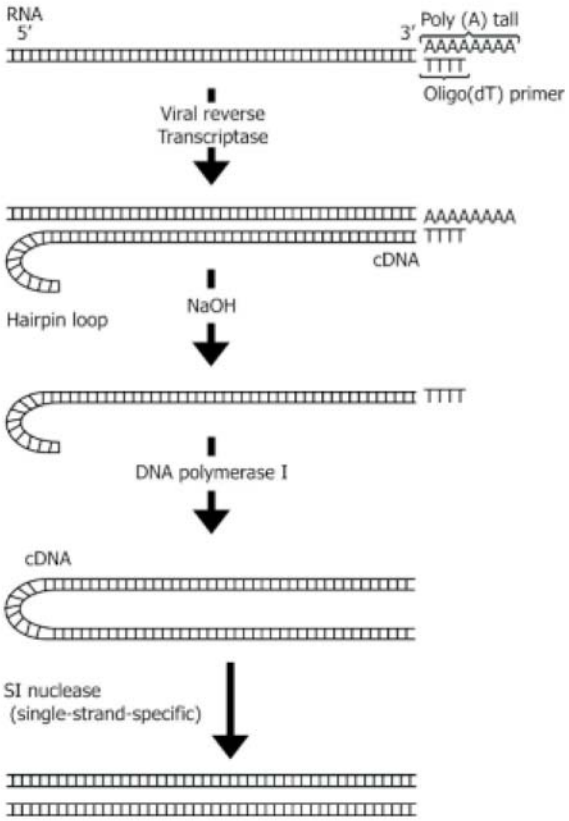


Figure 3.24: Formation of double stranded cDNA

When the mRNA strand has been degraded by treatment with NaOH, the hairloop becomes a primer for DNA polymerase I, which completes the paired DNA. The loop is then cleaved by S1 nuclease (which acts only on the single-stranded loop) to produce a double-stranded cDNA molecule.

Locating Genes for Important Traits: Identifying and locating genes for agriculturally important traits are currently the most limiting step in the transgenic process. Usually, identifying a single gene involved with a trait is not sufficient so it should be known that how the gene is regulated, what other effects it might have on the plant, and how it interacts with other genes active in the same biochemical pathway (Figure 3.25).

Figure 3.25: Simplified representation of a constructed transgene containing necessary components for successful integration and expression.

Designing Genes for Insertion

Once a gene has been isolated and cloned (amplified in a bacterial vector), it must undergo several modifications before it can be effectively inserted into a plant.

1. A promoter sequence must be added for the gene to be correctly expressed (i.e., translated into a protein product). The promoter is the on/off switch that controls when and where in the plant the gene will be expressed. To date, most promoters in transgenic crop varieties have been "constitutive", i.e., causing gene expression throughout the life cycle of the plant in most tissues. The termination sequence signals to the cellular machinery that the end of the gene sequence has been reached.
2. A selectable marker gene is added to the gene "construct" in order to identify plant cells or tissues that have successfully integrated the transgene. This is necessary because achieving incorporation and expression of transgenes in plant cells is a rare event, occurring in just a few percent of the targeted tissues or cells. Selectable marker genes encode proteins that provide

resistance to agents that are normally toxic to plants, such as antibiotics or herbicides.

Chromatography

Column chromatography is one of the most common methods of protein purification. Like many of the techniques on this site, it is as much an art form as a science. Proteins vary hugely in their properties, and the different types of column chromatography allow exploitation of those differences. Most of these methods do not require the denaturing of proteins. To be very general, a protein is passed through a column that is designed to trap or slow up the passing of proteins based on a particular property (such as size, charge, or composition).

There are three main steps to protein purification:

1. Capture: A protein is needed into a concentrated form. If, for example, it is tried to isolate a protein synthesized in an *E. coli* cell, it could be looking at a protein to junk ratio of 1:1,000,000. For capture purification a high capacity method is needed that is also fast.

2. Intermediate: Intermediate purification requires both speed and good resolution.

3. Polishing: For the final step of purification a system is needed that has both good resolution and speed. Capacity is usually irrelevant at this stage.

Some of the more common columns include:

IEX: Ion exchange chromatography. Good for capture, intermediate, and polish.

HIC: Hydrophobic interaction column. Good for intermediate purification.

AC: Affinity chromatography. Good for capture and intermediate purification.

GF: Gel filtration (size exclusion) chromatography. Good polishing step.

Ion Exchange Chromatography

Ion exchange chromatography is based on the charge of the protein to be isolate. If protein has a high positive charge, it should pass through a column with a negative charge. The negative charge on the column will bind the positively charged protein, and other proteins will pass through the column. Then a procedure is used called "salting out" to release positively charged protein from the negatively charged

column. The column that does this is called a cation exchange column and often uses sulfonated residues. Likewise, a negatively charged protein can be bound to a positively charge column. The column that does this is called an anion exchange column and often uses quaternary ammonium residues. Salting out will release, or elute, protein from the column. This technique uses a high salt concentration solution. The salt solution will compete the protein in binding to the column. In other words, the column has a higher attraction for the charge of salts than for the charged protein, and it will release the protein in favor of binding the salts instead. Proteins with weaker ionic interactions will elute at a lower salt, so it is often desirable to elute with a salt gradient. Different proteins elute at different salt concentrations.

Hydrophobic Interaction Chromatography

Where ion exchange chromatography relies on the charges of proteins to isolate them, hydrophobic interaction chromatography uses the hydrophobic properties of some proteins. Hydrophobic groups on the protein bind to hydrophilic groups on the column. The more hydrophobic a protein is, the stronger it will bind to the column.

Gel Filtration (Size Exclusion) Chromatography

Gel filtration, or size exclusion, chromatography separates proteins on the basis of their size. The column is packed with a matrix of fine porous beads. Affinity chromatography relies on the biological functions of a protein to bind it to a column. The most common type involves a ligand, a specific small biomolecule. This small molecule is immobilized and attached to a column matrix, such as cellulose or polyacrylamide. Target protein is then passed through the column and bound to it by its ligand, while other proteins elute out. Passing through the column a solution that has in it a high concentration of free ligand usually does elution of target protein. This is a very efficient purification method since it relies on the biological specificity of target protein, such as the affinity of an enzyme for a substrate.

Nucleic Acid Hybridization

The two strands of a DNA molecule are denatured by heating to about 100°C = 212°F. At this temperature, the complementary base pairs that hold the double helix strands together are disrupted and the helix rapidly dissociates into two single strands. The DNA denaturation is reversible by keeping the two single stands of DNA

for a prolonged period at 65°C = 149°F. This process is called DNA renaturation or hybridization.

Cloning into a Plasmid

Plasmids + Copies of DNA Fragment Produce Recombinant DNA: The recombinant DNA stew is allowed to transform a bacterial culture, which is then exposed to antibiotics (Figure 3.26). All the cells except those, which have been encoded by the plasmid DNA recombinant, are killed, leaving a cell culture containing the desired recombinant DNA.

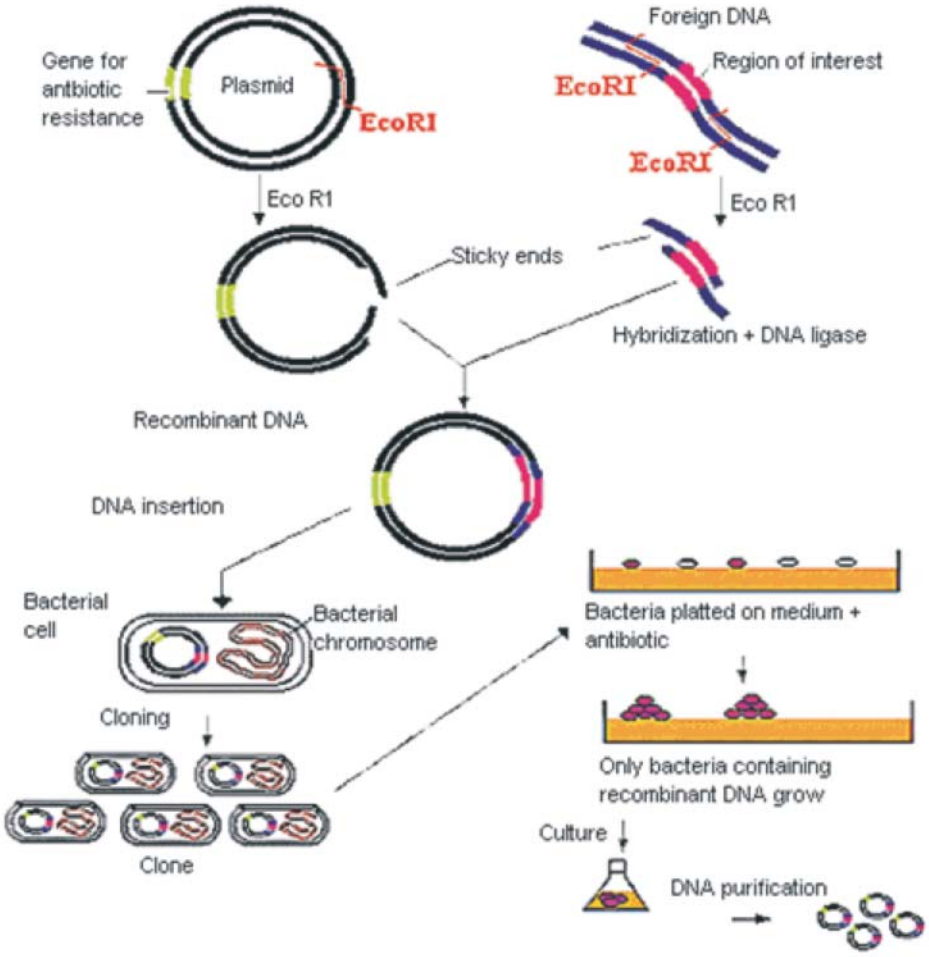


Figure 3.26: Plasmid Cloning

Transfer and Cloning of the Insulin Gene

Technique of DNA Cloning into Plasmid: Insertion of the gene coding for insulin into a bacterial plasmid, which in turn carries the gene into a replicating bacterial cell that produces human insulin (Figure 3.27).

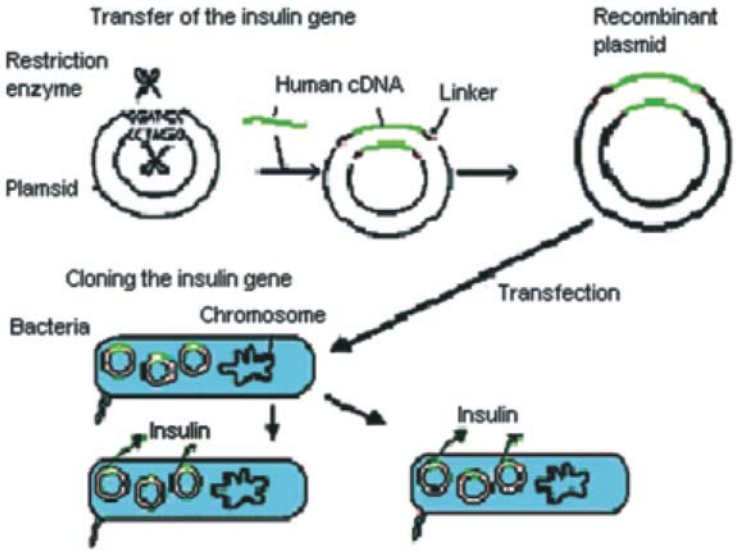


Figure 3.27: Transfer and Cloning of Insulin Gene

Transfer of the Insulin gene into a plasmid vector

- The plasmid is cut across both strands by a restriction enzyme, leaving loose, sticky ends to which DNA can be attached.
- Special linking sequences are added to the human cDNA so that it will fit precisely into the loose ends of the opened plasmid DNA ring.
- The plasmid containing the human gene, also called a recombinant plasmid, is now ready to be inserted into another organism, such as a bacterial cell.

The recombinant plasmids and the bacterial cells are mixed up. Plasmids enter the bacteria in a process called transformation. With the recombinant DNA molecule successfully inserted into the bacterial host, another property of plasmids

can be exploited - their capacity to replicate. Once inside a bacterium, the plasmid containing the human cDNA can multiply to yield several dozen copies. When the bacteria divide, the plasmids are divided between the two daughter cells and the plasmids continue to reproduce. With cells dividing rapidly (every 20 minutes), a bacterium containing human cDNA (encoding for insulin, for example) will shortly produce many millions of similar cells (clones) containing the same human gene.

Transgenic Mice

Genes responsible for particular traits or disease susceptibility are chosen and cloned. Next they are injected into fertilized mouse eggs (Figure 3.28). Embryos are implanted in the uterus of a surrogate mother. The selected genes will be expressed by some of the offspring. Since the first gene transfers into mice were successfully executed in 1980, transgenic mice have allowed researchers to observe experimentally what happens to an entire organism during the progression of a disease. Transgenic mice have become models for studying human diseases and their treatments.

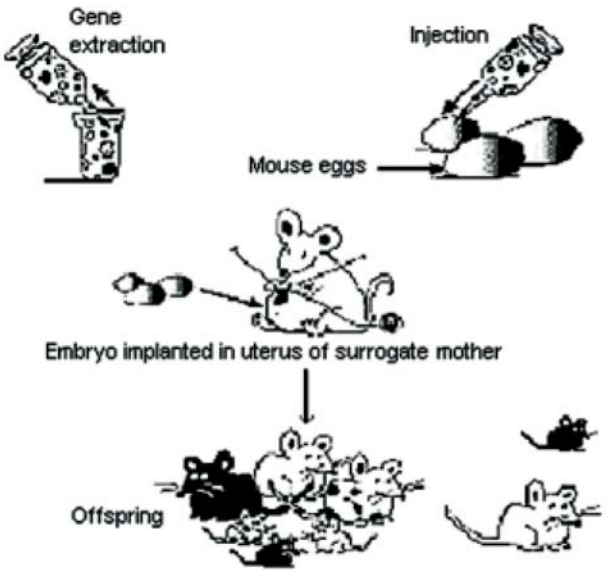


Figure 3.28: Transgenic Mice

Polymerase Chain Reaction (PCR)

The elegant technique of PCR, by which fragments of DNA can be made to replicate very rapidly, is illustrated. Polymerase chain reaction (PCR) is a common method of creating copies of specific fragments of DNA. PCR rapidly amplifies a single DNA molecule into many billions of molecules (Figure 3.29). In one application of the technology, small samples of DNA, such as those found in a strand of hair at a crime scene, can produce sufficient copies to carry out forensic tests.

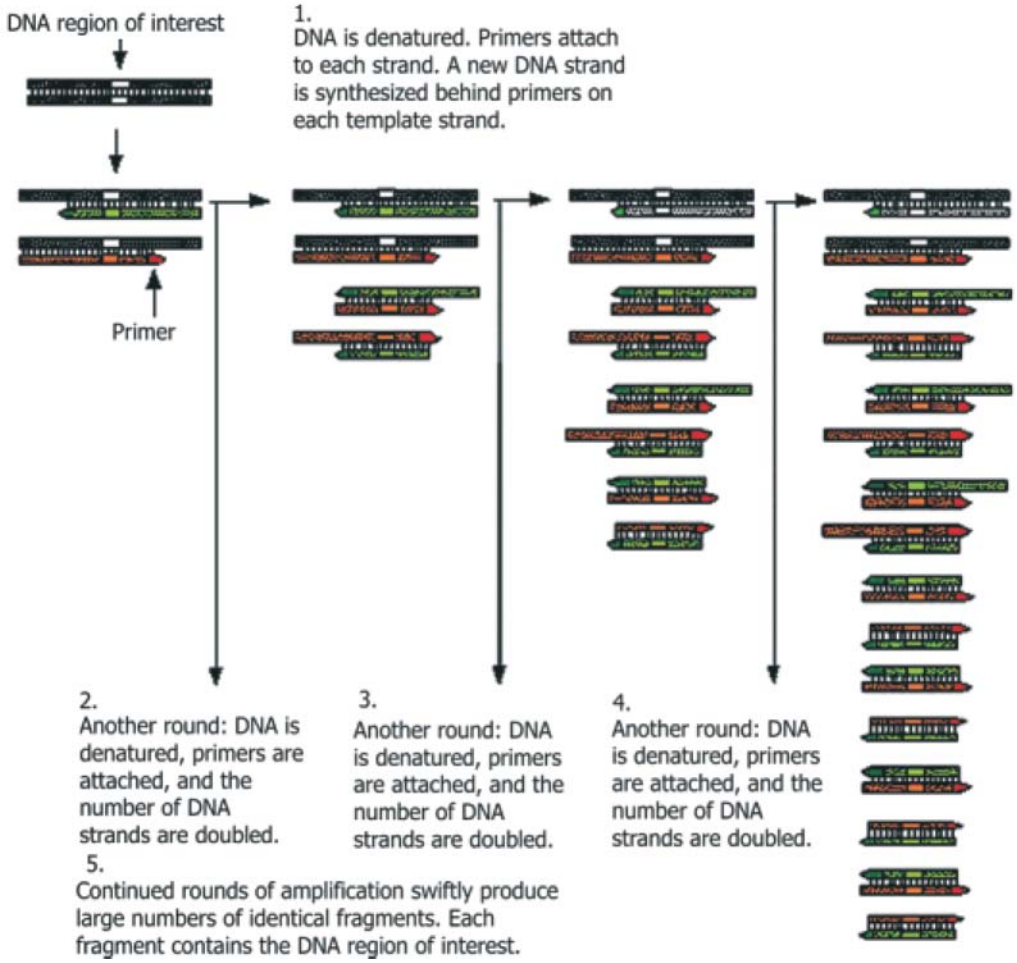


Figure 3.29: PCR Protocol

Gel Electrophoresis

Electrophoresis is used to separate molecules by some property. In the case of DNA we can separate the molecules based on their size. DNA has a negative charge in solution, so it will migrate to the positive pole in an electric field. In agarose gel electrophoresis the DNA is forced to move through a sieve of molecular proportions that is made of agarose i.e. based on size. The end result is that large pieces of DNA move slower than small pieces of DNA. The place in the gel that the DNA migrated to is observable under ultraviolet light when the current is turned off and the gel is stained with ethidium bromide.

SDS-PAGE

Where agarose gels are best for running larger molecules, like DNA, SDS-PAGE is better suited for running smaller ones, like proteins.

SDS-PAGE has a number of uses, which include:

- Establishing protein size
- Protein identification
- Determining sample purity
- Identifying disulfide bonds
- Quantifying proteins
- Blotting applications

SDS-PAGE stands for sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis. The SDS is a detergent. The purpose of the SDS detergent is to take the protein from its native shape, which is basically a big glob, and open it up into a linear piece. It's kind of like taking a wadded up ball of string and untangling it into one straight, long piece. This will allow it to run more efficiently down the gel.

In more scientific terms, it is an anionic detergent that binds quantitatively to proteins, giving them linearity and uniform charge, so that they can be separated solely on the basis if their size. The SDS has a high negative charge that overwhelms any charge the protein may have, imparting all proteins with a relatively equal negative charge. The SDS has a hydrophobic tail that interacts strongly with protein

(polypeptide) chains. The number of SDS molecules that bind to a protein is proportional to the number of amino acids that make up the protein.

The polyacrylamide gel electrophoresis works in a similar fashion to an agarose gel, separating protein molecules according to their size. In electrophoresis, an electric current is used to move the protein molecules across a polyacrylamide gel. The polyacrylamide gel is a cross-linked matrix that functions as a sort of sieve to help "catch" the molecules as they are transported by the electric current. The smaller molecules are able to navigate the mesh faster than the larger one, so they make it further down the gel than the larger molecules. This is how SDS-PAGE separates different protein molecules according to their size.

Once an SDS-PAGE gel is run, the proteins are needed to be fixed in the gel so they don't come out when stain the gel. Acetic acid 25% in water is a good fixative, as it keeps the proteins denatured. The gel is typically stained with Coomasie blue dye R-250, and the fixative and dye can be prepared in the same solution-using methanol as a solvent. The gel is then destained and dried.

Southern Blotting

Southern blotting was named after Edward M. Southern who developed this procedure at Edinburgh University in the 1970s. To oversimplify, DNA molecules are transferred from an agarose gel onto a membrane. Southern blotting is designed to locate a particular sequence of DNA within a complex mixture. For example, Southern Blotting could be used to locate a particular gene within an entire genome by using radiolabel or chemical label probe (Figure 3.30).

The amount of DNA needed for this technique is dependent on the size and specific activity of the probe. Short probes tend to be more specific. Under optimal conditions, It can be expected to detect 0.1 pg of the DNA for which probing has been done.

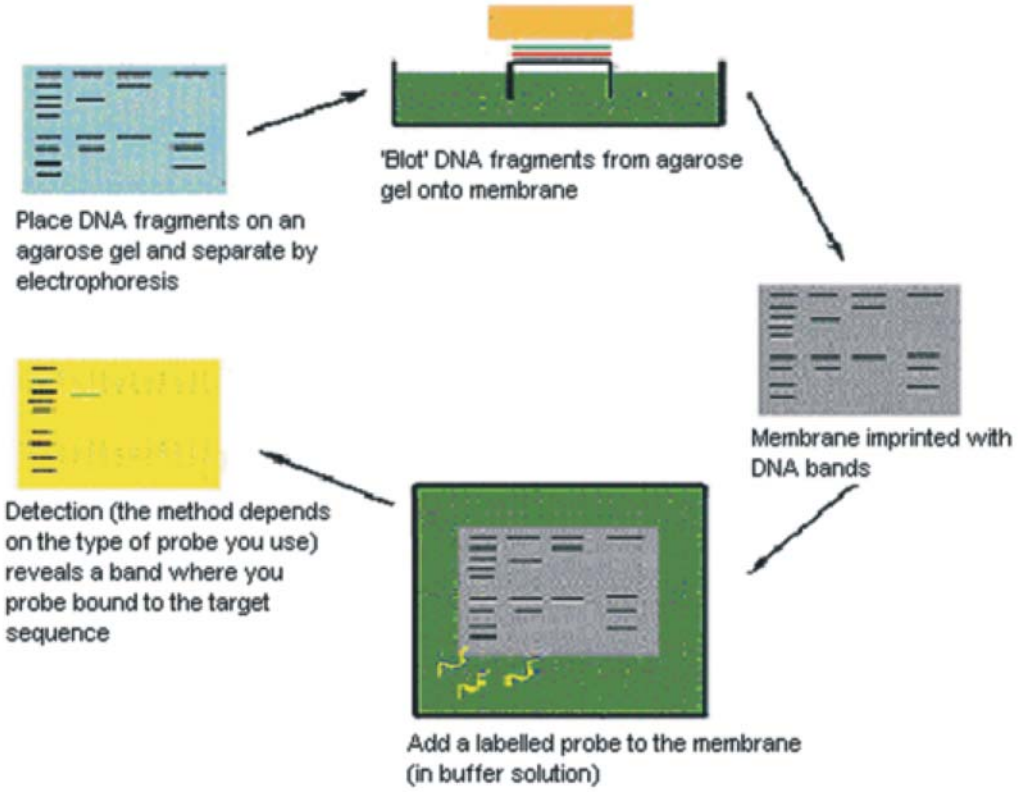


Figure 3.30: This diagram shows the basic steps involved in a Southern blot

Northern Blotting

Sometimes it's a bit hard to understand, but there is humor in science. In the 1970s, E.M. Southern developed a method for locating a particular sequence of DNA within a complex mixture. This technique came to be known as Southern blotting. In a tongue-in-cheek fashion, those who used a similar method for locating a sequence of RNA named it Northern blotting. It is also known as Northern hybridization or RNA hybridization. The procedure for and theory behind Northern blotting is almost identical to that of Southern blotting, except working with RNA instead of DNA.

Western Blotting

Western blot analysis can detect one protein in a mixture of any number of proteins while giving information about the size of the protein. It does not matter whether the protein has been synthesized in vivo or in vitro. This method is, however, dependent on the use of a high-quality antibody directed against a desired protein. Thus, this antibody can be used as a probe to detect the protein of interest.

Western blotting tells how much protein has accumulated in cells. If rate of synthesis of a protein has to be determined, Radio-Immune Precipitation (RIP) may be the best assay. Also, if a protein is degraded quickly, Western blotting won't detect it well then RIP is used.

DNA Sequencing

DNA sequencing is the method to define a complete base sequence of desired DNA strand for engineering purposes. In the automated Sanger sequencing method, the single-stranded DNA to be sequenced is "primed" for polymerization with a short complementary strand at one end. This preparation is then divided into four batches, and each is treated with a different replication-halting nucleotide (depicted here with a diamond shape), together with the four "usual" nucleotides. Each reaction then proceeds until a reaction-terminating nucleotide is incorporated into the growing strand, whereupon extension/polymerization stops. Thus, the "C" reaction produces new strands that terminate at positions corresponding to the G's in the strand being sequenced (Figure 3.31). (Note that when long strands are being sequenced the concentration of the reaction-terminating nucleotide must be carefully chosen, so that a "normal" C is usually paired with a G; otherwise, replication would typically stop with the first or second G). Gel electrophoresis, one lane per reaction mixture, is then used to separate the replication products, from which the sequence of the original single strand can be inferred.

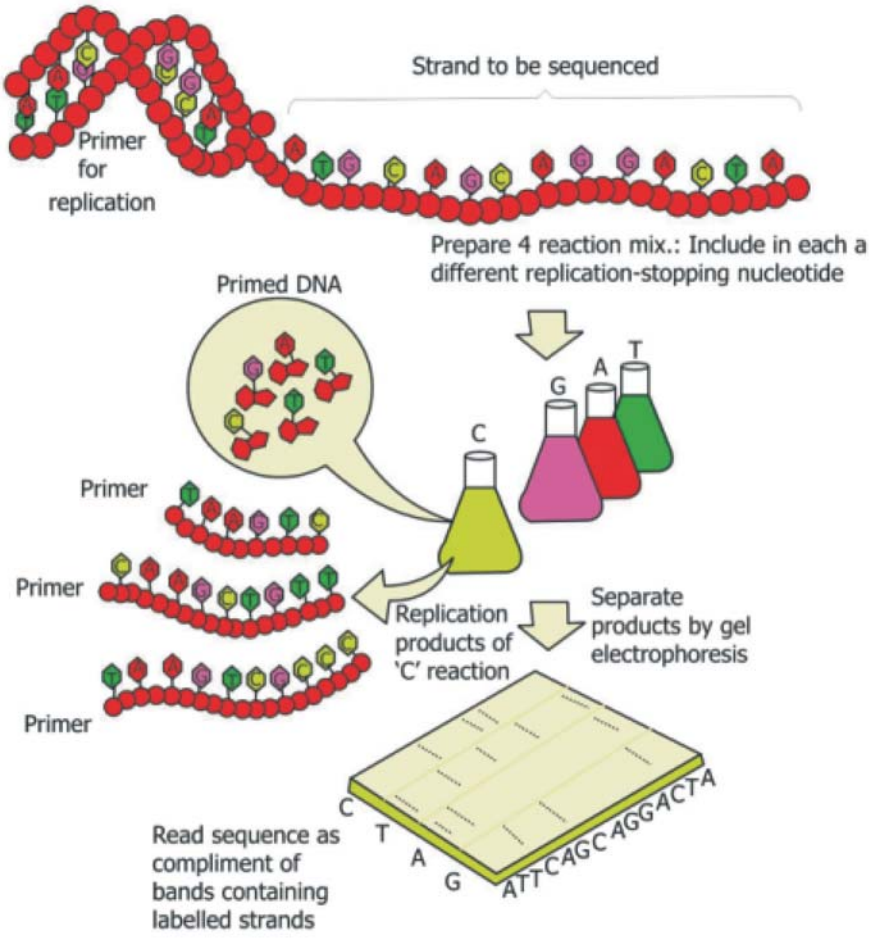


Figure 3.31: DNA Sequencing Procedure

Developments in Transgenic Technology

New techniques for producing transgenic plants will improve the efficiency of the process and will help resolve some of the environmental and health concerns. Among the expected changes are the following:

- More efficient transformation, that is, a higher percentage of plant cells will successfully incorporate the transgene.
- Better marker genes to replace the use of antibiotic resistance genes.

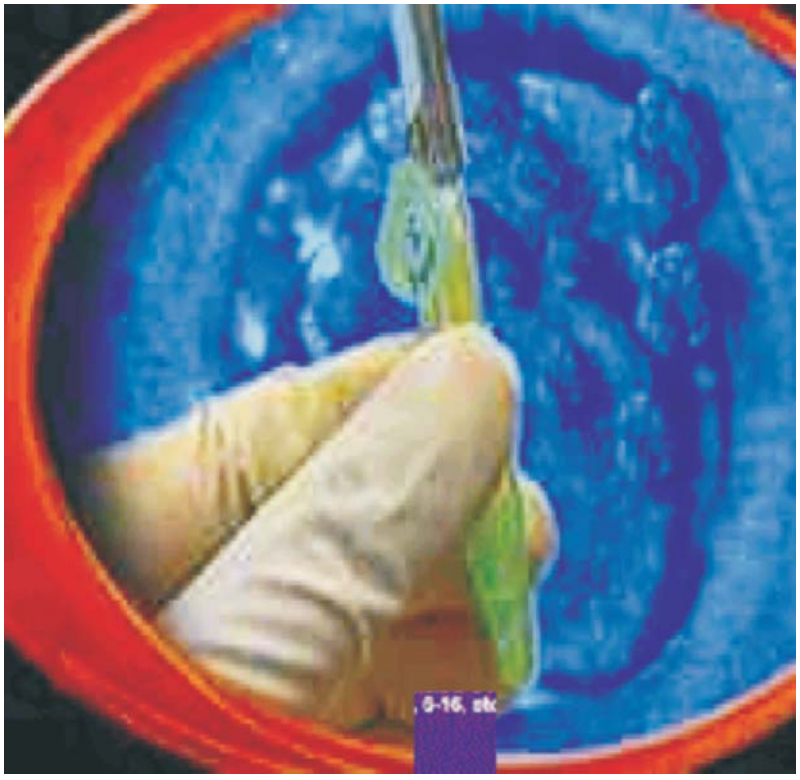
- Better control of gene expression through more specific promoters, so that the inserted gene will be active only when and where needed.
- Transfer of multi-gene DNA fragments to modify more complex traits.

Some transgenic tree species now being tested include:

Species	Trait
Poplar	Herbicide tolerance, insect resistance
Eucalyptus	Herbicide tolerance
Aspen	Reduced lignin
White spruce	Insect resistance

Chapter 4

Culture Techniques



Chapter 4

CULTURE TECHNIQUES

Tissue Culture

Plant Tissue and Cell Culture

Plant cell culture is viewed as a potential means of producing useful plant products such as conventional agriculture. Plant tissue culture (micropropagation) is a technique, which is used so extensively in the nursery business and in plant biotechnology. It is a fascinating and useful tool, which allows the rapid production of many genetically identical plants using relatively small amounts of space, supplies and time (Figure 4.1). Basically the technique consists of taking a piece of a plant i.e. explant (such as a stem tip, node, meristem, embryo, or even a seed) and placing it in a sterile, (usually gel-based) nutrient medium where it multiplies. The formulation of the growth medium is changed depending upon whether you are trying to get the plant to produce undifferentiated callus tissue, multiply the number of plantlets, grow roots, or multiply embryos for "artificial seed".

For many who become superficially aware of the technique it seems shrouded in mystery and is shrugged off as too technical to be of concern. Actually, it is no more of a mystery than taking a cutting of your favorite houseplant and growing it to share with a friend. As for being technical, you can begin plant tissue culture with as little as a cookbook approach and a feeling for sterile technique. Some people have visions of scientists doing plant tissue cultures in white gowns and masks in hospital-clean environments. Such conditions are excessive. While it is true that mold spores, bacteria, and other contaminants will grow and overrun a culture, air that is not moving has a minimum of contaminants.

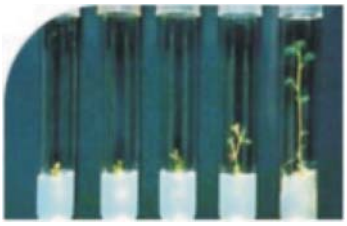


Figure 4.1: Plant shoot developed by tissue culture

In addition, disinfections of implements, work surface and nearby areas help to eliminate contaminants.

Animal Cell Cultures

Biotechnology is a multi-disciplinary science, which harnesses different areas of life sciences for the betterment of mankind. Of the different areas, animal cell biotechnology has developed into an important field in Biotechnology. The study of animal cells has helped us gain an insight not only in the structure and function of cells and tissues but also in different physiological, biochemical and immunological processes. Animal cells are known to produce many chemicals of great value to humans. There is an ever-increasing list of biological products available from animal cells. Some of the different products obtained from mammalian cells have been tabulated (Table 4.1).

Table 4.1: Products from Animal Cells

Product	**Example**
I.	
(1) enzymes	Urokinase, tissue plasminogen activator
(2) hormones	Growth hormone
(3) growth factors	
II. Viral vaccines	Human-rabies, mumps, rubella etc. Veterinary-FMD vaccine, New Castle's Disease etc.
III. Monoclonal antibodies	Diagnostic tools
IV. Insect virus	Baculovirus Bioinsecticides
V. Immunoregulators	Interferons and interleukins
VI. Whole cells	Toxicological testing

Ever since recombinant DNA technology came into being, it was easy to produce these products in bacteria or fungi on a large scale. Large-scale culture of bacteria and fungi is relatively easy. But the main drawbacks of prokaryotes is that they lack post-translational modifications such as phosphorylation, glycosylation, amidation, carboxylation etc. as a result of which the protein is different and may not be functional. Besides, bacteria and yeasts may not excrete their products. Very often, the wild type cells overgrow the recombinants. Animal cells soon came to be considered as alternatives to bacteria and yeasts for recombinant products. Mammalian cells possess post-translational systems and extraction of products from these cells is possible. As a result, cell lines are being preferred to bacteria and yeasts as cloning systems for developing recombinant products.

Methods of Plant Tissue and Cell Culturing

Types of Cells Grown in Culture

Tissue culture is often a generic term that refers to both organ culture and cell culture and the terms are often used interchangeably. Cell cultures are derived from either primary tissue explants or cell suspensions. Primary cell cultures typically will have a finite life span in culture whereas continuous cell lines are abnormal and often transformed cell lines.

Work Area and Equipment

a. Laminar Flow Hoods: There are two types of laminar flow hoods, vertical and horizontal. The vertical hood, also known as a biologically safe cabinet, is best for working with hazardous organisms since the aerosols that are generated in the hood are filtered out before they are released into the surrounding environment. Horizontal hoods are designed such that the airflows directly at the operator hence they are not useful for working with hazardous organisms but are the best protection for your cultures. Both types of hoods have continuous displacement of air that passes through a HEPA (high efficiency particle) filter that removes particulates from the air. The hoods are equipped with a short-wave UV light that can be turned on for a few minutes to sterilize the surfaces of the hood, but be aware that only exposed surfaces will be accessible to the UV light. All surfaces are wiped down with ethanol before and after each use.

b. CO_2 Incubators: The cells are grown in an atmosphere of 5-10% CO_2 because the medium used is buffered with sodium bicarbonate/carbonic acid and the pH must be strictly maintained. Culture flasks should have loosened caps to allow for sufficient gas exchange. Cells should be left out of the incubator for as little time as possible and the incubator doors should not be opened for very long.

c. Microscopes: Inverted phase contrast microscopes are used for visualizing the cells. Microscopes should be kept covered and the lights turned down when not in use. Before using the microscope or whenever an objective is changed, it is checked that the phase rings are aligned.

d. Preservation: Cells are stored in liquid nitrogen.

e. Vessels: Anchorage dependent cells require a nontoxic, biologically inert, and optically transparent surface that will allow cells to attach and allow movement for growth. The most convenient vessels are specially treated polystyrene plastic that are supplied sterile and are disposable. These include petri dishes, multi-well plates, microtiter plates, roller bottles, and screw cap flasks. Suspension cells are shaken, stirred, or grown in vessels identical to those used for anchorage-dependent cells.

Preservation and Storage

Liquid N_2 is used to preserve tissue culture cells, either in the liquid phase (-196^{o}C) or in the vapor phase (-156^{o}C). Freezing can be lethal to cells due to the effects of damage by ice crystals, alterations in the concentration of electrolytes, dehydration, and changes in pH. To minimize the effects of freezing, several precautions are taken. First, a cryoprotective agent, which lowers the freezing point, such as glycerol or DMSO, is added. The freezing medium we typically use is 90% serum, 10% DMSO. In addition, it is best to use healthy cells that are growing in log phase and to replace the medium 24 hours before freezing. Also, the cells are slowly cooled from room temperature to -80^{o}C to allow the water to move out of the cells before it freezes.

Maintenance

Cultures are examined daily, observing the morphology, the color of the medium and the density of the cells. A tissue culture log is maintained, the log should contain: the name of the cell line, the medium components and any alterations to the standard medium, the dates on which the cells were split and/or fed, a calculation of the doubling time of the culture and any observations relative to the morphology, etc.

a. Growth Pattern: Cells initially go through a quiescent or lag phase that depends on the cell type, the seeding density, the media components, and previous handling. The cells then go into exponential growth where they have the highest metabolic activity. Then enter into stationary phase where the number of cells is constant, this is characteristic of a confluent population (where all growth surfaces are covered).

b. Harvesting: Cells are harvested when the cells have reached a population density, which suppresses growth. Ideally, cells are harvested when they are in a semi-confluent state and are still in log phase. Cells that are not passaged and are allowed to grow to a confluent state can sometime lag for a long period of time and some may

never recover. It is also essential to keep your cells as happy as possible to maximize the efficiency of transformation. Most cells are passaged (or at least fed) three times a week.

i. ***Suspension Culture****:* Suspension cultures are fed by dilution into fresh medium.

ii. ***Adherent Cultures****:* Removing the old medium and replacing it with fresh medium can simply feed adherent cultures that do not need to be divided. When the cells become semi-confluent, several methods are used to remove the cells from the growing surface so that they can be diluted.

Mechanical Maintenance

A rubber spatula can be used to physically remove the cells from the growth surface. This method is quick and easy but is also disruptive to the cells and may result in significant cell death. This method is best when harvesting many different samples of cells for preparing extracts, i.e., when viability is not important.

Proteolytic Enzymes

Trypsin, collagenase, or pronase, usually in combination with EDTA, causes cells to detach from the growth surface. This method is fast and reliable but can damage the cell surface by digesting exposed cell surface proteins. The proteolysis reaction can be quickly terminated by the addition of complete medium containing serum

Samples

Plants: In theory, any part obtained from any plant species can be employed to induce callus tissue, however the successful production of callus depends upon plant species and their qualities. Dicotyledons are rather amenable for callus tissue induction, as compared to monocotyledons; the callus of woody plants generally grow slowly. Stems, leaves, roots, flowers, seeds and any other parts of plants are used, but younger and fresh ones are preferable as explant materials. Explants obtained must be sterilized using ethanol, sodium hypochlorite and/or other chemicals to remove all microorganisms from the materials and a typical sterilization procedure will be described later as an example.

Media

Inorganic Salts: To induce a callus from an explant and to cultivate the callus and cells in suspension, various kinds of media (inorganic salt media) have been designed. Agar or its substitutes is added into the media to prepare solid medium for callus induction. The significant feature of the mineral salt (MS) medium is its very high concentration of nitrate, potassium and ammonia.

Carbon Sources: Sucrose or glucose at 2 to 4% is suitable carbon sources which are added to the basal medium. Fructose, maltose and other sugars also support the growth of various plant cells. However, the most suitable carbon source and its optimal concentration should be chosen to establish the efficient production process of useful metabolites.

Vitamins: The basal media described above such as MS medium include myo-inositol, nicotinic acid, pyridoxine HCl and thiamine HCl. Among these vitamins, thiamine is an essential one for many plant cells and other vitamins stimulate the growth of the cells in some cases.

***Phytohormones*:** Phytohormones or growth regulators are required to induce callus tissues and to promote the growth of many cell lines. As an auxin, 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthaleneacetic acid (NAA) is frequently used. Other derivatives of auxin and kinetin are also used in some cases.

Organic Supplements: In order to stimulate the growth of the cells, organic supplements are sometimes added to the medium. These supplements include casamino acid, peptone, yeast extracts, malt extracts and coconut milk. Coconut milk is also known as a supplier of growth regulators.

Callus Induction: Explants are sterilized with 2% sodium hypochlorite solution and/or 70% ethanol solution. The period of time for submerging the plant materials in these solutions depends upon plant species, their parts and age. The stem or any other part of plants thus sterilized is cut to approximately 1 cm in length using a sterilized scalpel and each piece is transferred with tweezers to a solid medium in a flask or a petri-dish. The plant material is incubated aseptically at around 25° C on the solid medium for several weeks or more and a callus is produced. The callus is subcultured by transferring a small piece to fresh solid medium. After several subsequent transfers, the callus becomes soft and fragile.

Suspension Culture: The growth rate of the suspension-cultured cells is generally higher than that of the solid culture. The former is more desirable particularly in production of useful metabolites in a large-scale. A piece of the callus is transferred to a liquid medium in a vessel such as an Erlenmeyer flask and the vessel placed on a rotary or reciprocal shaker. By subculturing for several generations, a fine cell suspension culture containing small cell aggregates and single cells is established.

Scaling-Up: For commercialization, it is necessary to progress through several stages increasing the volume at each stage until the requisite bioreactor size is attained. For this purpose, different types of fermentors are used (Figure 4.2). In theory, it is anticipated that such large-scale suspension cultures will be suitable for industrial production of useful plant chemicals such as pharmaceuticals and food additives, in a manner similar to that of microbial fermentation.

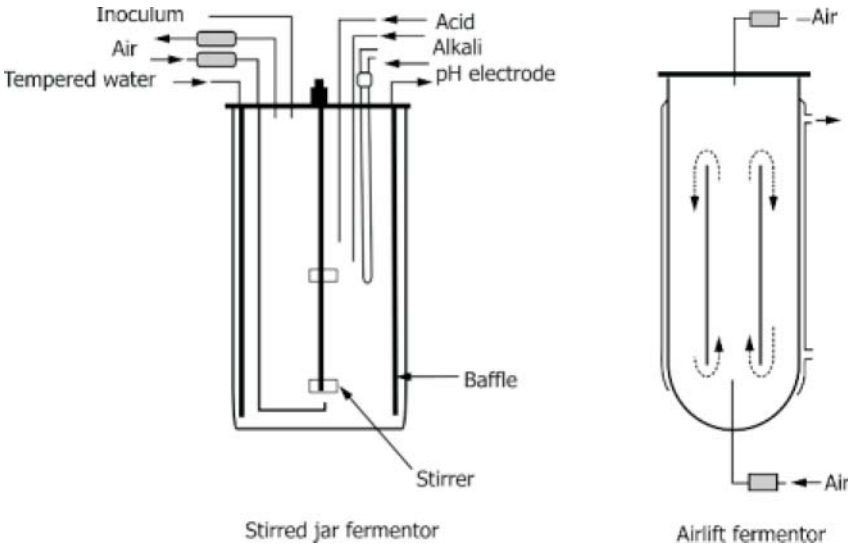


Figure 4.2: Different Fermentors for Plant Cell Culture

Methods of Cultivation of Animal Cells

Different methods of mass cultivation of animal cells have been devised to meet the growing demand for these cells. The choice of the method mainly depends on the cells to be used. Basically, cell lines may be roughly divided into those that grow as a suspension and those that are anchorage dependent.

Anchorage Dependent Culture: Anchorage dependent cell lines can be grown by the following methods.

Conventional Methods

These methods are followed routinely in laboratories. They include MD bottles, T flasks, Roux bottles and Rollers. If production is to be increased, the number of units has to be increased making the process time consuming and laborious. The result is that the product may not be cost effective. On the other hand, newer trends in manufacture aim at increasing productivity of a single unit.

Fermentation Technology (Growth of Animal or Plant Cells and their Products)

Fermentors have been used for the cultivation of bacteria and yeasts for a long time. Initially, fermentation was synonymous with alcohol production. Later, bacteriologists learnt to use the same principles for the preparation of vitamins, organic acids, antibiotics etc. This led to the rapid development of a variety of fermentors and methodologies (Figure 4.3).

Batch Fermentation

Batch fermentation (or batch culture) is a closed culture system, which will contain an initial, limited amount of nutrient. Six typical phases of growth are usually observed during a batch fermentation:

Lag phase, acceleration phase, logarithmic (log) phase or exponential phase, deceleration phase, stationary phase and death phase.

- ***Lag Phase:*** The microbial cells adapt to the new environmental conditions. The cells may have to adjust to a different pH or to a new level of available nutrients. The length of the lag phase corresponds to how long the inoculated cells were in the stationary phase, and differences in the composition between the previous and the new fresh medium.
- ***Logarithmic Phase:*** The cell mass undergoes several cell doublings, and the specific growth rate of the culture remains constant. In this phase there are excess amounts of substrate (nutrient supply).

- ***Stationary Phase:*** Following either the depletion of a critical growth substance, such as the carbon source, from the medium or the accumulation of metabolic end products that inhibit growth, the increase in cell mass eventually ceases and the cells enter the stationary phase.

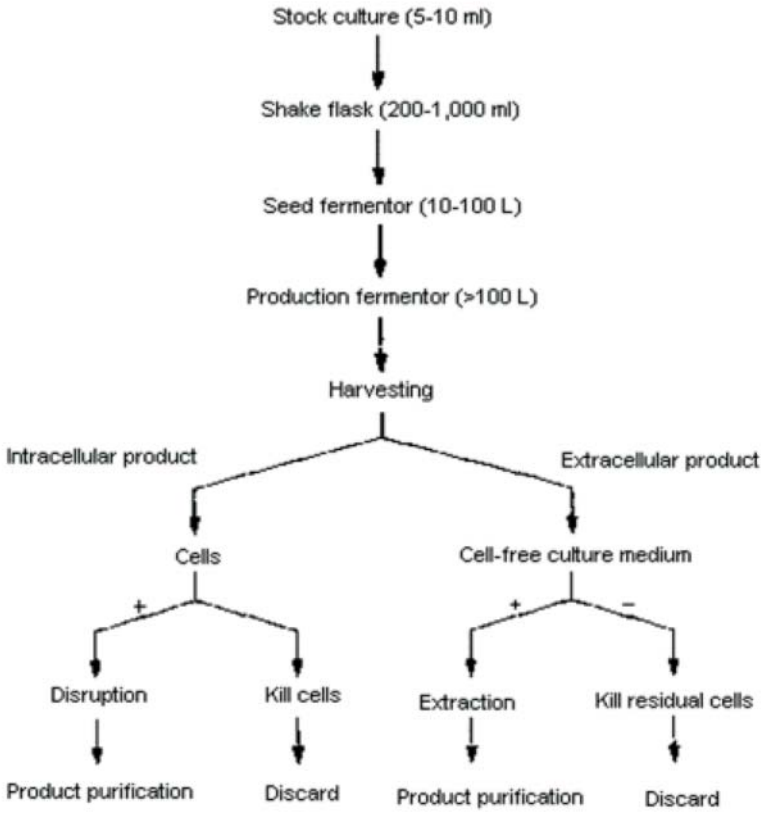


Figure 4.3: Fermentation Protocol

Fed-Batch Fermentation

Generally, Fed-Batch fermentations require greater monitoring and control than batch fermentations. In Fed-Batch Fermentation substrate is added in increments at various times throughout the course of the reaction. These additions prolong both the log and stationary phase and thereby increase the biomass and the amount of synthesis of stationary phase metabolites such as antibiotics. Microorganisms in the stationary phase often produce proteolytic enzymes, which will attack any protein

product synthesized by a genetically engineered microorganism. Therefore, when producing proteins from a recombinant microorganism, it is important to prevent the fermentation reaction from reaching this part of the growth cycle.

Another fed-batch type is cyclic fed-batch culture:

- Dilution of the culture by withdrawing a portion of the culture and using the residual culture as starting point for a further fed-batch process (variable volume).
- This will lead to periodic shift-up in growth-rate followed by a gradual shift-down.
- Dilution of the culture can also be achieved by withdrawing culture and refilling to original level with dH_2O or medium not containing the feed substrate (fixed volume).

Uses of Batch Fermentations

- Produce biomass
- Produce primary metabolites
- Produce secondary metabolites

Examples of the Use of Fed-Batch Culture

- Production of baker's yeast as early as 1915
- Production of recombinant proteins from yeast
- Penicillin (secondary metabolite)
- Enzymes
    - Cellulase
    - Lipase

Continuous Fermentation: In a continuous fermentation, a steady-state condition is attained when the total number of cells and the total volume in the bioreactor remain constant. In other words, under these conditions, the loss of cells due to outflow (product removal) is exactly balanced by the gain in new cells by growth (division).

Advantages: Continuous fermentations use smaller sized bioreactors than those needed for batch fermentations to produce the same amount of product. Equipment required for cell harvesting, cell breakage, and downstream processing can be much smaller, due to production "a little bit at a time". The physiological state of the cells during continuous fermentation is more uniform, so yields of product tend to be more consistent. In batch fermentations, small differences in the timing of cell harvest can lead to significant physiological differences. A fermentation to produce a metabolite is often more productive in continuous culture than batch culture.

Comparison of Batch and Continuous Culture as Investigative Tools

- Culture conditions are not constant even during the log phase (batch culture).
- Biomass, substrate and microbial products all change exponentially
- Study of physiologically effects of nutrient limitation nearly impossible to study in batch culture
- In continuous culture concentrations of biomass, substrate and product are constant over very long periods of time

Contamination: Contamination can be a serious problem in fermentation, especially in cultures that grows over a long period of time. If a foreign microorganism invades the fermentation the consequences can be:

a. Loss of productivity due to the fact that the medium has to support growth of both the production organism and the contaminant.

b. The contaminant can "outgrow" the production organism, and eventually displace it from the fermentation (continuous culture).

c. The foreign organism can contaminate the final product.

d. Production of compounds from the contaminant that makes extraction of the final product difficult.

e. The contaminant may degrade the desired product.

f. Lysis of the entire culture due to contamination with phage.

Therefore, it is essential that contamination is avoided, and this can be achieved by:

a. Using a pure inoculum to start the fermentation.

b. Sterilizing the medium to be employed.

c. Sterilizing the fermentor vessel.

d. Sterilizing all materials that are added to the fermentation during the whole process.

e. Maintaining aseptic conditions during the fermentation.

Sterilization Methods

Heat, filtration, radiation or chemical treatment is common sterilization methods.

- Moisture heat, steam:
  - Usually at 121C
- Dry heat:
  - Less effective in killing contaminating microorganisms than steam, and require therefore higher temperature (180C) and longer exposure time.
- Filtration:
  - Used on both fluid and gases
- Radiation:
  - Ionization and UV-radiation
- Chemical treatment:
  - Uses of disinfections solutions

Medium Sterilization

The far most used method for medium sterilization due to practical reasons is steam. The microorganisms "temperature death" caused by steam, are presumed to be due to denaturation of enzymes and nucleic acids. The survival of microorganisms is also time dependant. Some times the sterilization method can lead to degradation or destruction of medium components, and two types of reactions contribute to the loss of nutrient quality during sterilization.

I) Interaction between nutrient components of the medium.

II) Degradation of heat labile components such as certain vitamins and amino acids.

Filtration techniques are often employed when animal-cell-cultures are fermented because the media contain many heat labile components. If the browning reaction is a major problem then the sugar can be sterilized separately from the rest of the medium.

The sterilization method therefore often becomes a compromise to achieve the following:

- To achieve least possible risk of contamination.
- To avoid or minimize the decrease in nutrient value.

Batch Sterilization of Medium: Achieved either in the fermentor vessel itself or in a separate medium sterilization vessel. Consists of three phases; heating to the sterilization temperature, a holding time at the desired temperature, and a cooling period to restore the medium to the fermentation temperature. Highest temperature that seems to be feasible in batch sterilization is 121C, and is most commonly used.

Continuous Sterilization of Medium: The design of continuous sterilization cycles (warming up, holding and cooling) may be approached the same way as for the batch sterilization system. A much higher temperature can be achieved during a continuous process, thereby reducing the holding time and the nutrient degradation. The warming up and cooling time is much shorter than batch sterilization due to fact that only small increments of medium are treated at a time.

Sterilization of Gases

In fermentation processes, the need for gases are considerable. The most common and most practical method for sterilization is filtration. The most common gas to become filtrated is air, but also other gases are sometimes needed, such as oxygen, CO_2 and nitrogen. All are treated the same way when sterilized. The primary criteria when choosing a filter are its ability to remove microorganisms from the air-current. There are mainly two types of filters; depth filters (non-fixed pore filters) and absolute filters (fixed pore filters).

Depth Filters:

Material: Cotton, Steel wool, Fiberglass

Pore size: 0.5-15 m

Absolute Filters:

Material: Hydrophobic membrane making it resistant to wetting.

Pore size: 0.2 m

Factors that are Important for Deciding Sterilization Methods:

1. Type of fermentor and type of organism.
2. What is the Hazard Group category?
3. Type of medium and other fluids.
4. The size/type of fermentor.
5. Should the fermentor be sterilized empty or filled by medium?

The Size and Type of Fermentor: The most commonly used fermentor is a fermentor with agitation and aeration. The size and material used affects the choice. In other words the two commonly used ways to feed steam (either in a separate autoclave vessel or *in-situ* sterilization) will be more or less suited, dependent of fermentor design and material. Fermentors with agitation, which are less than 15-20 liters, are usually constructed with a glass body with a top and bottom of stainless steel. The preferred sterilization method for such small vessels is performed in an autoclave. Some fermentors of this size are equipped with in-situ sterilization facilities.

Autoclaving: If you have a choice you choose the autoclave with the shortest amount of time used to heating up to and cooling down from the sterilization temperature. Generally larger autoclaves use longer time than smaller ones.

In-situ Sterilization*:* The heating method is usually either by sending steam through the steel cap or through electrical heating. Fermentors that are sterilized *in situ* containing medium, usually has agitation to increase the heat transport and thereby decreasing the temperature gradients, which will be much higher when autoclaving. Larger vessels have larger gradients than smaller vessels, but good design/construction can improve this.

Inoculation Protocols

The Plate-to-Tube Transfer: For this and all other transfer situations, the inoculating loop and needle should be flamed slowly from the base to the tip (Figure 4.4a). Note that the petri dish cover is opened just enough to allow entry of the needle (Figure 4.4b). Always when inoculating tubes, the cap or plug is held as shown in the same hand as the loop or needle, and the tube is always held at an angle in the hand, never straight up and down nor standing in the test tube rack (Figure 4.4c).

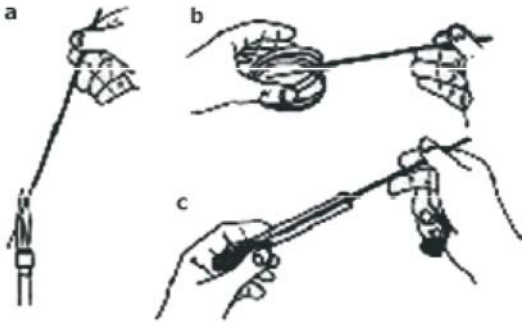


Figure 4.4: Inoculation Procedure

The Tube-to-Tube Transfer: Note that the tubes are held together at an angle. Again, never straight up and down nor standing in the test tube rack (Figure 4.5).

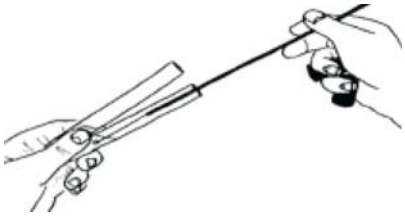


Figure 4.5: Inoculum Transfer

The Streak-Plate Procedure: For each "phase," the plate is streaked from the "far edge" toward the center (Figure 4.6). The plate is just opened enough to get the loop in, and keep the plate on the table.

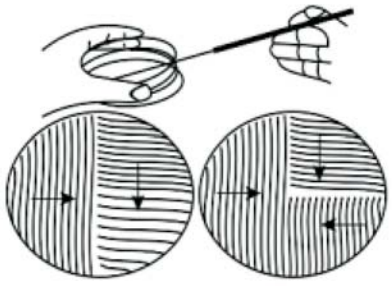


Figure 4.6: Streak Plate Method

The streak plate method is a rapid and simple technique of mechanically diluting a relatively large concentration of microorganisms to a small, scattered population of cells over the surface of the plated medium. The primary purpose is to obtain well-isolated colonies on a large part of the plate surface, each arising hopefully from a colony-forming unit (hopefully consisting of not much more than one cell), such that pure cultures of each desired species in a mixture can be established. A fluid suspension, well shaken to homogenize the cell concentration, is prepared for streaking plates from materials with mixed microbial populations. After flaming the entire wire to redness in a Bunsen burner flame and then allowing it to cool without touching anything, the loop is tipped into the suspension. If the number of bacteria in the suspension is believed to be small, a loopful is transferred to the plate and the streaking process is begun immediately. After depositing this primary inoculum, the loop is flamed and cooled again before starting the streaking pattern. (Whenever desired, a flamed loop may be cooled rapidly by applying it to an uninoculated area of medium at the side of the plate). To streak the plate, the top lid is opened just enough to allow the insertion of the loop from the right (from the opposite direction if you are left-handed). See the figure above the streaking patterns. With a wrist motion, the loop handle is moved back and forth over the surface of the medium, starting from the far left (from the opposite direction if you are left-handed) and moving toward the center of the plate. (Looking directly down at the plate, the loop should appear to be moving in an up-and-down direction.) A very gentle and even pressure is used. The loop should almost retrace its path with each swing as it moves across the agar surface. In the multiphase streaking patterns, the loop is flamed after the first phase is completed, cooled, and (rotating the plate 90° to again streak from the left edge toward the center) recharged by streaking over the first phase a few times. One does not go back to the culture tube for additional inoculum. This is repeated for any subsequent phases. The plate should not be left uncovered unnecessarily. The lid is replaced immediately when streaking the plate, and flame the loop carefully, as discussed above before setting it down.

Fermentation Media

The media used in shake flasks does differ from the standard media used in a fermentation vessel. Shake flask media is generally of a much simpler composition. Culture media are employed in the isolation and maintenance of pure cultures of bacteria and are also used for identification of bacteria according to their biochemical

and physiological properties. Following types of culture media are used usually for fermentation (Table 4.2):

Liquid vs. Solid Medium: The manners in which microorganisms are cultivated, and the purpose of culture media, vary widely. Liquid media are used for growth of pure batch cultures while solidified media are used widely for the isolation of pure cultures, for estimating viable bacterial populations, and a variety of other purposes. The usual gelling agent for solid or semisolid medium is agar, a substance derived from red algae. Agar is used because of its unique physical properties (it melts at 100 degrees and remains liquid until cooled to 40 degrees, the temperature at which it gels) and because it cannot be metabolized by most bacteria. Hence as a medium component it is relatively inert; it simply holds (gels) nutrients that are in aqueous solution.

Chemically Defined vs. Complex Media: A chemically defined (synthetic) medium is one in which the exact chemical composition is known. A complex (undefined) medium is one in which the exact chemical constitution of the medium is not known. Defined media are usually composed of pure biochemical off the shelf. A defined medium is a minimal medium if it provides only the exact nutrients (including any growth factors) needed by the organism for growth.

Complex media usually contain complex materials of biological origin such as blood or milk or yeast extract or beef extract, the exact chemical composition of which is obviously undetermined. Complex media usually provide the full range of growth factors that may be required by an organism so they may be more handily used to cultivate unknown bacteria or bacteria whose nutritional requirement are complex (i.e., organisms that require a lot of growth factors).

Selective, Differential, Enrichment Media:

1. Selective media is designed to suppress the growth of some microorganisms while allowing the growth of others (i.e., they select for certain microbes). Examples of selective media include:

i. Mannitol salts agar selects against non-skin flora

ii. MacConkey agar selects against gram-positives

iii. Eosin-methylene blue agar (EMB agar) selects against gram-positives

iv. Phenylethyl alcohol agar (PEA agar) selects against gram-negatives

2. Differential media allows the growth of more than one microorganism of interest but with morphologically distinguishable colonies. Examples of differential media include:

i. Mannitol salts agar (mannitol fermentation = yellow)

ii. Blood agar (various kinds of hemolysis)

iii. MacConkey agar (lactose fermentation = yellow)

iv. Eosin-methylene blue agar (various kinds of differentiation)

3. Enrichment medium (or enrichment culture) differs from selective medium in suppressing the growth of non-enriched microorganisms (e.g., no selective poisons are employed). Enrichment culture is used to increase the relative concentration of certain microorganisms in the culture prior to plating on solid selective medium.

Table 4.2: Types of Culture Media

Type	Purpose
Chemically defined	Growth of chemoautotrophs and photoautotrophs, and microbiological assays.
Complex	Growth of most chemoheterotrophic organisms.
Reducing	Growth of obligate anaerobes.
Selective	Suppression of unwanted microbes; encouraging desired microbes.
Differential	Differentiation of colonies of desired microbes from others.
Enrichment	Similar to selective media but designed to increase numbers of desired microbes to detectable levels.

Antifoam: Constant stirring in stirred fermentors or in shake flasks produces foams. To avoid oxygen limitation in medium using antifoam should treat this foam timely. Antifoam such as silicon oil, Dow Corning's Antifoam 2210 or Compound A are usually suitable for use in fermentation.

Concentration and Type of Acid and Base: The acid solution is 2-3 N H_2SO_4. The base solution is either 5 N NaOH or NH_4OH ~ 29% (which is the standard commercially available concentration).

Shakers: Shakers are used for submerged fermentation (Figure 4.7). Solid-state fermentation needs only the incubators with controlled temperature and light optima.

Figure 4.7: Incubator Shaker and Incubator Water Bath

Bioreactors (or Fermentors) in Biotechnology

Bioreactors are the places where proteins for biotechnology are made during upstream processing. Today, bioreactors can be vessels made of plastic, glass or steel in which cells are cultured or, alternatively, whole plants or animals can be genetically engineered to produce a particular protein (transgenics). There are many modifications of bioreactors, currently being used in industry.

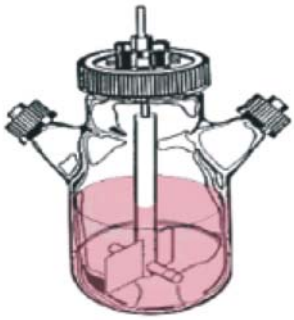


Figure 4.8: A Stirred Bioreactor Vessel.

- Stirred tank bioreactor (Figure 4.8)
- Airlift bioreactor

For industrial upstream processing of proteins various types of plastic bioreactors are also in use including fixed cells in a fluidized-bed bioreactor. In this bioreactor the cells are fixed in a compartment and media is constantly pumped to the cells in the fixed compartment and constantly withdrawn and pooled for downstream processing. Another plastic bioreactor is the hollow fiber bioreactor in which cells grow inside hollow fibers or tubes and media is pumped either through the hollow fibers or over the surface of the hollow fibers and constantly withdrawn and pooled for downstream processing (Figure 4.9). Another sort of bioreactor is the perfusion bioreactor.

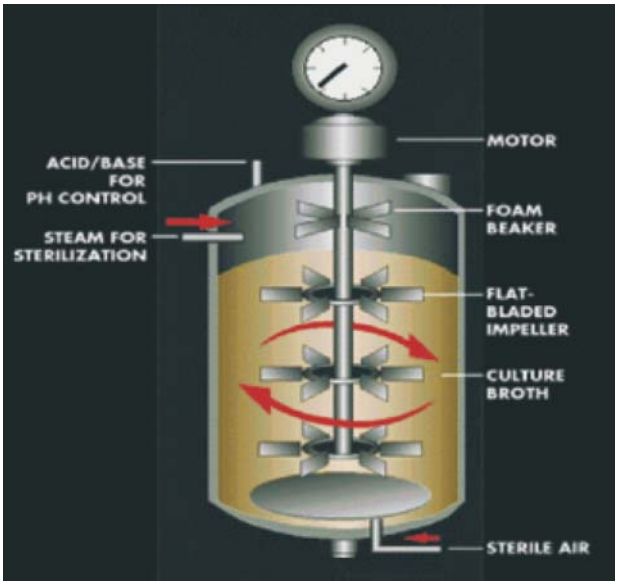


Figure 4.9: A Fermentor / Bioreactor and its Parts

Microcarriers

In 1967, Van Wezel made microcarriers composed of cross-linked dextran beads charged with tertiary amine groups (DEAE) having an exchange capacity of 3.5 milli equivalent / gm dry weight. He demonstrated the growth of primary cells and cells from a human diploid cell strain on them as well as the propagation of poliomyelitis virus in the cells grown on microcarriers.

It was Levine *et al.* 1979 who developed microcarriers of reduced ion exchange capacity (1.5 meq./gm dry weight). These could be used at concentrations as high as 6 gms/liter with no deleterious effect on cell growth. These low charged beads could be used to cultivate primary, diploid as well as established cell lines. Subsequently a lot of work was done to improve the quality of the beads. Different types of microcarriers were developed and patented.

Advantages of Microcarrier Cultures

This system has the following advantages over other methods of large-scale cultivation:

- High surface area to volume ratio can be achieved which, can be varied by changing the microcarrier concentration. This leads to high cell densities per unit volume with a potential for obtaining highly concentrated cell products.
- Cell propagation can be carried out in a single high productivity vessel instead of using many low productivity units, thus achieving a better utilization and a considerable saving of medium.
- Since the microcarrier culture is well mixed, it is easy to monitor and control different environmental conditions such as pH, pO_2, pCO_2 etc.
- Cell sampling is easy.
- Since the beads settle down easily, cell harvesting and downstream processing of products is easy.

Microcarrier cultures can be relatively easily scaled up using conventional equipment like fermentors that have been suitably modified. A wide range of cells has been cultured on microcarriers (Figure 4.10). For instance, cells from invertebrates, from fish, birds and cells of mammalian origin have been cultivated on microcarriers. Transformed and normal cell lines, fibroblastic and epithelial cells

and even genetically engineered cells have been cultivated on microcarriers for various biologicals such as for the production of immunologicals like interferons, interleukins, growth factors etc. Cells cultured on microcarriers also serve as hosts for a variety of viruses that are used as vaccines like foot and mouth disease or rabies.

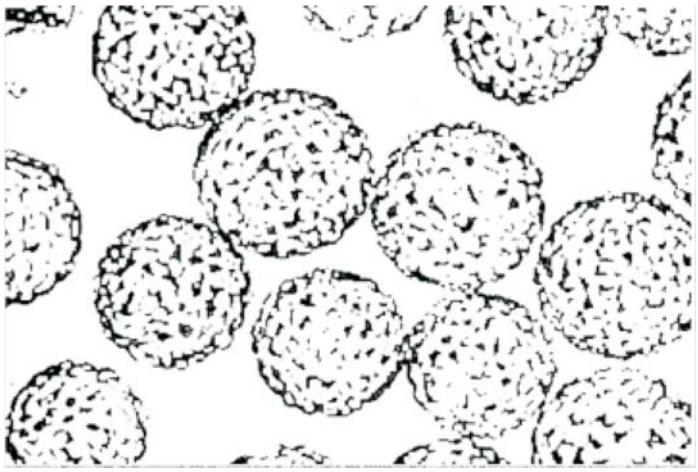


Figure 4.10: Cells Grown on Microcarriers

Applications of Microcarriers

Microcarrier cultures have found a wide number of applications other than mass cultivation as well. Cells growing on microcarriers serve as an excellent tool for studying different aspects of cell biology such as cell-to-cell or cell-to-substratum interactions. Cell differentiation and maturation, metabolic studies have also been carried using microcarriers. Such cells can also be used for electron microscopic examinations or for the isolation of cell organelles such as the cell membrane.

Stem Cells

Stem cells are one of the most fascinating areas of biology today. But like many expanding fields of scientific inquiry, research on stem cells raises scientific questions as rapidly as it generates new discoveries. Stem cells have two important characteristics that distinguish them from other types of cells. First, they are unspecialized cells that renew themselves for long periods through cell division. The second is that under certain physiologic or experimental conditions, they can be

induced to become cells with special functions such as the beating cells of the heart muscle or the insulin-producing cells of the pancreas.

Scientists primarily work with two kinds of stem cells from animals and humans: embryonic stem cells and adult stem cells, which have different functions and characteristics that will be explained in this document. Scientists discovered ways to obtain or derive stem cells from early *mouse* embryos more than 20 years ago. Many years of detailed study of the biology of mouse stem cells led to the discovery, in 1998, of how to isolate stem cells from *human* embryos and grow the cells in the laboratory. These are called human embryonic stem cells. The embryos used in these studies were created for infertility purposes through in vitro fertilization procedures and when they were no longer needed for that purpose, they were donated for research with the informed consent of the donor.

Embryonic Stem Cells Stimulated to Differentiate:

Stem cells are unspecialized and are capable of dividing and renewing themselves for long periods. Stem cells can give rise to specialized cells. Stem cells are important for living organisms for many reasons. In the 3 to 5 day old embryo, called a blastocyst, a small group of about 30 cells called the inner cell mass gives rise to the hundreds of highly specialized cells needed to make up an adult organism. In the developing fetus, stem cells in developing tissues give rise to the multiple specialized cell types that make up the heart, lung, skin, and other tissues.

In some adult tissues, such as bone marrow, muscle, and brain, discrete populations of adult stem cells generate replacements for cells that are lost through normal wear and tear, injury, or disease. As long as the embryonic stem cells in culture are grown under certain conditions, they can remain undifferentiated (unspecialized). But if cells are allowed to clump together to form embryoid bodies, they begin to differentiate spontaneously. They can form muscle cells, nerve cells, and many other cell types (Figure 4.11). Although spontaneous differentiation is a good indication that a culture of embryonic stem cells is healthy, it is not an efficient way to produce cultures of specific cell types.

So, to generate cultures of specific types of differentiated cells: heart muscle cells, blood cells, or nerve cells, for example scientists try to control the differentiation of embryonic stem cells. They change the chemical composition of the culture medium, alter the surface of the culture dish, or modify the cells by inserting specific genes.

Through years of experimentation scientists have established some basic protocols or "recipes" for the directed differentiation of embryonic stem cells into some specific cell types.

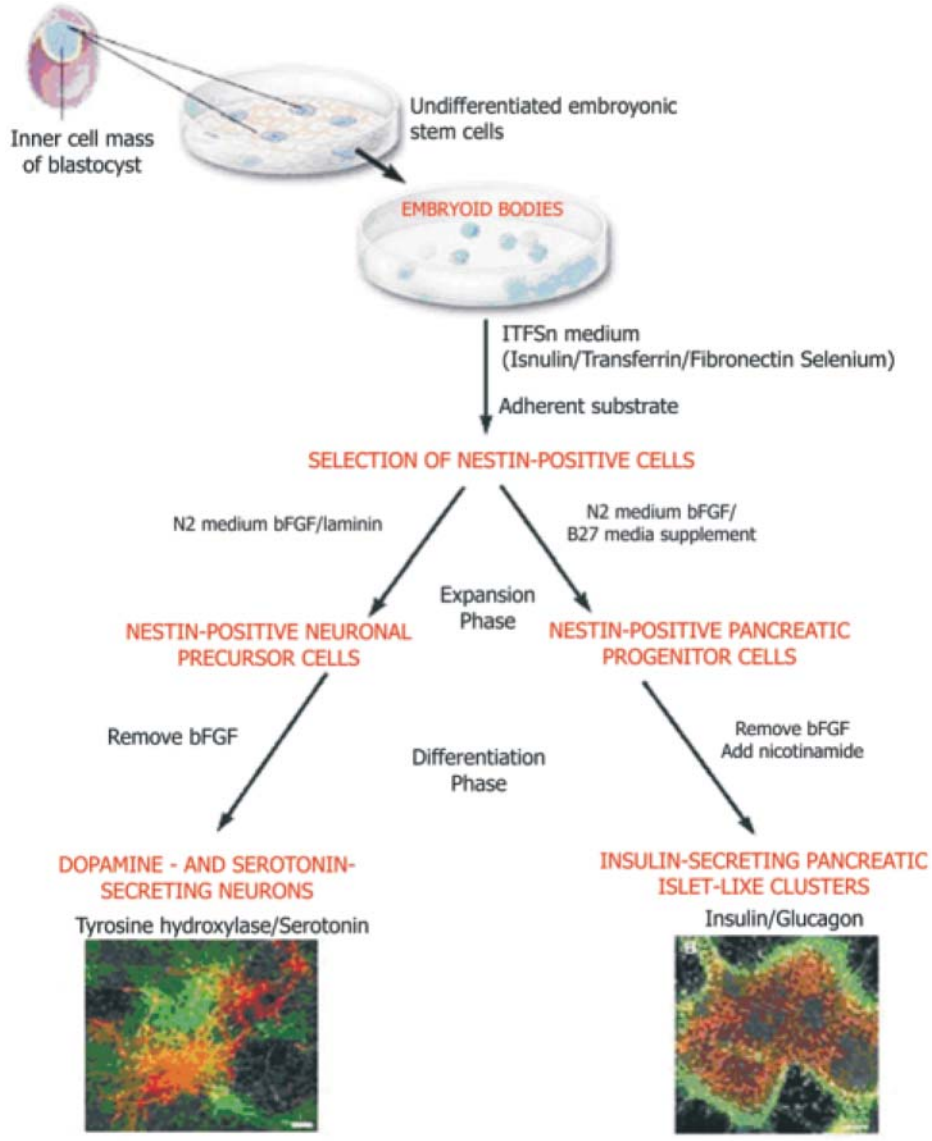


Figure 4.11: Directed Differentiation of Mouse Embryonic Stem Cells

Potential Uses of Human Stem Cells

It has been hypothesized by scientists that stem cells may, at some point in the future, become the basis for treating diseases such as Parkinson's disease, diabetes, and heart disease. Studies of human embryonic stem cells may yield information about the complex events that occur during human development. A primary goal of this work is to identify how undifferentiated stem cells become differentiated. Scientists know that turning genes on and off is central to this process. Some of the most serious medical conditions, such as cancer and birth defects, are due to abnormal cell division and differentiation. A better understanding of the genetic and molecular controls of these processes may yield information about how such diseases arise and suggest new strategies for therapy.

Human stem cells could also be used to test new drugs. For example, new medications could be tested for safety on differentiated cells generated from human pluripotent cell lines. Other kinds of cell lines are already used in this way. Cancer cell lines, for example, are used to screen potential anti-tumor drugs. But, the availability of pluripotent stem cells would allow drug testing in a wider range of cell types. However, to screen drugs effectively, the conditions must be identical when comparing different drugs.

Perhaps the most important potential application of human stem cells is the generation of cells and tissues that could be used for cell-based therapies. Today, donated organs and tissues are often used to replace ailing or destroyed tissue, but the need for transplantable tissues and organs far outweighs the available supply. Stem cells, directed to differentiate into specific cell types, offer the possibility of a renewable source of replacement cells and tissues to treat diseases including Parkinson's and Alzheimer's diseases, spinal cord injury, stroke, burns, heart disease, diabetes, osteoarthritis, and rheumatoid arthritis. For example, it may become possible to generate healthy heart muscle cells in the laboratory and then transplant those cells into patients with chronic heart disease. Preliminary research in mice and other animals indicates that bone marrow stem cells, transplanted into a damaged heart, can generate heart muscle cells and successfully repopulate the heart tissue. Other recent studies in cell culture systems indicate that it may be possible to direct the differentiation of embryonic stem cells or adult bone marrow cells into heart muscle cells (Figure 4.12).

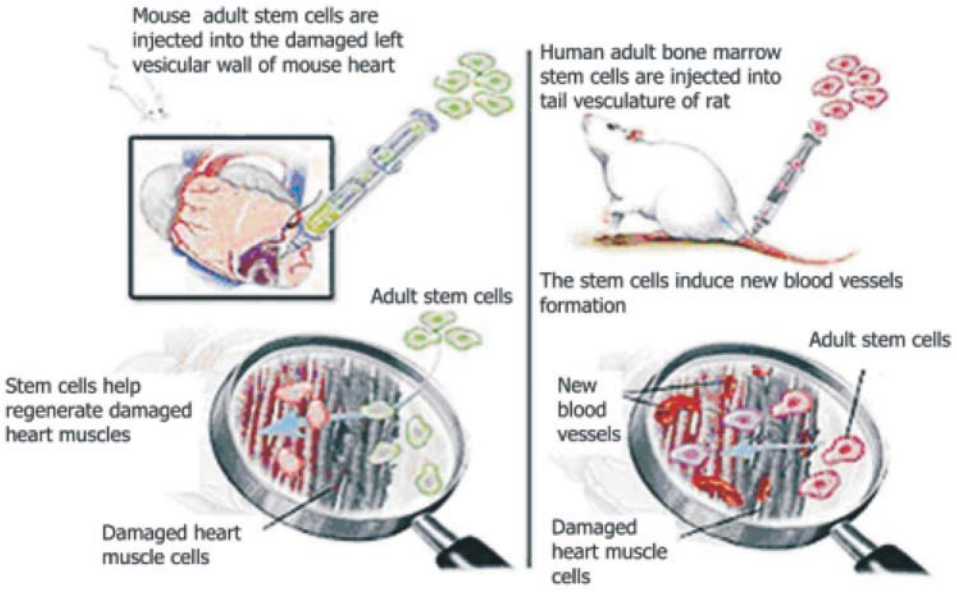


Figure 4.12: Heart Muscle Repair with Adult Stem Cells

Interferon

Interferon alpha is a man-made copy of a substance that is made naturally by some types of white blood cell. Or any of a family of proteins made by cells in response to virus infection that prevent the growth of the virus. Some interferons can prevent cell growth and have been tested for use in cancer therapy. The body makes interferon as part of the immune response, when the body reacts to infection or to cancers.

There are two main types of interferon: type I represented by the interferons a (lymphocyte interferon) and b (fibroblasts interferon) and type II (or immune interferon) represented by the interferon γ (IFNg). In fact, IFNγ belongs to the group of lymphokines, and for historical reasons only is called interferon. Human interferon gamma (hIFN γ) is endowed with multiple biological functions. Besides its strong antiviral activity, it plays a key role in the modulation of immune system and is responsible for the defense of the organism against bacteria, intracellular parasites and various xenobiotics.

Recombinant Human Interferon-γ

Interferon-γ is a product of activated T lymphocytes and natural killer cells, which was originally described as an antiviral agent. The x-ray crystal structure of recombinant human interferon-γ has been determined with the use of multiple-isomorphous-replacement techniques. The biological actions of IFNs are mediated by IFN-inducible gene expression. interferon-α therapy has in general proven to be beneficial for many superficial bladder cancers, but some tumors do not respond well to this form of therapy. IFNs were initially discovered as antiviral compounds, and many viruses have evolved strategies to inhibit the action of IFNs.

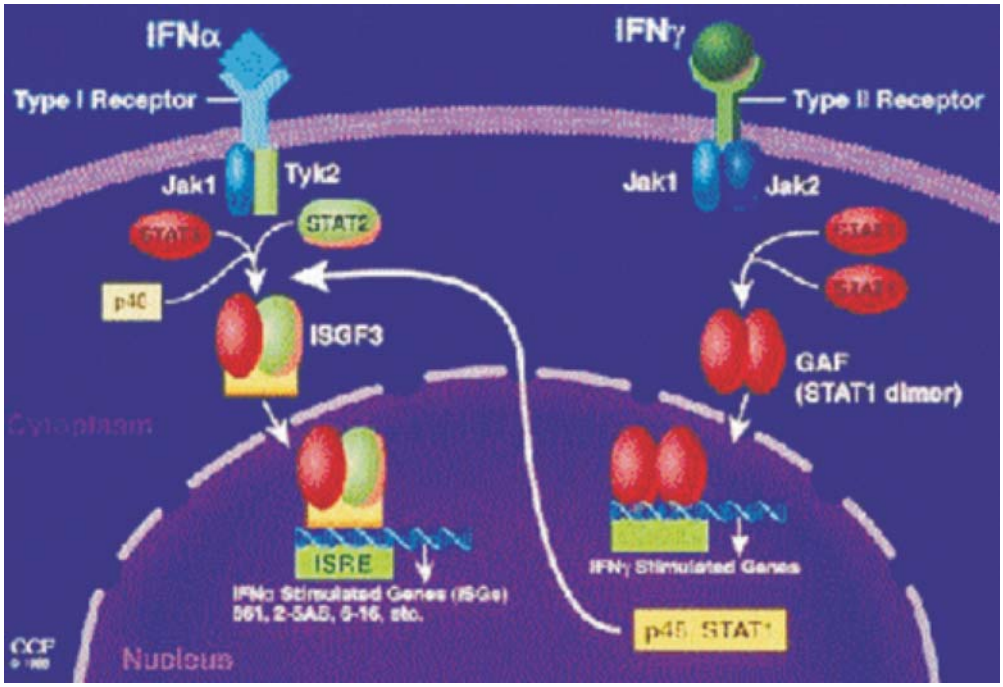


Figure 4.13: Bladder Cancer Cells Resistant to the Biological Activities of Interferon-α may be Overcome by First Exposing the Cells to Interferon-δ.

Like many viruses, some malignant cells may have the capabilities of inhibiting the action of interferon, either by altered expression of essential components of interferon-α signaling molecules (Figure 4.13) or by expressing proteins that interfere with interferon-α signaling molecules.

Biotechnology and Microbes

Fermentation has always been an important part of our lives: foods can be spoiled by microbial fermentations, foods can be made by microbial fermentations, and muscle cells use fermentation to provide us with quick responses. Fermentation could be called the staff of life because it gives us the basic food, bread. Fermentation is the process that produces alcoholic beverages or acidic dairy products. For a cell, fermentation is a way of getting energy without using oxygen. In general, fermentation involves the breaking down of complex organic substances into simpler ones. The microbial or animal cell obtains energy through glycolysis, splitting a sugar molecule and removing electrons from the molecule. The electrons are then passed to an organic molecule such as pyruvic acid. This results in the formation of a waste product that is excreted from the cell. Waste products formed in this way include ethyl alcohol, butyl alcohol, lactic acid, and acetone the substances vital to our utilization of fermentation.

Today, many other chemicals are made by fermentation (a term technically restricted to processes that occur in the absence of air, such as alcohol production by yeast, though it is often used more broadly). Products include oxalic acid, used in printing and dyeing, propenoic acid (acrylic acid) used as an intermediate in the production of plastics, lactic acid for acidifying foods, and antifreeze. Microbes also make many different enzymes, which are catalysts that promote chemical changes under much milder conditions than would otherwise be required. Their applications range from the removal of stains by enzymes, incorporated in detergents that attack fats and proteins to the conversion of corn flour to high-fructose syrup, used to sweeten soft drinks, biscuits, and cakes. Plant and animal cells can also be cultivated in vast quantities, like microbes, to produce useful substances (Figure 4.14).

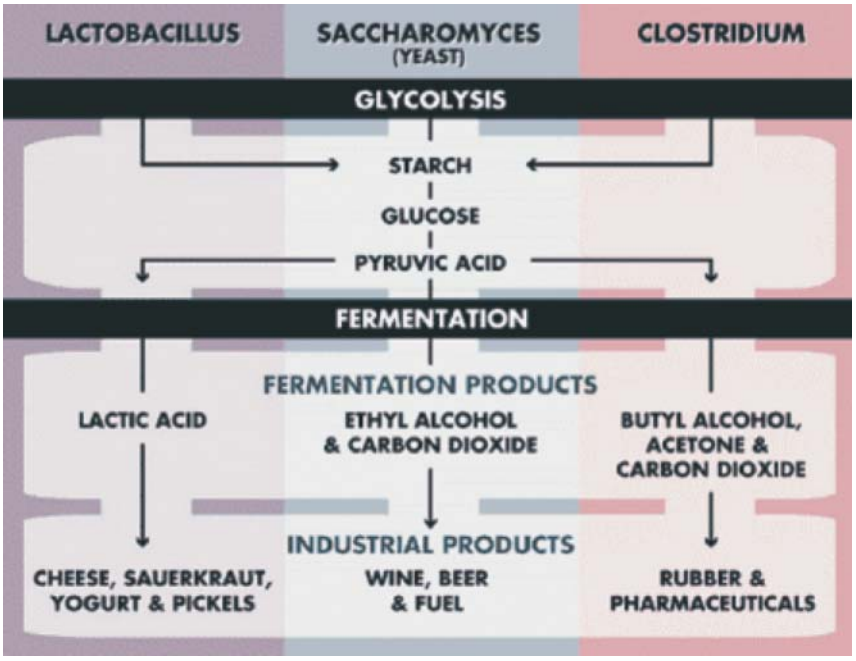


Figure 4.14: Microbial Fermentation Process

Some Important Microbial Fermentation Products and their Production Technologies

Citric Acid

Citric acid ($CH_2COOH.COH.COOH.CH_2COOH$), a tricarboxylic acid was first isolated from lemon juice and crystallized in 1784 by Schelle (Figure 4.15). Today most of the citric acid used in food and other industries comes from fungal fermentation. Citric acid has a variety of uses. About 70% of the citric acid is used in the food and beverage industry, about 12 % in pharmaceuticals and about 18% in other industrial applications. The increasing use of citric acid in a variety of industries has demanded a steady increase in citric acid production. Current estimates suggest that the world wide demand of this acid is about $3.5x10^5$ million ton per year and this is bound to increase in coming years if this organic

Figure 4.15: Citric acid crystals (viewed through microscope)

acid finds use in other industries.

The Citric Acid Cycle is one of 3 stages of cellular respiration (Figure 4.16).

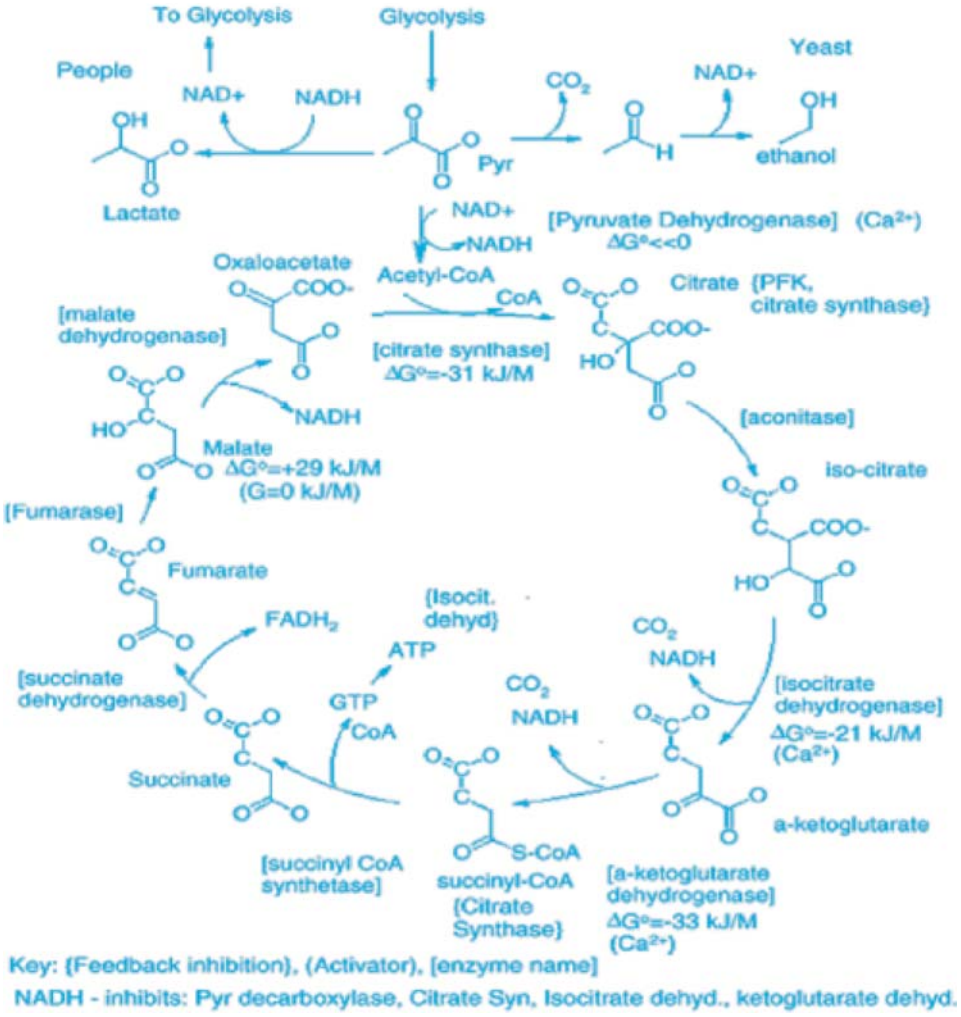


Figure 4.16: Citric Acid Cycle.

The others are glycolysis and electron transport/oxidative phosphorylation. Glycolysis breaks 1 glucose into 2 pyruvate, producing 6 ATP. Pyruvate is used to make acetyl-CoA, the starting product for the citric acid cycle. Each turn of the cycle oxidizes 1 pyruvate, so it takes 2 turns to completely oxidize 1 glucose. Two turns

produce 8 $NADH_2$, 2 $FADH_2$, and 2 ATP. NADH and $FADH_2$ are then oxidatively phosphorylated, resulting in 28 more ATP. The 3 stages together produce 30 to 38 ATP.

The net reaction of the cycle:

Acetyl-CoA + 3 NAD^+ + FAD + GDP + P_i + $2H_20$ <==> CoASH + 3 NADH + $FADH_2$ + GTP + $2CO_2$ + $3H^+$

$DG^{o'}$ = -49 kJ/mol DG ~ -115 kJ/mol

Citric Acid Production by Fungi

Fungal Strains: Since the early demonstration by Wehmer "1893" of the presence of citric acid in culture media containing sugars and inorganic salts with species of *Penicillium*, a variety of fungi have been screened for citric acid production. The various fungi, which have been found to accumulate citric acid in their culture media, include strains of *Aspergillus niger, A. awamori, A. wentii, A. usami, A.flavus, Penicillium restrictum, P. janthinellum, Trichoderma viridae, Mucor piriformis*, and species of *Botrytis, Ascochyta, Absidia*, *Talaromyces*, *Acromonium* and *Eupenicillium*. Among all of these *A. niger* is the organism of choice for citric acid production because:

- The ease with which it can be handled.
- The cheap raw material that it can utilize for acid production.
- High and consistent yields, thereby making the process economical.

Types of Fermentations: The development of process for citric acid fermentation can be divided into 3 phases:

a. In the first phase citric acid production was confined to species of *Penicillium* and *Aspergillus* under stationary or surface culture conditions.

b. The second phase beginning in the 1930's consisted of the development of submerged fermentation processes for citric acid production using *A. niger*.

c. The third stage, which is of recent origin, involves the development of the solid-state culture, continuous culture and multistage fermentation techniques for citric acid production.

Cultural Conditions

Carbon, Nitrogen and Phosphorus: Cultural conditions for citric acid production by fungi vary from strain to strain and also depend upon the type of the process. This has been so since a strain that produces citric acid effectively under surface culture conditions.

Molasses: It is a by-product of sugar industry. It is the syrupy liquid left after the removal of sugar from the mother syrup. The average composition of molasses is given in the Table 4.3.

Table 4.3: Molasses composition.

Constituents	Beet molasses (%)	Cane-molasses (%)
Water	16.5	20.0
Sugars	53.0	62.0
Non-sugars	19.0	10.0
Inorganic (ash)	11.5	8.0

Trace Elements: A. niger needs a variety of divalent trace elements such as Fe^{++}, Cu^{++}, Zn^{++}, Mn^{++} and Mg^{++}, etc., for growth and citric acid production. However citric acid production is very sensitive to the concentration of these metals in the fermentation media. In fact successful citric acid production depends to a great extent on the control of the concentration of these trace elements.

Pre-treatment of Raw Material: Since the concentration of trace elements affects citric acid production profoundly, various techniques have been used to minimize the concentration of these metals in fermentation media. Complete elimination of trace metals is practically impossible, especially when raw materials such as molasses are used. In recent years therefore, two approaches have been used to overcome this difficulty;

1. Pretreatment of raw materials with chemical, ion exchange resins etc., to reduce the trace element concentration.
2. The development of the strains of fungi that have the ability to produce citric acid in the presence of high concentration of trace elements.

pH Control: The maintenance of a favorable pH is very essential for the successful production of citric acid. The initial pH required depends on the carbon source used

e.g. when sucrose or glucose or clarified molasses are use; a low pH (3.0) is desirable. When crude molasses is used as a carbon source, a high pH is desirable.

Inoculum Development: The inoculum for citric acid fermentation can be either the spores or pre-grown mycelia. When spores are used as the inoculum, a suspension of spores is freshly prepared in sterile water and this is used to inoculate the fermentation medium. The initial concentrations of spores vary between $1x10^5$ and $1x10^7$ ml of fermentation media.

Aeration, Temperature and Incubation Period: The citric acid fermentation is essentially an aerobic fermentation and the organism needs an abundant supply of oxygen beyond that required for growth. Aeration and agitation are primarily employed in liquid fermentations to ensure an appropriate oxygen supply as well as to maintain ventilation and to prevent contamination by providing a positive pressure inside the working vessel of the fermentor. The degree of agitation and aeration will depend upon the organism, the medium, and the size of the fermentor. It is generally agreed that the oxygen demand of a fermenting culture is so high that the amount of the oxygen in the saturated aqueous medium is inadequate.

Citric acid Production by Yeast

The variety of yeasts known to produce citric acid from various sources are species of *Candida, Pichia, Hansenula, Debaromyces, Trichosporon, Torula, Sporobolomyces, Endomyces, Nocardia, Nematospora* and *Saccharomyces*. A variety of substrates like glucose, acetate hydrocarbons, alcohol fatty acids and natural oils have been used for producing citric acid by yeast.

Citric acid Production by Bacteria

Some bacteria such as *Bacillus licheniformis*, *Bacillus subtilis* and *Brevibacterium flavum* have been found to produce citric acid either from glucose, isocitric acid or from hydrocarbons.

Baker's Yeast

Yeasts are simple fungi. The term yeast refers to the unicellular phase of the life cycle of many different fungi, but it is usually used more commonly as a generic term for fungi that have only a unicellular phase. The organism is most often called “yeast”.

Yeast Strain

Baker's yeast strain is *Saccharomyces cerevisiae* (Figure 4.17). The strain can be propagated by pure culture methods in the laboratories of yeast producers. Attempts have also been made to hybridize *Saccharomyces cerevisiae* with *Saccharomyces rosei* in order to confer better osmotolerance to the yeast.

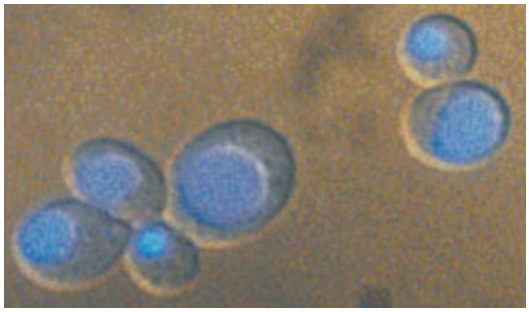


Figure 4.17: Yeast Cells

Reproduction

Yeast is unusual in that it has two methods of reproduction, budding and sporulation.

Budding: Both haploid and diploid cells divide by budding (Figure 4.18). The cell division cycle begins with a single unbudded cell. A bud grows on this yeast cell's nucleus splits into two, one half of which enters the bud. When the bud has grown to half the size of the mother cell, it separates, leaving the mother cell with a scar. The same cycle repeats for several times resulting in exponential increase in the number of cells with a doubling times equals to the mean of cell division cycle time (Figure 4.19).

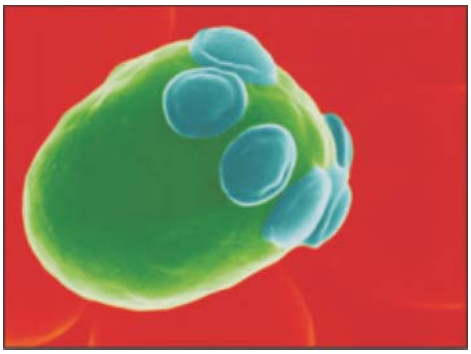


Figure 4.18: Yeast Cells Showing Buds

Sporulation: This is a sexual method of reproduction enabled by cell fusion. Two cells merge, and then split into four spores. Budding may also take place between fusion and sporulation.

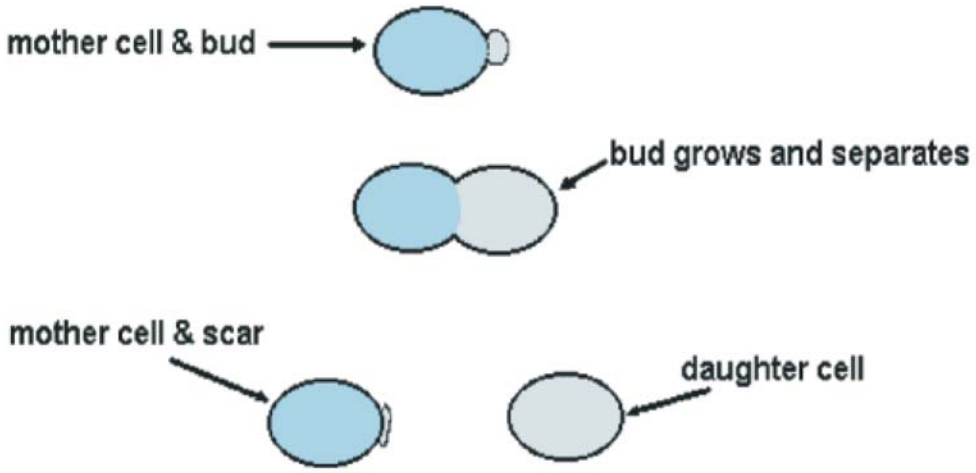


Figure 4.19: Budding Pattern in yeast cells

The Yeast Life Cycle: A Complete Sexual Cycle

In sexual organisms, the life cycle is composed of a series of events that alternate between a haploid and a diploid phase (Figure 4.20) and results from mating (sexual conjugation) between the gametes.

Sexual Conjugation

Mating in yeast is mediated by diffusible molecules, the "pheromones". Each type of cell secretes its pheromone in to the medium in response to the one produced by the opposite type by differentiating into specialized functional form, a gamete. The cell stops dividing, elongate and become pear shaped. These distinctive cells have been termed "shmoos"**.** Cells of opposite mating types that are in contact join at the surface and fuse together forming a characteristic "peanut" shape with a central constriction (fusion of two shmoos). The two haploid nuclei within each joined pair fuse into a diploid nucleus, forming a true zygote. The diploid suddenly buds at the constriction forming a characteristic "clover leaf" structure.

Meiosis: Meiosis is a part of the process of sporulation, which is initiated when diploid cells are transferred to nutritionally unbalanced medium. The ascus results from meiosis usually having four ascospores but may also contain two or three. The ascus has a lumpy shape much like oranges inside a cloth bag. When the spores either within the ascus or after being liberated are returned to a nutritionally adequate environment they germinate and undergo vegetative growth in a stable haploid phase.

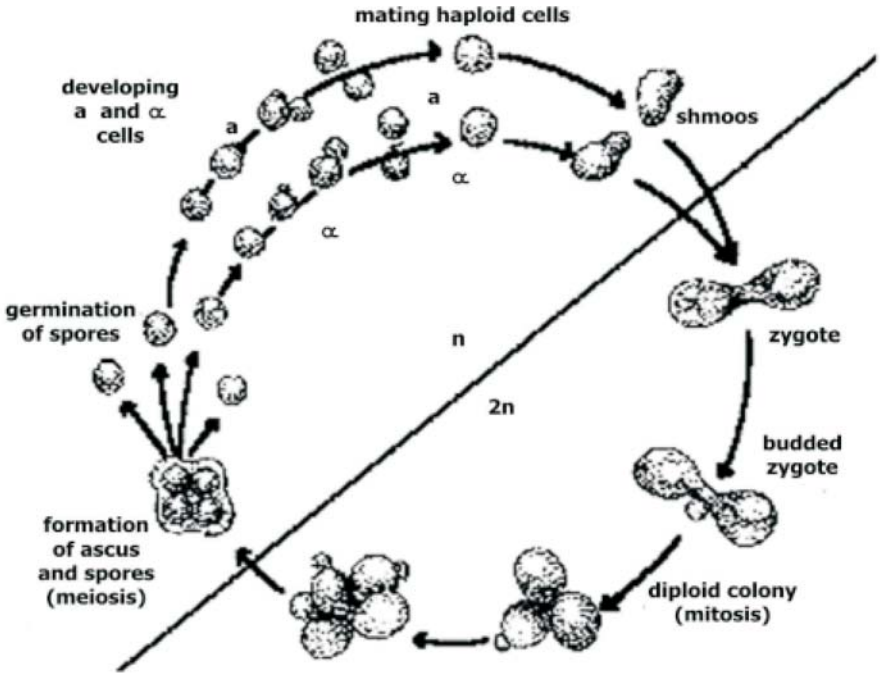


Figure 4.20: Life Cycle of Yeast

Production of Baker's Yeast

Two Production Phases of Baker's Yeast: The production of baker's yeast comprises of two phases:

(i) Fed-batch Production Phase: The fed-batch production phase of baker's yeast is an aerobic process where sugar and nitrogen sources are limited.

The general equation for the fermentation reaction is:

Substrate + Glycolytic Enzymes ® Ethyl Alcohol (C_2H_5OH) + CO_2 + ATP

(ii) Dough Phase: The dough phase is similar to anaerobic batch culture where sugars and nitrogen sources are in excess. As the dough rise period is very short (3 hours) in order to obtain optimal leavening of bread dough the rapid adaptation of yeast to this new environment is favorable.

Requirements for the Baker's Yeast Production

The principle carbon and energy source for the production of baker's yeast is cane or beet molasses. Any sugary or starchy material that can be hydrolyzed to sugars may serve as a carbon and energy source for the production of bakers yeast. Lactose is not fermented by bakers yeast, and galactose is fermented slowly. The nitrogen sources are ammonia, ammonium salts and urea; phosphorus source is orthophosphate or phosphoric acid. The fermentation medium is also supplemented with minerals (magnesium and trace metals) and vitamins (biotin and thiamin).

Alcohols

The production of alcohol for use as a source of energy has been included because of the growing interest in the use of the fermentation alcohol as a fuel for motorcars. Approximately 80% of the world supply of alcohol is met through fermentation, preferably the ethanol, although in countries with advanced technology, such as United States. Almost all of the industrial ethanol is made from ethylene derived from petroleum sources.

Raw Materials

As a general rule it can be assumed that 45kg (100lb) of fermentable sugar (as glucose) yield from 18 to 23 kg (40 to 50 lb) of ethanol [or 23 to 28 liters (6 to 7.5 gal)]. Types of raw materials can be usefully divided into sugar-containing materials, which can be fermented directly, starchy materials, which can be easily hydrolyzed by enzymes or acids to fermentable sugars, and cellulosic materials, which are difficult to hydrolyze.

Cellulosic Raw Materials: The utilization of cellulosic materials for the production of ethanol is ultimately the most promising endeavor, but it also presents the most difficult technological and economic problems. The cellulosic residues contains the

variable portions of the á-cellulose, hemicellulose, and lignin, and comprised such varied materials as, for example, saw mill residue, paper mill residue, news print, potato peelings, rice straw, corn stover, peanut shells, cocoa and coffee husks, tobacco stalks, wheat straw, etc (Figure 4.21).

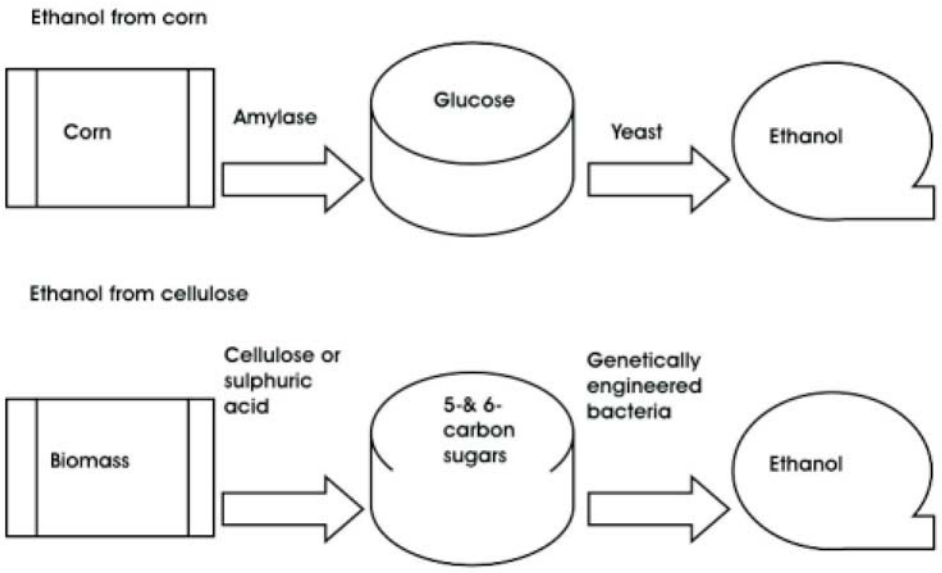


Figure 4.21: Ethanol Production from Corn and Cellulose

Sugar-containing Raw Materials: Ethanol may be produced from any sugar-containing fruits, fruit juices, or extracts, such as grape juice, apple juice, honey, date syrup, or sugar containing effluents of canneries. Such sources are usually too costly in comparison with sugar beets, sugarcane or sweet sorghum. The production of crystalline sucrose, yields a by-product, molasses, which until recently has been the cheapest source of fermentable sugar.

Starchy Raw Materials: Starch, which has been gelatinized by heating, can be readily hydrolyzed to fermentable sugars by enzymes. Such starches occur in cereal grains (rice, wheat, corn), root crops (cassava), or tubers (potatoes). All of these materials have been used for the production of distilled alcoholic beverages; the use of wheat or corn for the production of whiskey, and the use of potatoes for production of vodka are well known.

Microorganisms

Most of the experimental work, pilot scale work, and commercial production is carried out *Saccharomyces cerevisiae*. This is the organism traditionally used for the leavening of doughs and for the production of ale and distilled beverages. *Saccharomyces uvarum* (formerly *Saccharomyces carlsbergensis*), the larger beer yeast, has also been used. This species is closely related to *S. cerevisiae* and may have certain advantages because it is more flocculent and sediments faster than *S. cerevisiae*. *Candida utilis* is used for the fermentation of waste sulfite liquor since it also ferments pentoses. For *Saccharomyces cerevisiae* the optimum temperature for growth and ethanol fermentation is 30ºC but sometimes-higher temperatures (35º-38ºC) are tolerated. However, at such higher temperatures growth rate, yield of ethanol, and the death rate may be affected. The fermentation of whey requires the use of "dairy" yeast. Suitable species are *Kluyveromyces fragilis* and *K. lactis* (formerly classified in the genus *Saccharomyces*).

Fermentation Nutrients

In most traditional fermentation, the use of yeast nutrients is not required. However, in some instances the supply of such nutrients may be limiting. For instance, in production of fruit wines (other than grapes) the supply of nitrogen is often inadequate. Yeast requires the following nutrients for growth per 45 kg (100 lb) of yeast solids: About 3-3.6 kg (7-8 lb) of nitrogen from an assimilable nitrogen source such as ammonia, ammonium salts, urea, or amino acids; about 1.1 kg (2.5 lb) of phosphorus (as P_2O_5), usually in the form of orthophosphate; and minor quantities of potassium, magnesium, and calcium salts as well as trace minerals. The addition of vitamins is rarely required but thiamin often accelerates the rate of fermentation (Figure 4.22).

Fermentation

Batch Fermentation: The total fermentation time varies between 48 and 72 hr, and final alcohol concentrations of 6-8% by vol. are obtained. Batch fermentations are carried out without the need for establishing pure culture conditions, that is, with out the need for complete sterilization of media (although pasteurization is advantageous) and without maintenance of complete sterility of equipment. However, this presupposes a rapid start of the yeast fermentation. This fermentation inhibits the growth of other microbes by depleting the available nutrients, by the

lowering of the pH, and most importantly by the formation of ethanol.

Continuous Fermentation: Most of the experimental work has been carried out with homogenous fermentations, that is, with a single stirred fermentor. Fresh medium is continuously pumped into the fermentor and an equal volume of the fermentor liquid is continuously pumped out for recovery of ethanol and yeast.

Cell Recycle: Cell recycle is a method for maintaining a high yeast cell population in the continuous fermentation. Yeast cells are separated from the withdrawn fermentor liquid by means of a centrifuge (or by a process of settling) and returned to the fermentor while the supernate essentially free from yeast cells is sent to the still.

Aeration: Continuous fermentations definitely the introduction of air into the fermentor to maintain the viability of cells and an efficient fermentation. The extent of aeration by sparging is often expressed as volumes of air per fermentor volume per minute (VVM).

Distillation: The distillation process may be divided into the distillation proper, which separates the volatile components of the fermentor liquid from the insoluble solids and which concentrate the ethanol to a distillate containing 30-96% ethanol by weight, and the rectification, which separated the ethanol from other volatile components. Ethanol has a lower boiling point than water and the vapor phase in equilibrium with an alcoholic solution has a higher concentration of ethanol.

Commercial Plants: Most of the plants producing industrial ethanol are designed to operate conventional processes, which have been successful for the production of distilled beverages such as whiskey, rum, etc. There is no doubt that fermentation of wort containing fermentable sugars can be accomplished more rapidly, even in batch processes, by using large cell concentrations. Continuous fermentation processes with high cell counts can reduce the fermentation time to 10 hr or less (dilution rate of $0.1\ hr^{-1}$).

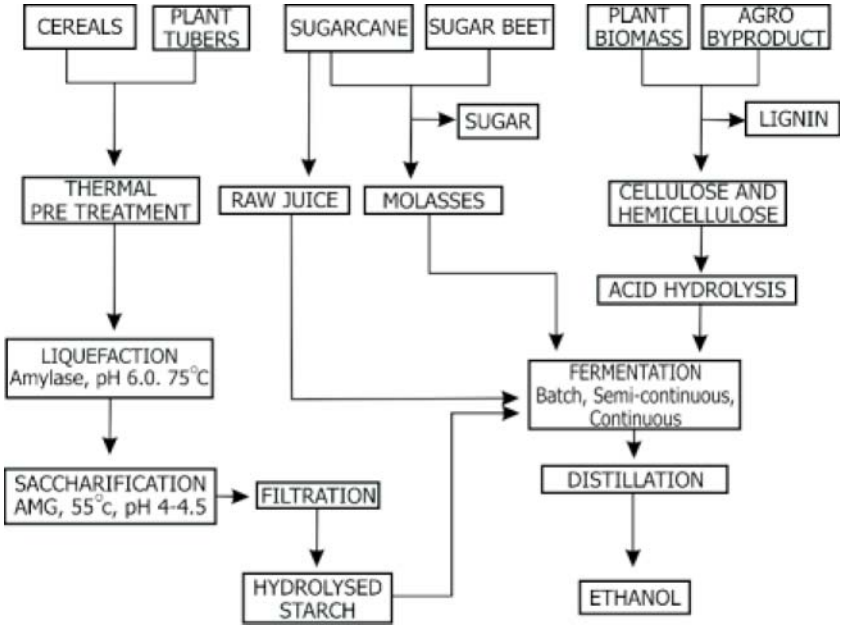


Figure 4.22: Ethanol Fermentation

Vinegar

Vinegar is the product obtained by acetic acid fermentation of alcohol-containing solutions. It must contain at least 4g of acetic acid per 100 ml (at 20°C) and may not contain more than 0.5% by vol. ethanol. Since vinegar fermentation is carried out at higher concentrations, appropriate level can be achieved by dilution with water.

Properties: Vinegar is an aqueous clear liquid which is either colorless or has the colour of the raw material. The content of solute depends on the compounds of the raw material used for the fermentation. The density, boiling point, freezing point, surface tension and viscosity of vinegar may vary with the concentration of the acetic acid and the type of raw material used. The pH is between 2 and 3.5.

Production

Basics of Vinegar Fermentation: The Vinegar fermentation is an aerobic process in which *Acetobacter* oxidizes diluted solutions of ethanol to acetic acid and water. The oxidation proceeds according to the equation:

$C_2H_5OH + O_2$ $CH_3COOH + H_2O$

The alcohol-containing solution is called a mash.

Raw Material: All mashes must contain ethanol, water and nutrients for the acetic acid bacteria. By far the largest percentage of vinegar is distilled vinegar, which is produced from diluted, purified ethanol or from fuel oil containing crude spirit. Other common names for the same product are white vinegar, spirit vinegar, alcohol vinegar or grain vinegar. It is customary in almost all countries to denature ethanol, which serves as raw material for vinegar production.

Malt vinegar is the product obtained by alcoholic and subsequent acetous fermentation, without distillation, of an infusion of barley malt or cereals whose starch has been converted to malt. It is well known in USA, UK and South Africa. Whey vinegar is produced by the alcoholic and subsequent acetous fermentation of concentrated whey. Fruit vinegar is produced from fruits, which are abundant in some countries e.g., dates, citrus fruit, or bananas. Sugar vinegar is produced by the alcoholic and subsequent acetous fermentation of sugar syrup, molasses, or refiner's syrup. Glucose vinegar is produced by the alcoholic and subsequent acetous fermentation of glucose solutions. Rice vinegar is produced by saccharification of rice starch, followed by alcoholic and subsequent acetous fermentation.

Water for Processing: The water used for the preparation of mashes must be clear, colorless, odorless, and free of sediments or suspended particles. It may be hard or soft but frequent changes in hardness as they usually occur in municipal water supplies may interfere with the fermentation.

Acetobacter: The producer organism

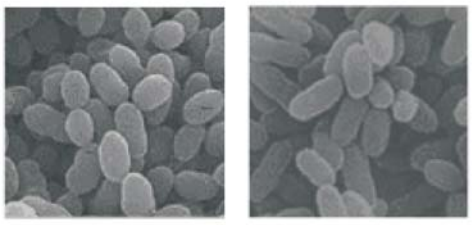


Figure 4.23: *Acetobacter* (useful), *Acetobacter*(useless)

Biochemistry of Vinegar Fermentation

In satisfactory vinegar fermentation, ethanol is quantitatively oxidized to acetic acid. Yields between 95 and 98% are normal, and the remainder is mainly lost in effluent gas. Acetic acid should not be oxidized further to water and carbon dioxide (overoxidation). Oxidation of ethanol takes place in two steps with acetaldehyde as the intermediate product.

Special Behavior of Acetobacter During Vinegar Fermentation: Sensitivity toward lack of oxygen: *Acetobacter* is very sensitive toward lack of oxygen. It is suggested that *Acetobacter* has high aspyrase activity so that the ATP that accumulates during the oxidation of ethanol is rapidly hydrolyzed and therefore only poorly available for other metabolic activities of the cell. When aeration is interrupted, the ATP pool disappears so quickly that the cell does not have the ability to adjust to the changed conditions. ATP pool is required to prevent the entry of ethanol or acetic acid into the cell interior. Thus oxygen deficiency rapidly cause cell death.

Overoxidation: Overoxidation is the undesirable oxidation of the acetic acid to carbon dioxide and water. Continued aeration in the absence of ethanol may trigger a change in cell metabolism, which occurs more rapidly at lower total concentrations than at higher ones. Overoxidation is always correlated with bacterial growth; and the reaction is faster at lower concentrations. Overoxidation once started can proceed simultaneously with the oxidation of ethanol to acetic acid. It can be recognized by lower yields, which are caused by the drop in total concentration.

The Frings Aerator: It consists of a hollow body turbine surrounded by a stator. The hollow body has 6 openings for the escape of air, which are arranged radially and which are open against the direction of rotation. The rotor has an upper and lower ring, stator has also an upper and a lower ring The rings are connected with the vertical baffles which form an angle of 30^{o}C with the radius. These baffles receive the air-liquid emulsion very close to the rotor and direct it outward. The whole aerator works by self-aspiration that is without need for compressed air (Figure 4.24).

The Biggest Acetator in Europe

Small-Scale Acetator

Figure 4.24: Fermentors

The Frings Defoamer: Foam, which occasionally forms, must be prevented from leaving the fermentor. It must be removed at the end of the fermentation cycle. The frings aerator is equipped with a mechanical defoamer. It consists of a spiral housing in which a rotor turns at 1000 or 1450 rpm.

The Frings Alkograph: An automatic instrument for measuring the percentage of alcohol for control of the fermentation has been introduced.

Trickling Processes

Older Process: The equipment used for surface fermentation of the New Orleans method can only be seen in museums of factories. The next step in the development of equipment for the fermentation of the vinegar was so called trickling process in which the bacteria adhere to the large surface areas of carrier material which is surrounded by air. The carrier material upto this date is still beech wood shavings, birch twigs, or corn cobs.

This equipment can be enlarged by addition of collection tank from which the liquid is pumped continuously over the column, with cooling of the liquid and for opening of the aspiration of air or blowing air in by means of ventilators. This scheme leads to *generator process*. The process has some basic disadvantages in comparison with equipment using the submerged fermentation processes. It is impossible to distribute

the liquid trickling over the carrier material so uniformly that the ethanol content is everywhere the same. There is, therefore, always danger that the ethanol content will drop to zero at the same points of the fermentor and lead to loses of fermentation capacity and to overoxidation. However, once the fermentation rate has been reduced it cannot be as quickly reestablished as with the submerged process, since the recolonization of those parts of the columns, where the *Acetobacter* have died is a slow process .It is difficult to stop the fermentation completely; and switching from one raw material to another produces mixed type of vinegar for some time. Yields are also lower than with the submerged process. Today quite a number of generators are still in operation, which were designed and built by vinegar factories (Figure 4.25).

Figure 4.25: Frings Aerator

Treatment of Raw Vinegar

After completion of the vinegar fermentation, a rest period of several months is required for precipitation of insoluble particles for all kinds of vinegars produced from natural raw materials. This does not apply to distilled vinegar. It is well-recognized fact that quality of vinegar improves on storage. Residual ethanol, which forms esters, plays an important part in this improvement of quality. During storage of vinegar, precipitated materials settle. This facilitates subsequent operations.

Refining: Raw vinegar contains acetic acid bacteria, which make it opaque. This is also true to a lesser extent for vinegar produced in generators. A portion of acetic acid bacteria settles during storage period. Subsequent filtration is facilitated if the bentonite is used to further refine the vinegar.

Filtration: Filtration is usually carried out with suspensions of diatomaceous earth whether the vinegar has been stored for some time or not. Filtration removes acetic acid bacteria and occasionally occurring “vinegar eels”. The latter occur often during production by trickling process, but rarely during submerged fermentation. For the filtration of the distilled vinegar simple plate and frame filters are also satisfactory. Membrane ultrafiltration process used today also eliminates the need for fining process and simplifies the filtration.

Uses

Vinegar used in home for preparation of salads and vegetables. In food industry it is used for the production of pickles, other vegetables, fish, mustard, mayonnaise, and salad dressings.

Antibiotics

Antibiotics are strong medicines that can cure many bacterial illnesses and infections. The standard definition states that an antibiotic is a substance produced by microorganisms that kills or inhibits other microorganisms. Antibiotics are medicines that help body to fight against bacteria and viruses, either by directly killing the offending bugs or by weakening them so that immune system can fight and kill them more easily. The vast majority of antibiotics are bacteria fighters; although there are millions of viruses, we only have antibiotics for half-a-dozen or so of them. Bacteria, on the other hand, are more complex (while viruses must "live" in a "host" (us), while bacteria can live independently) and easier to kill.

The first antibiotic, penicillin, was discovered in 1929 by Sir Alexander Fleming who observed the inhibition of staphylococci on a plate contaminated by a Penicillin mold. By the mid 1940's antibiotics were available for treatment against many bacterial infections including strep throat, pneumonia, skin infections, wound infections, scarlet fever, toxic shock syndrome and other bacterial infections. By the early 1950's the discovery and introduction of streptomycin, tetracycline and other antibiotics led to effective treatment of a vast array of formerly life-threatening

Bacterial Weaponry*:* Disease-causing microbes antibiotics by interfering with their mechanism of action. For example, penicillin kills bacteria by attaching to their cell walls, then destroying the main part of the wall. The wall falls apart, and the bacterium dies. Resistant microbes, however, either alter their cell walls so penicillin can't bind or produce enzymes that dismantle the antibiotic. In another scenario, erythromycin attacks ribosomes. Resistant bacteria have slightly altered ribosomes to which the drug cannot bind. The ribosomal route is also how bacteria become resistant to the antibiotics tetracycline, streptomycin and gentamycin.

Antibiotic Resistance: Antibiotic resistance results from gene action. Bacteria acquire genes conferring resistance in one of three ways. In spontaneous DNA mutation, bacterial DNA (genetic material) may mutate (change) spontaneously (indicated by starburst). Drug-resistant tuberculosis arises this way. In a form of microbial reproduction called transformation, one bacterium may take up DNA from another bacterium. Penicillin-resistant gonorrheae results from transformation.

Most frightening, however, is resistance acquired from a small circle of DNA called a plasmid, which can flit from one type of bacterium to another. A single plasmid can provide a slew of different resistances. In 1968, 12,500 people in Guatemala died in an epidemic of *Shigella diarrhoea*. The microbe sheltered a plasmid which carrying resistance to four antibiotics.

If this bacterium could be shown four times bigger, it would be the right relative size to the virus beneath it. (Both are microscopic and are shown many times larger than life). Although bacteria are single-celled organisms, viruses are far simpler, consisting of one type of biochemical (a nucleic acid, such as DNA or RNA) enclosed in another (protein). Most biologists do not consider viruses to be living things, but instead, infectious particles. Antibiotic drugs attack bacteria *not* viruses.

Kinds of Antibiotics

Penicillins and Cephalosporins: In the first part of this century, Alexander Fleming discovered that a mold called *Penicillium* (the cells are pencil-shaped when you look at them under a microscope) produces chemicals, which kill most of the bacteria nearby. The mold is green when it grows in large amounts, and is often found on bread (Figure 4.26). There are other things produced by molds. He was able to isolate these chemicals, which are now known as "penicillins". Sometime later, another mold was also found which produced a bacteria-killing chemical, and this chemical's

molecule was found to be very similar to the penicillin molecule; this chemical and its cousins were called "cephalosporins" after the mold it came from. The vast majority of antibiotics are either penicillins or cephalosporins; chemical changes have been made to the molecules over the years to improve their bacteria-fighting abilities and to help them overcome breakdown and "immunity" of resistant bacteria.

The bacterial cell has a double layer on its outside. The outermost layer the "cell wall" is similar to the outer layer of plant cells, but is missing in human and animal cells. This wall must grow along with the cell, or the growing cell will eventually become too big for the wall and burst and die. Penicillin kills by preventing some bacteria from forming new cell walls. One by one, the bacteria die because they cannot complete the process of division that produces two new "daughter" bacteria from a single "parent" bacterium. The new cell wall that needs to be made to separate the "daughters" is never formed. Some bacteria are able to resist the action of antibiotic drugs, including penicillin.

Taking antibiotics for viral illnesses like colds can also cause antibiotic resistant bacteria to develop. Antibiotics have no effect on viruses, but it will kill off harmless and even the beneficial bacteria living in the patient's body. The surviving resistant bacteria, free from competition, will live and multiply and may eventually cause disease. There are a very few bacteria that don't have cell walls. Most bacteria do have cell walls, but many have changed their wall-building systems so that penicillins can't interfere, or have come up with ways to break down the medicines before the medicines can work.

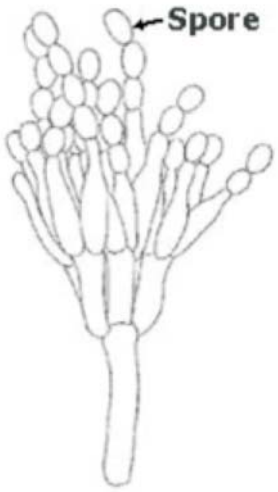


Figure 4.26: *Penicillium sp.*

Cephalexin: The full chemical name for cephalexin is 7-Alpha-D-Phenylglyclamino-3-methyl. Cephalexin is a member of the cephalosporin group of antibiotics, and is prescribed for a variety of mild infections. It is not as wide ranging in its action as some other antibiotics, but it is useful for treating infections of the respiratory tract as well as skin conditions (including acne) and soft tissue infections.

Tetracycline: Tetracyclines (tetracycline, doxycycline, minocycline, oxytetracycline) are antibiotics, which inhibit the bacterial growth by stopping

protein synthesis (Figure 4.27). They have been widely used for the past forty years as therapeutic agent in human and veterinary medicine. Tetracyclines cause Gastric mucosa cramps, burning, Nausea and vomiting.

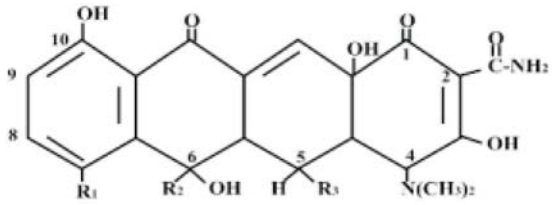


Figure 4.27: Tetracycline Nucleus

Cyclosporin prevents the rejection of transplanted organs. Without it, transplant operations would be impossible. Knowing the full life history of the cyclosporin-producing fungus may make it easier to find related moulds. Even people who see no special beauty or value in the world's biodiversity may one day benefit from the currently unknown and powerful substances, produced by fungi and other microbes, that are waiting for discovery in familiar places. Humans can slow the creation of antibiotic resistant diseases by understanding the uses and limits of antibiotics. Take all of an antibiotic, and only take them when prescribed by a doctor. Research to develop new antibiotics to treat resistant bacteria continues, but research takes time.

Amino Acids

Amino acids are the monomer units from which the polypeptide chains of proteins are constructed. Most proteins contain, in varying proportions, the same 20 L--amino acids. Many specific proteins contain L--amino acids derived from some of the basic 20 amino acids by the processes that occur after formation of the polypeptide bone. These unusual amino acids fulfill highly specific functions for the protein in question. The three dimensional structure and biological properties of simple as well as complex proteins are determined by the kind of amino acids, the order in which they are joined together, and their mutual spatial relationships.

Necessities of Amino Acids: The human diet must contain adequate quantities of ten essential L--amino acids. Humans and other higher animals cannot synthesize these amino acids in amounts adequate to support infant growth or to maintain health in

adults. Amino acids perform a multitude of structural, hormonal and catalytic functions essential to life in the form of proteins. The genetic defects in the metabolism of amino acids can result in severe illness.

Properties of Amino Acids: The genetic code specifies 20 L- -amino acids. Although over 300 different amino acids occur in nature but a subset of only 20 constitute the monomer units, which are used to make polypeptide backbones of proteins. While a triplet genetic code accommodate over 20 amino acids the redundancy of the universal genetic code limits available amino acids codons to the 20 L- -amino acids. Consequently all proteins contain varying proportions of these 20 L- -amino acids. Certain proteins contain "unusual" L- -amino acids that have arisen from some of the basic 20 by processing that occurs after formation of the polypeptide backbone (post translational processing). Different types of posttranslational modifications include methylation, formylation, acetylation, prenylation, carboxylation, and phosphorylation.

Production of Amino Acid: Amino acids are commercially produced by optimization of scale-up conditions. Some commercially produced amino acids are given in the table 4.4.

Table 4.4: World Production of Amino Acids and Their Applications.

Amino acid	**Annual production** (Metric tons)	**Application**
L-Alanine	130	Flavor enhancer
L-Arginine	1000	Therapy, Cosmetics
L-Asparagine	50	Therapy
L-Aspartic acid	4000	Aspartame production,
L-Cystein	700	Antioxidant
L-Glutamic acid	370,000	Flavor enhancer
L-Glutamine	500	Therapy (ulcer)
L-Glycine	5000-6000	Sweetener
L-Histidine	200	Therapy (ulcer)
L-Leucine	150	Infusions
L-Lysine	70,000	Feed additive

Methods of Production: In the commercial manufacture of amino acids, there are three processes:

1) Extraction of amino acids from protein hydrolysates: This method is used to obtain L-cystein, L-leucine, L- tyrosine and L-asparagine.

2) Chemical synthesis: The production of glycine, D, L-alanine, D, L methionine and D, L tryptophan always involves chemosynthesis. Chemical method is cheaper than microbial production but the chemical product is the optically inactive mixture of the D- and L- isomers.

3) Microbial production (Table 4.5):

Table 4.5: Production of Amino Acids by Fermentation.

Amino acid	Strain used	Yield (g/l)	Carbon source
L-Alanine	*Pseudomonas sp*	17.5	Glucose
L-Aspartic acid	*Escherichia coli*	56	Fumaric acids
L-Glutamine	*Corynebacterium glutamicum*	58	Glucose
L-Histidine	*Serratia marcescens*	40	Sucrose
L-Leucine	*Brevibacterium lactofermentum*	28	Glucose
L-Lysine	*Brevibacterium lactofermentum*	70	Glucose
L-Serine	*Brevibacterium lactofermentum*	4.5	Glucose
L-Tryptophan	*Escherichia coli*	23.5	Glucose
L- Tyrosine	*Corynebacterium glutamicum*	18	Glucose

There are three approaches to microbial production.

(i) Direct fermentation of amino acids: It takes place by using different carbon sources as glucose, fructose, molasses, starch hydrolysates, n-alkanes, ethanol, glycerol and acetate.

(ii) Converting inexpensive intermediate products: This process has been done through biosynthesis. For example glycine that is inexpensive can be converted to L-serine.

(iii) Immobilized enzymes or cells: These are used for the microbial production of amino acids, sometimes in continuous processes involving enzyme-membrane reactors.

Strains for Amino Acid Production: Although microorganisms, which excrete glutamic acid, are easily obtained from nature, it has been more difficult to obtain natural isolates, which excrete other amino acids for large-scale production.

Process Control

Amino acid production runs for 2-4 days in batch processes in vessels with a capacity of up to 450 m^3. Methods using continuous processes have been developed, but not yet implemented in commercial plants. Following aspects are considered important during process control:

1) *Infections Due to Bacteriophage:* These may cause considerable loss in the production. Phage-resistant mutants and the chemical agents, which inhibit phage reproduction, may be used to avoid phage infection.

2) *Oxygen Requirement:* Because of the high rate of the sugar breakdown and the high respiratory activity during the fermentation, a high oxygen requirement is exhibited. The optimal aeration rates for individual processes depend on the strain, the substrate and the biosynthetic pathway.

3) *Temperature:* Excess heat must be simultaneously dissipated, since the processes are carried out at temperatures between 28 - 30 C.

4) *pH:* The pH is kept constant between 6.8- 8.0 depending on the process; gaseous NH_3 is frequently used for pH control and is simultaneously metabolized as a nitrogen source.

5) *Amino Acid Concentration:* Biosensors have been developed which permit continuous measurement of the amino acid concentration in the fermentor.

Production of Individual Amino Acids

L-Glutamic acid: It is manufactured predominantly by microbial means, although it is also manufactured chemically. L-glutamic acid production was found to occur on a

wide variety of bacteria, streptomycetes, yeasts and fungi. The isolation of *Corynebacterium glutamicum* was accomplished in 1957 and was immediately used industrially due to its high excretion of glutamic acid.

L-Lysine: Lysine is essential for animal and human nutrition. It occurs in plant proteins only in low concentrations. Addition of lysine can therefore increase the quality of plant foods. Lysine is produced today only by microbial processes by several approaches. Lysine can be produced via diaminopimelic acid (DAP) on molasses medium with a yield of 19-24 g/l by using *Aerobacter aerogenes* at 35 C.

L-Tryptophan: Tryptophan has traditionally been produced through chemical synthesis or through fermentative or enzymatic conversion of chemically synthesized intermediates. The disadvantage of the enzymatic processes is the high cost of the starting materials, indole, serine, pyruvate, or anthranillic acid.

Commercial Uses of Amino Acids

Amino acids have extensive industrial applications. About 66% of the amino acids produced are used in the food industry, 31% as feed additives, and 4% in medicines, cosmetics and as starting material in the chemical industry.

Food Industry: In food industry amino acids are used alone or in combination to enhance flavors. The flavor enhancing effect of sodium glutamate has been known. Sodium aspartate and D'L-alanine are added to fruit juices. L-glycine is used as sweetener. L-cysteine improves the quality of bread during baking process and acts as an antioxidant in fruit juices.

Chemical Industry: In the chemical industry amino acids are used as starting materials for the manufacture of polymers, such as polyalanine fibers and lysine isocyanite resins. Poly--methylglutamate is used as surface layer in the manufacture of synthetic leather. Glycine is used as a starting material for the production of the herbicide glyphosphate and threonine serves a similar purpose for azthreonam.

Pharmaceuticals: Many amino acids are used in the medicine, particularly as ingredients of infusion solutions in post-operative treatment.

Enzymes

Enzymes are proteins produced by living cells and utilized by these cells to catalyze

specific chemical reactions. The biological function of enzyme is to bring about and regulate the metabolic processes in the organism. The characteristic feature of enzymes is that that they are highly specific in their action. Enzymes work at high conversion rates, and exhibit their action under physiological conditions of low pressure, low temperature and in aqueous solutions. Usually enzymes are intracellular (interior of living cells) but extracellular enzymes are excreted to prepare nutrients which otherwise could not enter the cell.

In fermentation processes microbial enzyme reactions have been conducted for many centuries. The most obvious advantages are easier handling, greater specificity in catalytic function and greater predictability of activity.

Rules for the Working of Enzymes as Biological Catalysts:

(i) The enzymes speed up the chemical reaction without affecting the results of overall reaction.

(ii) The enzymes react with the compound called the substrate and is regenerated at the end in its original form (Figure 4.28).

(iii) The enzyme-substrate complex has energy of formation lower than that of the activated intermediates of the uncatalysed reactions. Thus a large number of molecules react at a given temperature.

(iv) The formation of an enzyme-substrate intermediate is temporary and has no effect on the equilibrium of the reaction.

(v) Enzymes do not shift the equilibrium but merely increase the rate at which the equilibrium is reached.

(vi) Enzymes are very specific and act at very low concentrations.

Principles of Industrial Enzymology: Commercial production and utilization of enzymes are based on two factors:

(i) Enzymes are produced by living cells.

(ii) Enzymes can exert their specific action independent of living cells.

Enzyme Activation: The enzymes are not always found in active state but before their action they become activated, such enzymes include the proteolytic or protein

digesting varieties, which are present in cells. The inactive enzyme such as trypsinogen is converted into an active enzyme trypsin by a number of enzymes including trypsin itself. The activation involves certain structural changes.

Cofactors: Some enzymes are inactivated by certain other small, non-protein, heat stable substances called cofactors. The cofactors are of following three types:

(i) ***Prosthetic Group:*** Some enzymes, called apoenzymes are unable to perform their functions unless they are firmly attached with a co-factor, called prosthetic group. Thus they form holoenzymes (active form).

Apoenzymes + Co-factor Holoenzyme

(ii) ***Coenzymes:*** Coenzymes are the groups, which are covalently bonded with the apoenzymes and can easily be separated by dialysis. They are heat stable and are generally responsible for the transfer of electrons and functional groups from one substrate to an accepter such as nicotinamide adenine dinucleotide phosphate (NADP) are present in a group of enzymes called dehydrogenases, whereas NADH and NADPH are coenzymes of reductases.

(iii) ***Metal Activators:*** Some enzymes, function only in the presence of specific metals like Mn^{++}, Ca^{++}, Mg^{++}, K^{+}, Zn^{+} and Co^{++}, thus they are called metal activators.

Properties of Enzymes

(i) Specific Nature of Enzyme: Enzyme catalysis involves a close interaction of enzyme and substrate. The structural changes brought about in the molecule of enzyme like denaturation reduces the enzyme activity. This structural change suggests the specific secondary, tertiary and quaternary structure of enzyme molecule to carry on catalytic action.

(ii) Active Site: There are one or more regions on enzyme at which the temporary union between the enzyme and the substrate takes place and this region is known as active site. The active site is composed of R groups brought in to specific position by the coiling, folding and association of the secondary, tertiary and quaternary structure of enzyme respectively. This arrangement of the R-group modifies their reactivity so as to make the active site not only specific in its configuration but also its chemical reactivity.

(iii) Substrate Specificity: This term refers to the characteristic property of enzymes

to selectively activate certain chemical compounds or types of compounds. At present 1000 enzymes are known. According to their action they are classified into 6 main groups:

(a) Oxidoreductases: Enzymes, which catalyze oxidation-reduction reactions.

(b) Transferases: Enzymes that transfer groups (e.g. methyl-, glycosyl-groups) from one substrate (donor) to another substrate (acceptor).

(c) Hydrolases: Enzymes that catalyzes the splitting of bonds, such as C-O, C-N, C-C etc. by hydrolytic action.

(d) Lyases: Enzymes that cleave bonds as C-O, C-N, and C-C etc. with the formation of double bond or catalyze the binding of groups to double bonds.

(e) Isomerases: Enzymes that catalyze conformational changes of a molecule.

(f) Ligases: Enzymes that catalyze coupling of two molecules in conjunction with hydrolysis of an energy rich triphosphate.

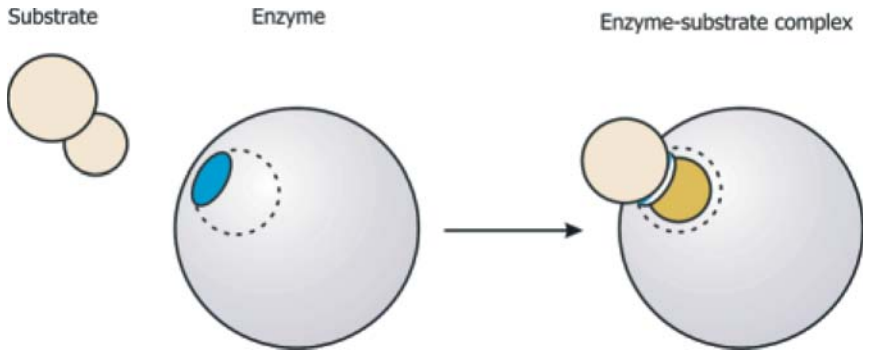


Figure 4.28: A Cartoonish View of the Formation of an Enzyme-Substrate Complex

(iv) ***Attachment of Substrate to Active Site:*** Two mechanisms are known to exist to explain attachment of a substrate to active site of an enzyme.

(a) *Lock and Key Hypothesis:* Enzymes are very specific and it was suggested by Fischer in 1890 that this was because the enzyme had a particular shape in to which the substrate or substrates fit exactly. This is called "lock and key hypothesis", where the substrate is the key whose shape is complimentary to the enzyme or lock (Figure 4.29).

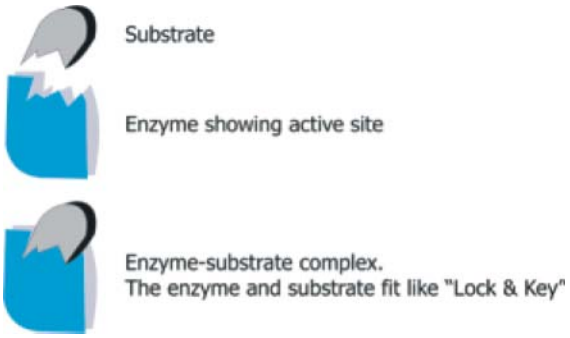


Figure 4.29: Enzyme Substrate 'Lock and Key'.

(b) *Induced-Fit Hypothesis:* In 1959, Koshland suggested when a substrate combines with an enzyme, it induces changes in the enzyme structure. The amino acids, which constitute the active site, are molded in to a precise formation that enables the enzyme to perform its catalytic function most effectively. This is called “induced-fit hypothesis” or hand and glove model because the hand changes the shape of a glove as the glove is put on. In some cases substrate also changes the shape slightly before binding (Figure 4.30).

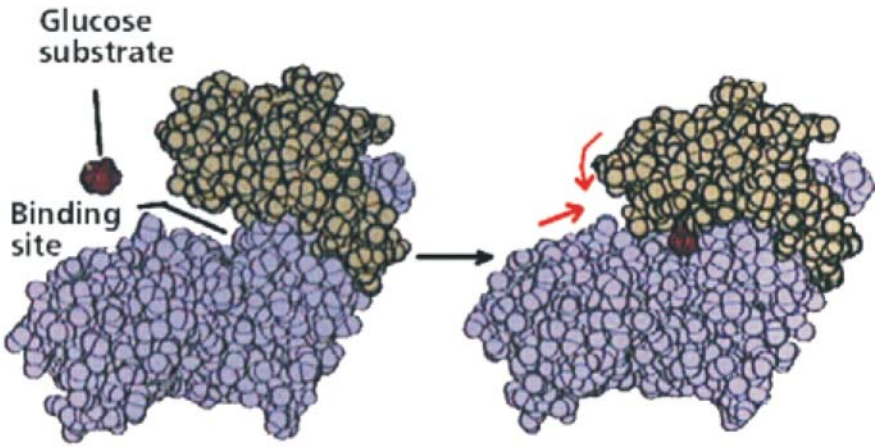


Figure 4.30: Induced-fit Model of an Enzyme Working on Glucose

(v) *Enzyme Action:* In 1913, Michaelis Menten outlined general theory of enzymatic catalysis on the basis of an enzyme substrate complex (ES) formed by a reversible action between enzyme (E) and substrate (S):

An enzyme acts as a catalyst for a certain chemical reaction. This means that the enzyme decreases the activation energy and reaction time for the reaction without changing the equilibrium. The enzyme is neither consumed nor modified in the reaction. An enzyme-catalyzed reaction starts with the formation of an enzyme-substrate complex, ES. The complex has two possible outcomes. It can dissociate to free enzyme (E) and substrate (S) with a rate constant k_2, or it can proceed to form product (P) with a rate constant k_3. At a fixed concentration of the enzyme the product is formed at a rate linearly proportional to the substrate concentration. However, after saturation of the active site of each enzyme molecule with its substrate the rate of product formation is independent of the substrate concentration, [S]. The equilibrium in (1) can be pushed towards product formation by increasing the substrate concentration.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \quad (1)$$

The rate of product formation depends on the concentration of enzyme-substrate complex.

$$V = k_3\,[ES] \quad (2)$$

The rates of formation and consumption of ES can be written as:

rate of formation of ES

$$= k_1[E][S] \quad (3)$$

rate of consumption of ES

$$= (K_2 + K_3)[ES] \quad (4)$$

At steady state, [ES] is constant and the rates in (3) and (4) are equal:

$$k_1[E][S] = (K_2 + K_3)[ES] \quad (5)$$

Rearrangement of (5) gives an expression for [ES] at steady state:

$$[ES] = \frac{\dfrac{[E][S]}{K_2 + k_3}}{K_1} \quad (6)$$

The Michaelis constant, Km, is defined below:

$$Km = \frac{K_2 + k_3}{K_1} \quad (7)$$

The concentration of free enzyme [E] equals the total enzyme concentration minus the concentration of enzyme-substrate complex:

$$[E] = [E_{kd}]\ [ES] \quad (8)$$

Substitution of (7) and (8) into (6) gives:

$$[ES] = \frac{([E_{kd}]-[ES])[S]}{K_m} = E_{kd}\ \frac{[S]}{[S]+K_m} \quad (9)$$

Substituting this expression for [ES] in (2) gives:

$$V=k_3[E_{kd}]\ \frac{[S]}{[S] + K_m} \quad (10)$$

The reaction reaches its maximal rate, Vmax, when all active sites of the enzyme are occupied, i.e. when [S] is much larger than K_m, which means that [S]/[S]+Km approaches 1. Thus,

$$V_{max}=k_3[E_m] \quad (11)$$

these conditions product molecules are formed at a constant and maximal velocity. Equation (11) can also be written as:

$$K_3 = \frac{V_{max}}{[E_{kd}]} = K_{cs} \quad (12)$$

k_3 reflects the turnover number, which is the maximal number of substrate molecules that can be converted to product per time unit, dimension time^{-1}.

The Michaelis-Menten equation (13) is obtained from (10) and (11) and explains the relationship between reaction rate and substrate concentration as shown in Figure 4.31.

$$V = V_{max}\ \frac{[S]}{[S]+K_m} \quad (13)$$

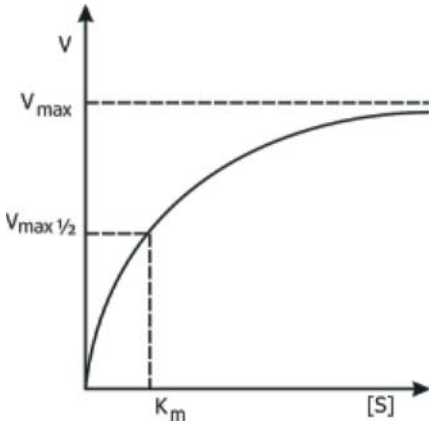


Figure 4.31: Reaction rate V versus substrate concentration [S] for an enzyme-catalyzed reaction.

At a low substrate concentration ([S] << K_m), then

$$V = V_{max} \frac{[S]}{K_m} \quad (14)$$

i.e. the rate is directly proportional to [S].

This means that an increase in substrate concentration will cause an increase in the reaction rate. On the other hand, at a high substrate concentration ([S] >> K_m), then

$$V = V_{max} \quad (15)$$

i.e. the reaction rate is independent of [S].

A special situation can be identified when the substrate concentration equals K_m ([S] = K_m); then

$$V = \frac{V_{max}}{2} \quad (16)$$

which means that the Michaelis constant K_m is equal to the substrate concentration at which the reaction rate is exactly half the maximum rate or $V_{max/2}$. The Michaelis-Menten equation (13) is transformed into an equation giving a straight-line plot by taking the reciprocal of both sides of the equation.

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}[S]} \quad (17)$$

A plot of 1/V versus 1/[S] is called the Lineweaver-Burk plot or double-reciprocal plot, and has the intercept $1/V_{max}$ and the slope K_m/V_{max}. Thus, the kinetic parameters K_m and V_{max} are readily derived by measuring the rate of catalysis at different substrate concentrations.

Multiplication of both sides of equation (17) with the substrate concentration [S] gives

$$\frac{[S]}{V} = \frac{[S]}{V_{max}} + \frac{K_m}{V_{max}} \quad (18)$$

The plot of [S]/V versus [S] is sometimes referred to as the Hanes plot (Figure 4.32). The advantage of the Hanes plot over the Lineweaver-Burk plot is that the experimental errors in V give in the former a more or less constant contribution over a wide range of [S] values (15). Consequently, more accurate results are obtained using the Hanes procedure for data treatment.

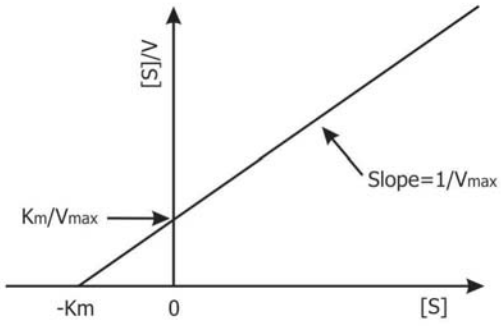


Figure 4.32: The Hanes Plot

Enzyme Inhibition: There are certain chemical substances, which have the ability to bind with the enzymes in different fashions and thereby block the catalysis. Such substances are called inhibitors or enzyme inhibitors and the process as enzyme inhibition. Inhibitors are the compounds that reduce the rate of an enzyme action.

Types of Inhibitors: According to their mode of action inhibitors are classified into several types:

(i) *Competitive Inhibitors:* These are compounds that act on the binding site of an enzyme molecule and need to be similar in size, shape and charge distribution to the substrate (Figure 4.33).

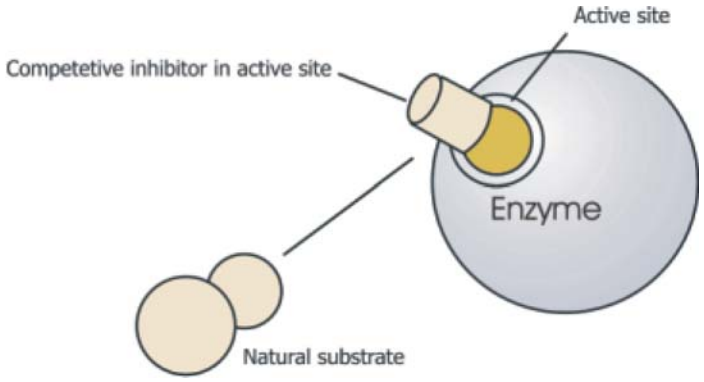


Figure 4.33: General Diagram Showing Competitor in the Active Site Normally Occupied by the Natural Substrate.

(ii) *Noncompetitive Inhibitors:* These inhibitors depend only on the concentration of the inhibitors. They may be added to a reaction mixture to rapidly reduce the enzyme activity to zero when the reaction has proceeded to the desired stage.

(iii) *Uncompetitive Inhibitors:* These inhibitors act on the intermediate enzyme-substrate complex.

(iv) *High Substrate Concentration:* High concentrations of substrate can also reduce the rate of enzyme reactions (substrate inhibition).

(v) *End Product Inhibitors:* These inhibitors are the metabolites having no similarity to the substrate, coenzyme or product of the enzyme i.e. these are allosteric, affecting the enzyme activity. They are usually end products of a synthetic chain and acts through feedback inhibition.

Enzyme Assay: The amount of enzyme is not determined on the base of its chemical constitution but on the basis of its catalytic activity, the rate of the substrate conversion, which measures the enzyme activity. The formation of an intermediate enzyme-substrate complex during the reaction makes the reaction suitable for the application of Michaelis Menten equation.

To obtain the comparable values of a given enzyme the Enzyme Commission of the International Union of Biochemistry defined the “international unit”(U) as the amount of enzyme that catalyzes the conversion of 1M substrate per minute under standardized conditions of substrate concentration, optimum pH, absence of

inhibitors, presence of activators. A list of substrates that have been used for the assay of hydrolases is presented:

(i) For amylases: Raw starch, Lintner starch, blue starch,

(ii) For cellulases: Ground bran, cotton fibers, cellulose powder, CM cellulose

(iii) For proteases: Hide powder, raw meat, casein, peptone.

Large Scale Production: Fermentation

Sources of Enzymes: Industrial enzymes are produced from plants, animals and microorganisms, but the production from first two groups is limited due to several reasons. Plant cultivation is restricted to areas where climate is suitable and is generally seasonal responsible for steady enzyme production. As the concentration of enzymes in plant tissues is generally low, processing of large amounts of plant material is necessary.

Microbial Enzymes: The microbial enzymes can be produced in amounts meeting all demands of the market. The use of microorganisms as a source material for enzyme production has developed for several important reasons. There is normally a high specific activity per unit dry weight of product, seasonal fluctuations of raw materials and possible shortages due to climatic changes or political upheavals do not occur, in microbes, a wide spectrum of enzyme characteristics, such as pH range and high temperature resistance, is available for selection. Environmental and genetic manipulations of bacteria and fungi give increased yields of desired enzymes in a way not possible with higher organisms. The diversity of enzymes from microorganisms is very great and more specific in applications. Industrial genetics has greatly increased the possibilities for optimizing enzyme yield and type through strain selection, mutation, induction and selection of growth conditions and, more recently, by using the innovative powers of gene transfer technology and protein engineering.

Selection of Microorganisms: The first step in the production of an enzyme involves the selection of an organism suitable to produce the desired enzyme in amounts as large as possible. The general aspects of procedure is given below:

(i) Extracellular enzymes are preferred than intracellular because difficult and costly methods of cell disruption are not necessary.

(ii) High yields of enzyme should be obtained with an economical time of culture production.

(iii) The strain must be stable with respect to productivity and requirement of cultural conditions.

(iv) The organism should be able to grow on cheap substrates.

(v) Synthetic activity must be in direction of desired enzyme avoiding interference of by-products.

(vi) Clarification of extract must be without difficulties.

(vii) The strain must not produce toxic substances and should be free from antibiotic activities.

Inducible Enzymes

There are very few enzymes synthesized in substantial amount under all conditions of growth. Many commercially used enzymes are inducible in nature. Their biosynthesis requires presence of substrate in the medium. Some inducible enzymes are given in Table 4.6:

Table 4.6: Inducible Enzymes

Enzyme	Organism	Inducer
Amylase	*Bacillus* spp.	Starch, dextrin, amylose
Cellulase	*Aspergillus niger*	H_2O_2, O_2
Invertase	*Pullularia pullulans*	Sucrose
Lipase	*Candida cylinderacea*	Tripalmitin,
	Candida lipolytica	Sorbitan monooleate
	Candida paralipolytica	Cholesterol

Fields of Application

Enzymes are of very much industrial importance. Simple enzymes are usually preferred for their application in the food and other industries.

Uses of Microbial Enzymes: Uses of microbial enzymes in food processing falls roughly in to three categories:

(i) Enzymes used as an essential part of a process: This category involves enzymes, which forms an essential part of a process e.g. in production of cheese, beer, spirits.

(ii) Enzymes used to improve the economics of a process: Enzymes which are used to improve the economics of a process e.g. extraction of fruit juice and essential oils are involved in this category.

(iii) Enzymes used to improve the product quality: Enzymes which are used to improve the product quality e.g. meat tenderization, loaf volume etc. falls in this category.

So these are some essential microbial fermentation products that utilize all the basic techniques for production.

Chapter 5

Downstream Processing

Chapter 5

DOWNSTREAM PROCESSING

Introduction

After the organism has been grown and the product produced, the most important step is to get the purified product. Possibly the most expensive part of the entire process is extraction and purification of the product. Product must be separated from cells, cell fragments, cell by-products, medium constituents. It is not enough to grow the required cells in a bioreactor, extraction and purification of the desired end product (the so-called downstream processing) from the bioprocess is equally important.

The design and efficient operation of downstream processing operations are vital elements in getting the required products into commercial use and should reflect the need not to lose more of the desired product than is absolutely necessary. An example of the effort expended in downstream processing is provided by the plant Eli Lilly built to produce human insulin (Humulin). Over 90% of the 200 staff is involved in recovery processes. Thus, the downstream processing of the biotechnological systems represents a major part of the overall costs but is also the least glamorous aspect of biotechnology. Improvements in downstreaming techniques will benefit the overall efficiency and costs.

Downstream processing is primarily concerned with initial separation of the bioreactor broth into a liquid and a solid phase and subsequent concentration and purification of the product. Processing normally involves more than one stage.

Choice of downstream process is based upon:

- Location of the product in the organism intracellular or extracellular
- Concentration of the product in the growth medium
- Physical and chemical properties of the product
- Intended use of the product
- Minimal standard of purity

- Biohazard level of medium, cells, or product
- Impurities present
- Market price of the product

One can modify the upstream processes to aid in downstream purification by:

- Selection of organisms that do not produce undesirable pigments or metabolites
- Modify the fermentation conditions so that undesirables are not produced
- Precise timing of harvest
- pH control after harvesting
- Temperature control after harvest
- Addition of flocculating agents
- Addition of antifoams that do not cause purification problems
- Use of lytic enzymes to aid in cell wall disruption

Unit operations in downstream processing are individual processes that operate separately from each other. Optimization of downstream processing costs can be achieved by as much integration of unit operations like:

Cell separation

Flocculation

Centrifugation

Filtration

Cell disruption

Homogenizers

Hydrolytic enzymes

Clarification

Centrifugation

Filtration

Concentration

Precipitation

Chromatography

Ultrafilteration

Partitioning

Distillation

High resolution techniques

Chromatography

Electrophoresis

Dialysis

Finishing/packaging

Crystallization

Filtration

Gel chromatography

Drying

Methods in use or proposed range from conventional to the arcane, including distillation, centrifugation, filtration, ultrafilteration solvent extraction, adsorption, selective membrane technology, reverse osmosis, molecular sieves, electrophoresis and affinity chromatography. It is in this area that several potential industrial applications of modern biotechnology have come to grief wither because the extraction has defeated the ingenuity of the designers or, more probably, because the extraction process has required so much energy as to render it uneconomic.

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

The role of downstream processing will continue to be one of the most challenging and demanding parts of many biotechnological processes. Purity and stability are the hallmarks of most high value biotechnological products.

Enzyme Recovery

In enzyme production, product output is very small as compared to input of raw material. Thus, involvement of adequate concentration procedures is needed. For commercial application of enzymes, a concentration of 10-fold is usually required. For example, enzyme products employed in detergents contain about 5-10% protease while amylase preparations for use in flour treatment contain only about 0.1% pure alpha amylase. However, in applications where high purity enzymes are required, e.g., in enzymic analysis, 1000-fold purification is quite common. In some applications, such as baking and dextrose manufacture, the removal of contaminations and by-products is necessary to avoid undesirable effects.

Considering enzyme stability there is another reason for treatment of crude enzyme preparations. Since the trend in enzyme applications is toward use of liquid preparations, stabilization is an important procedure. Figure 5.3 is a diagrammatic presentation of some treatments used for the preparation of enzyme on commercial scale. Techniques for large-scale isolation and purification of enzymes from microbial sources make use mainly of traditional procedures. Nearly all process operations are carried out at low temperatures (0-10ºC), with the exception of drying.

Separation procedures are usually carried out in batches rather than continuously. However, at large scale, batch operations extend processing times resulting in increased loss of enzyme activity due to denaturation of enzyme protein. For this reason the application of continuous operations seems to be useful, but it requires highly reliable machines and ingenious process control.

Downstream Processing for Enzymes

Extraction Methods

The first step in the isolation of enzymes is their extraction. Techniques that fall into this group are employed either to separate enzymes from solid substrate culture or to release enzymes from the interior of microbial cells. Most industrial fermentations involved well-mutated or engineered strains of microorganisms that produce large amounts of the required enzyme activity. In industrial enzymology therefore the methods of purification (known as "downstream processing") are directed first at separation of the enzyme from it's producer cells and secondly to removal of the excess water. This is in order to enable storage and transport of these enzymes in an economically viable way. This applies to bulk enzymes such as protease, pectinase, cellulase, xylanase, glucanase, phytase and alpha-amylase. Figure 5.1 shows a complete commercial downstream processing unit.

The method of production determines the methods of separation and purification required. There are two types of fermentation used to produce enzymes. The first and more common method is via submerged fermentation in large tanks. These tanks have significant engineering requirements in order to keep the fermentor cells oxygenated and stirred. A common producer in this is *Bacillus* species. The second method is surface or solid-state fermentation, where the microorganisms are grown on beds or layers on solid substrates such as sugar beet pulp and wheat bran. This is more suited to organisms such as *Aspergillus* that develop a mycelium structure.

Figure 5.1: Complete Downstream Processing Unit

Most industrially produced enzymes are actually excreted into the surrounding liquid (extracellular) but if the enzymes are intracellular a preliminary stage involves the disruption or "lysing" of the producer cells. This cell disruption is usually done by physical means. Examples of equipment used to physically disrupt microbial cells include bead mill homogenisers, rotor-stator homogenisers, high-pressure homogenisers and ultrasonic disintegrators. Lysing can also be induced by killing the cells with various chemicals or by radically altering the pH, temperature or osmotic properties of the growing medium.

The next stage in downstream processing is to remove the bacterial or fungal cells. After submerged fermentation, this is done usually by centrifugation, although in the case of more solid mycelial fermentation the mycelium literally may be pressed to extract the liquid or "juices". The remaining liquid is then subjected to ultrafilteration, which removes most of the water and other residual small molecules (amino acids, sugars etc.). If the enzyme is to remain in a liquid format for subsequent use, usually all that is added at this stage are some stabilizers such as potassium sorbate or glycerine. If the enzyme is to be sold as a powder, then the ultrafiltered liquid is spray-dried and blended with carriers to the correct activity. Speciality enzymes, or those requiring greater purity, are subjected to large-scale chromatography such as ion exchange, gel filtration or affinity chromatography.

Extraction from Solid Substrate Culture: Enzymes produced by solid substrate cultivation used to be of the extracellular type. It is therefore easily conceived that extraction of mold bran is rather a washing out process. Countercurrent techniques of percolation are the most frequently used unit operation.

In many cases, the mold bran is dried prior to extraction. This is convenient when the utilization of the particular enzyme preparation is seasonal. The cultures can be produced in relatively small equipment all the year round, while the extraction is conducted in times of enzyme demand. Extraction from dried bran will yield solutions with higher enzyme concentrations. In addition, drying avoids interference caused by the activity of living cells of fresh cultures. This argument, however may not apply in continuously operated culture plants. In all cases, the extractant is water, which however, may contain acids (inorganic or organic), salts, buffer or other substances to facilitate solubilization of the enzyme or to improve its stability in solution, or to exclude or minimize undesired effects caused by contaminating by-products or microorganisms.

Extraction of Cells: The decision on whether to employ whole cells for a biochemical process or to use isolated enzymes depends on many factors. Technical difficulties and cost of large-scale isolation play an important role. There are a number of methods for cell disruption. Chemical and biochemical methods, such as autolysis, treatment with solvents, detergents or lytic enzymes are difficult to standardize and optimize, whereas, mechanical methods involving homogenization are more recommendable.

Separation Methods

It is possible here to give only outline of methods applied for large-scale production of enzymes.

Solids Separation Techniques: Such methods are involved in the clarification of culture liquors and extracts, in the separation of precipitates, and in the sterilization of liquid enzyme preparation by mechanical methods. The solids to be separated may have a number of properties, which make separation process difficult. For instance, they may be greasy, sometimes colloidal, and often density differences between solid particles and liquid phase are very small. Therefore, pretreatment of the liquor is usually inevitable, as conducted by acidification, addition of water miscible solvents or liquid polyions, mild heating etc.

The problems of large-scale solid-liquid separation are complex and diverse. There are two approaches, centrifugation and filtration. Industrial centrifuges are not ideal for removal of finely divided biological solids. Disc type centrifuges are efficient in separation of easily settling suspensions of greasy particles. Decanters are used in cases where solids content is high but easily settling, e.g., in the production of dried acetone precipitates, hollow bowl centrifuges are employed for separation from low solids suspensions as obtained during fractionated enzyme precipitation. Cooling devices are used to increase precipitate settling rate. Filters are more suitable for separation of biological particles.

Membrane Separation Techniques: Solutes can be separated from solvents without any phase change or interphase mass transfer. There are many different kinds of membrane processes, which are classified on the basis of driving forces that cause transfer of solutes through the membrane. Such a force may be transmembrane differences in concentrations, as in dialysis; electric potential, as in electrodialysis; or hydrostatic pressure, as in microfilteration, ultrafilteration and reverse osmosis.

At present, ultrafilteration is mostly used in large-scale enzyme production. The Technique of ultrafiltration has advantage of combining both separation of impurities and concentration of the desired enzyme.

Gel Filtration: Gel filtration is diffusional partitioning of solute molecules between the readily mobile solvent phase and that confined in spaces within porous gel particles that make up the stationary phase. Separation of molecules from each other can be obtained by gel filtration, but in many cases, its application is not economically feasible.

Adsorption Techniques: Adsorption processes are increasingly used because of the possibility of highly selective separation. Properties of enzyme molecules like lipophily, electric charge, specificity etc., are the basis of separation. Different types of adsorbents are used e.g., active carbon, hydroxyapatite, ion exchangers, carrier fixed substrate analogs etc. Separation is achieved by adsorption and elution of either enzyme or impurities. Two methods are available, batchwise separation and column chromatography.

Precipitation Techniques: Separation from solution by salting out is one of the oldest end yet most important methods for concentration and purification of enzymes. Commonly used salt for precipitation is ammonium sulphate because it is highly soluble, non-toxic, has low price, and act as a stabilizer.

Conversion to Storage Form: Commercial enzyme products are available as solutions or in solid state, solutions are preferred for their easy handling, but enzymes are very unstable in aqueous solutions. For this reason stabilization of dissolved enzymes is very important step in the manufacture of liquid enzyme preparations. Enzyme stability is affected by two factors i.e., microbial deterioration of the enzyme and denaturation of the enzyme protein.

Various methods are used to prevent growth of microorganisms, which include incorporation of chemical preservatives, salts, sugars and polyhydric alcohols, pasteurization and irradiation. Storage at low temperatures and at suitable pH is inevitable.

Stabilization is achieved by following techniques:

- Conformational or charge stabilization by using buffers, glycerol, substrates, or inhibitors.

- Protection of active site by thiols, redox dyes, oxygen binding agents, or chelating agents.

Miscellaneous methods include, inhibition or removal of proteolytic enzymes; protection from light by photosensitive dyes; lowering activity of water by viscosity effectors, salts or sugars; lowering surface energy by antifoams; cooling and crystallization protection by antifreeze; removal of harmful agents and sterilization for protection against microbial attack (Figure 5.2).

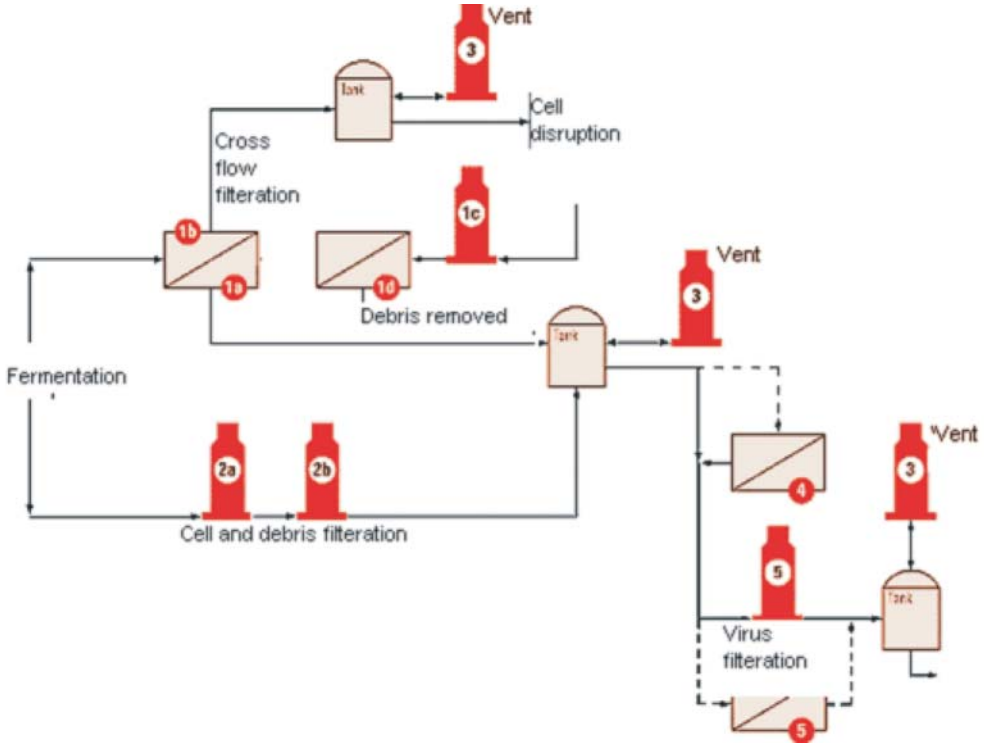


Figure 5.2: Downstream Processing: Clarification -- Schematic Flow

Recovery Methods for Acids

Downstream Processing of biological products produced by fermentation or cell culture begins with the clarification and separation of the product from the harvest fluid. For example, the crude fermented liquor containing citric acid is filtered to

remove mycelia or cells and other suspended impurities. The waste mycelia are pressed to recover most of the fermented liquor. The mother liquor is heated at 80-90°C by the addition of small amount of hydrated lime to allow precipitation of oxalic acid. Citric acid is then precipitated as calcium citrate using 1 part of hydrated lime for every two parts of liquor added over a 1-hour period while the temperature is raised to 95°C. Although this is a generally accepted procedure, a precipitation temperature of 50°C for 20 minutes has been reported to be optimum for calcium citrate precipitation and citric acid recovery. The product obtained at this temperature is easy to filter. The precipitation is slightly slower but complete at this temperature.

The precipitated calcium citrate is filtered and washed with water several times. It is then transferred to acidulators and treated with H_2SO_4. The citric acid is decolorized by charcoal and passed through ion exchange resin columns. The liquid is concentrated in vacuum and finally run into low temperature crystallizers where citric acid crystallizes as citric acid monohydrate. The quality of citric acid can be improved by using $Ca(OH)_2$ free of contaminating metals such as Mg^{++}, Fe^{++}, Al^{+++}, etc. Also by addition of 9-12% calcium ferrocyanide to mother liquor at 95-97°C for 5-8 minutes, the recovery of citric acid is found to improve.

Electrodialysis: It is an electrochemical membrane process, by which the charged particles of an electrolyte solution (ions) are separated from a crude solution in an electrical field using ion-selective membranes. Through the combination of the product separation step with the electrodialytically forced water dissociation on bipolar membranes, aqueous ionogenic solutions are transferred into the respective inorganic or organic acid and base. Applications for bipolar electrodialysis in downstream processing, e.g.

- In biotechnology and food technology
- Separation and purification of organic acids and amino acids from fermentation broths by combined electro-dialysis and e.g. crystallization, distillation, and nanofiltration (experiences in: acetic acid, formic acid, citric acid, malic acid, fumaric acid, itaconic acid, succinic acid, gluconic acid, galacturonic acid, glycine, alanine, glutamic acid, methionine)
- Separation of amino acids from racemic mixtures
- Pasteurization of hydrous solutions at room temperature

- Combined deacidification of fruit juices and recovery of the organic acid
- Gentle adjustment of the pH value of aqueous solutions without increasing the ion potential, e.g. flavor enhancing.
- Partial inversion of sucrose or inversion of potato starch

Further applications of bipolar electrodialysis are the recovery of process chemicals and the saving of chemicals in manufacturing industries, e.g. for the recovery of EDTA from electroless copper plating bath, for the recycling and regeneration of battery acid or the recovery of exhausted alkali from process solutions for the manufacture of nickel-cadmium batteries.

Bipolar electrodialysis is used for the recovery of lactic acid for the production of lactic acid from acid whey, which is a residual material of milk processing. In the first step of this process a selected strain of lactic acid bacteria produces lactic acid from lactose with high efficiency. After the fermentation the organisms are removed by cross-flow-filtration (cell recycle), and the lactate ions are separated from the fermentation broth and converted to free lactic acid by bipolar electrodialysis.

In conclusion, it can be said that biotechnological processes will, in the most part, need to be contained within a defined area or system and to a large extent, the ultimate success of most of the processes will depend on the correct choice and operation of these systems. On the industrial side, the scale of operation will, for economic reasons, mainly be very large, and in almost all cases, the final success will require the closest cooperation between the bioscientist and the process engineer in this way demonstrating the truly interdisciplinary nature of biotechnological processes.

Chapter 6

Applications of Biotechnology

Chapter 6

PRACTICAL APPLICATIONS OF BIOTECHNOLOGY

Contrary to its name, biotechnology is not a single technology, rather it is a group of technologies that shares two characteristics, working with living cells and their molecules and having a wide range of practical uses that can improve our lives. The applications of biotechnology are so broad and the advantages so compelling that virtually every industry is using this technology. Developments are underway in areas as diverse as pharmaceuticals, diagnostics, textiles, aquaculture, forestry, chemicals, household products, environmental cleanup, food processing and forensics. Biotechnology is enabling these industries to make new or better products, often with greater speed, efficiency and flexibility. Biotechnology holds significant promise for the future but certain amount of risk is associated with any area. Biotechnology must continue to be carefully regulated for maximum benefits are received with the least risk.

Traditional biotechnology has been practiced since the beginning of recorded history. It has been used to bake bread, brew alcoholic beverages, and breed food crops or domestic animals. But recent developments in molecular biology have given biotechnology new meaning, new prominence, and new potential. It is modern biotechnology that has captured the attention of the public as that can have a dramatic effect on the socioeconomic standards.

One example of modern biotechnology is genetic engineering that envisages transferring individual genes between organisms or modifying the genes in an organism to remove or add a desired trait or characteristic. Examples of genetic engineering are described later in this document. Through genetic engineering, genetically modified (GM) crops or organisms are formed. These GM crops or GMOs are used to produce biotech-derived foods. It is this specific type of modern biotechnology that seems to generate the most attention and concern by consumers and consumer groups. Modern biotechnology is far more precise than traditional forms of biotechnology and so is viewed by some as being far safer.

If one looks at modern biotechnology, based on recombinant DNA technology, a similar distribution can be find in the use of recombinant organisms to serve in the production of food and medicines and to solve environmental problems. This leads to the division of biotechnology into three areas: Agricultural Biotechnology, Pharmaceutical Biotechnology, and Environmental Biotechnology. A fourth area of importance is Industrial Biotechnology.

Functions of Modern Biotechnology

All organisms are made up of cells that are programmed by the same basic genetic material, called DNA (deoxyribonucleic acid). Each unit of DNA is made up of a combination of nucleotides i.e. adenine (A), guanine (G), thymine (T), and cytosine (C) as well as a sugar and a phosphate. These nucleotides pair up into strands that twist together into a spiral structure call a "double helix." This double helix is DNA. Segments of the DNA tell individual cells how to produce specific proteins. These segments are genes. It is the presence or absence of the specific protein that gives an organism a trait or characteristic. More than 10,000 different genes are found in most plant and animal species. This total set of genes for an organism is organized into chromosomes within the cell nucleus. The process by which a multicellular organism develops from a single cell through an embryo stage into an adult is ultimately controlled by the genetic information of the cell, as well as interaction of genes and gene products with environmental factors.

When cells reproduce, the DNA strands of the double helix separate, because nucleotide A always pairs with T and G always pairs with C. Each DNA strand serves as a precise blueprint for a specific protein. Except for mutations or mistakes in the replication process, a single cell is equipped with the information to replicate into millions of identical cells. Because all organisms are made up of the same type of genetic material (nucleotides A, T, G, and C), biotechnologists use enzymes to cut and remove DNA segments from one organism and recombine it with DNA in another organism. This is called recombinant DNA (rDNA) technology, and it is one of the basic tools of modern biotechnology. In recombinant DNA technology DNA, or fragments of DNA from different sources, are cut and recombined using enzymes. This recombinant DNA is then inserted into a living organism. This technology, usually used synonymously with genetic engineering, allows researchers to move genetic information between unrelated organisms to produce desired products or

characteristics or to eliminate undesirable characteristics.

Major advances also have been made through conventional breeding and selection of livestock, but using biotechnology can still make significant gains. Diseases such as hog cholera and pests such as screwworm have been eradicated. Uses of biotechnology in animal production include development of vaccines to protect animals from diseases, production of several calves from one embryo (cloning), increase of animal growth rate, and rapid disease detection.

Modern biotechnology has offered opportunities to produce more nutritious and better tasting foods, higher crop yields and plants that are naturally protected from disease and insects. Modern biotechnology allows for the transfer of only one or a few desirable genes, thereby permitting scientists to develop crops with specific beneficial traits and reduce undesirable traits. Traditional biotechnology such as cross-pollination in corn produces numerous, non-selective changes. Genetic modifications have produced fruits that can ripen on the vine for better taste, yet have longer shelf lives through delayed pectin degradation. Tomatoes and other products containing increased levels of certain nutrients, such as vitamin C, vitamin E, and or beta-carotene, and help protect against the risk of chronic diseases, such as some cancers and heart disease. Similarly introducing genes that increase available iron levels in rice three-fold is a potential remedy for iron deficiency, a condition that effects more than two billion people and causes anemia in about half that number. Most of the today's hard cheese products are made with a biotech enzyme called chymosin.

In 1992, Monsanto Company successfully inserted a gene from a bacterium into the Russet Burbank potato. This gene increases the starch content of the potato. Higher starch content reduces oil absorption during frying, thereby lowering the cost of processing french fries and chips and reducing the fat content in the finished product. Biotechnical methods may be used to decrease the time necessary to detect food borne pathogens, toxins, and chemical contaminants, as well as to increase detection sensitivity. Enzymes, antibodies, and microorganisms produced using rDNA techniques are being used to monitor food production and processing systems for quality control.

Industrial Biotechnology

Industrial biotechnology uses modern molecular biology to improve the efficiency and reduce the environmental impacts of industrial processes like textile, paper and pulp, and chemical manufacturing. For example, industrial biotechnology companies develop biocatalysts, such as enzymes, to synthesize chemicals. Enzymes are proteins produced by all organisms. Using biotechnology, the desired enzyme can be manufactured in commercial quantities.

Commodity chemicals (e.g., polymer-grade acrylamide) and specialty chemicals can be produced using biotech applications. Traditional chemical synthesis involves large amounts of energy and often-undesirable products, such as HCl. Using biocatalysts, the same chemicals can be produced more economically and more environment friendly. An example would be the substitution of protease in detergents for other cleaning compounds. Detergent proteases, which remove protein impurities, are essential components of modern detergents. They are used to break down protein, starch, and fatty acids present on items being washed. Protease production results in a biomass that in turn yields a useful byproduct- an organic fertilizer. Biotechnology is also used in the textile industry for the finishing of fabrics and garments. Biotechnology also produces biotech-derived cotton that is warmer, stronger, has improved dye uptake and retention, enhanced absorbency and wrinkle or shrink-resistance.

Some agricultural crops, such as corn, can be used in place of petroleum to produce chemicals. The crop's sugar can be fermented to acid, which can be then used as an intermediate to produce other chemical feedstock for various products. It has been projected that such renewable resources in the first half of the next century could supply 30% of the world's chemical and fuel needs. It has been demonstrated, at test scale, that biopulping reduces the electrical energy required for wood pulping process by 30%.

Use of Microbes in Biotechnology

Several features of microorganisms make them the most exploited group of organisms in biotechnology to date. Among those features are the ease of culture, rapid growth cycles, the very diverse types of metabolism found which give a wide range of useful commercial products, and ease with which the relatively simple

genome can be manipulated to enhance their usefulness. A wide range of bacteria, yeasts and filamentous fungi already are in use to produce food products, industrial chemicals, hormones and antibiotics. There is, however, a constant search for organisms that produce new products, and biologists are sampling all kinds of habitats for new microorganisms.

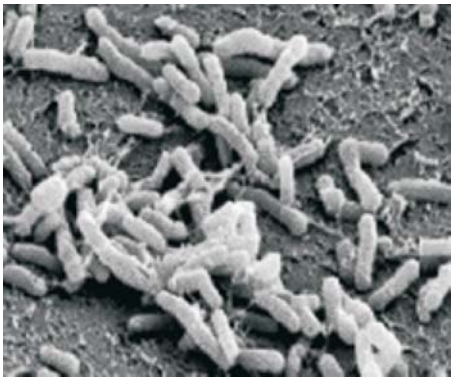


Figure 6.1: *Agrobacterium sp.*

Agrobacterium is like a mercenary commando (Figure 6.1). This bacterium invades a plant, commandeers its genetic machinery, throws the plant's cells into anarchy, and forces the plant to produce K rations for the invading bacterium and its legions in the soil. Different species of *Agrobacterium* form different types of tumors. *Agrobacterium tumefaciens* causes a tumor called a crown gall. *Agrobacterium rhizogenes,* like the name implies, causes the sprouting of root tissue from an infection site, a condition known as hairy root. *Agrobacterium rubi* causes cane gall of raspberries.

Microbes are used as workhorses in the production of many compounds, from fuel, to pharmaceuticals, to chemicals. They are also used in mining; insects and disease control, genetic engineering and some are even used to make computer biochips.

Fuel Production - ethanol production for automobile fuel

Mining - leaching of metals from ore-bearing rocks by microbes.

- 5% of world's copper ore is produced by bio-leaching
- Uranium is mined with the help of bacteria

Biocontrol- Using microbes to combat pests are called biocontrol. One of the most popular forms of biocontrol is the use of *Bacillus thuringenesis*, a bacterium that produces a toxin that kills over 40 problem pests such as the gypsy moth.

Computer Biochips- Microorganisms may some day be used to produce protein-based microprocessors with more switches than conventional microchips.

Microbial Fermentation Products

Pyruvic Acid, Lactic acid, CO_2, Butanol, Acetone, Lactic Acid, Ethanol, Acetic acid

Some Common Products of Microorganism Activities

Scotch, Wine, Beer, Yogurt, Pickles, Cheese, Amino acid, Vitamins, Insulin (first recombinant DNA product).

Yeasts and Fermentation: Yeasts are microscopic, single-celled fungi, which are found naturally in the air, soil and on the surface of some fruit. They are produced commercially on a large scale for the food and drink industries. Under the right conditions, yeast will produce the gas carbon dioxide and alcohol in a series of chemical reactions known as fermentation; these reactions are essential for making bread rise, and alcohol in wine and beer.

Bread: In bread making, fermentation takes place while the dough is rising or 'proving' in a warm place. During fermentation, sugars that are naturally present in the flour are converted to glucose, which is then fermented by the yeast to carbon dioxide and alcohol. The sugars include the disaccharide maltose, which is produced when enzymes called amylases, also present in the flour, break down the starch.

Wine: In winemaking, the fermentation of grape juice is a complex microbiological process involving interactions between yeasts, bacteria and filamentous fungi, but it is the yeasts that play the central role. During fermentation, yeasts use the sugars and other components of grape juice as 'food', converting them into ethanol, carbon dioxide and other end products that contribute to the chemical composition and taste of wine.

Yogurt: Yogurt is a milk product made using bacterial cultures. It originated in West Asia and Eastern Europe, and is now eaten all over the world. In the UK, it is usually made from cow's milk, although goat's or ewe's milk can also be used. Whole or skimmed milk is fermented by the carefully controlled addition of a 'starter culture'

of special, harmless bacteria (usually the lactic acid bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus*). Given the right conditions, these bacteria ferment lactose, the disaccharide sugar in milk, producing lactic acid, which curdles the milk and gives yogurt its characteristic tangy taste and texture. Yogurts that have not been pasteurized to destroy the fermentation bacteria are known as 'live' yogurts.

Bio-Yogurts: Recently, manufacturers have developed 'bio-yogurts', which contain extra bacteria that are not found naturally in our digestive system (most often *Lactobacillus acidophilus* and *Bifidobacterium bifidum)*. Bio-yogurts are milder and creamier than conventional yogurts. Some manufacturers also claim extra health benefits for them. Bio-yogurts are claimed to enhance the growth of beneficial bacteria that are naturally present in our intestines. The balance of these bacteria is believed to be important in maintaining intestinal health, and/or might help to protect against major diseases such as cancers and coronary heart disease.

Cheese: As is the case for yogurt, the production of cheese is initiated by the addition of a special starter culture to the milk, to convert the lactose to lactic acid, which helps to preserve the cheese. Another important 'player' in cheese making is the enzyme chymosin (or rennin), which is required to make the milk set. Traditionally, this was added in the form of calf rennet, although non-animal sources of this enzyme are available, including that produced by genetic modification and used in the manufacture of 'vegetarian cheese'.

Blue Cheeses: Yet another type of microorganism is called upon to give blue-veined cheeses (e.g. Stilton and Danish Blue) their characteristic appearance and flavor. Such cheeses are produced by inoculating the curd with a specially produced, harmless mould (*Penicillium roquefortii*), which then grows during ripening.

Mycoprotein from 'Waste' Carbohydrate: The production of mycoprotein represents an economical means of converting any surplus carbohydrate into foods of much higher nutritional and commercial value. For example, the mould *Fusarium graminearum*, which is related to mushrooms and truffles, is odorless and tasteless. It contains 45% protein and 13% fat, a composition similar to that of grilled beef. It is also high in fiber, and has a low nucleic acid content and an 'ideal' (or complete) amino acid content. The feedstock for such a mould might be based on, say, wheat in the UK, potato in Ireland, and cassava, rice or sugar in tropical countries.

Environmental Biotechnology

Environmental biotechnology is used in waste treatment and pollution prevention. Environmental biotechnology can more efficiently clean up many wastes than conventional methods and greatly reduce our dependence on methods for land-based disposal. Every organism ingests nutrients to live and produces by-products as a result. Different organisms need different types of nutrients. Some bacteria thrive on the chemical components of waste products. Environmental engineers use bioremediation, the broadest application of environmental biotechnology, in two basic ways. They introduce nutrients to stimulate the activity of bacteria already present in the soil at a waste site, or add new bacteria to the soil. The bacteria digest the waste at the site and turn it into harmless byproducts. After the bacteria consume the waste materials, they die off or return to their normal population levels in the environment.

Environmental pollution can harm the fauna and flora of affected habitats, results in the uptake of toxic chemicals into and accumulation in food chains and, in some instances, causes serious health problems and/or genetic defects in humans. Though substantial progress to reduce chronic industrial pollution has been made over the last years, major accidents still occur, an enormous number of known polluted sites exist, and new ones are being continuously discovered. Some of these sites are a source of contamination of air and groundwater drinking supplies. The public has an inalienable right to be protected from such hazards and it is imperative that critical cases of pollution be remediated as quickly as possible. Remediation by biotechnology constitutes an attractive alternative to physico-chemical methods of remediation, or removal to "safer" sites. In particular, it can be less expensive, achieve complete destruction of organic pollutants, and can be used *in situ* for pollutants at low but nevertheless environmentally relevant concentrations. Whereas some instances of pollution can be readily bioremediated using existing technology, others involving toxic, chemically stable compounds require the development of new innovative technology.

Bioremediation is an area of increasing interest. Through application of biotechnological methods, enzyme bioreactors are being developed that will pretreat some industrial waste and food waste components and allow their removal through the sewage system rather than through solid waste disposal mechanisms. Waste can

also be converted to biofuel to run generators. Microbes can be induced to produce enzymes needed to convert plant and vegetable materials into building blocks for biodegradable plastics. In some cases, the byproducts of the pollution-fighting microorganisms are themselves useful. For example, methane can be derived from a form of bacteria that degrades sulfur liquor, a waste product of paper manufacturing. This methane can then be used as a fuel or in other industrial processes.

Metal Extraction from Solid Waste with Microorganisms: Solid industrial waste often contains high concentrations of heavy metals. Many microorganisms (bacteria and fungi) are capable of extracting such substances from solid materials through a process where acids and other metabolic products are used to oxidize or reduce metal (bioleaching). These microorganisms can thus be used for innovative recycling processes for metallic waste, which are based on natural geochemical cycles.

Various materials such as ashes, slag, sludge, electronic scrap and foundry sand are treated with bacteria and fungi to extract a range of metals. About 95% of the aluminium, lead, nickel and zinc, as well as approximately 65% of the copper and tin, in electronic waste can be eliminated with the aid of fungi (*Aspergillus niger*, *Penicillium simplicissimum*), depending on the organism used and the growing conditions.

The metals (e.g. copper and aluminium) are reclaimed through a precipitation process. With the aid of bacteria (*Acidithiobacillus* sp.), 80% to 90% of the cadmium, copper and zinc can be reclaimed (Figure 6.2). Such processes can quite possibly be used to remove metals from polluted soil.

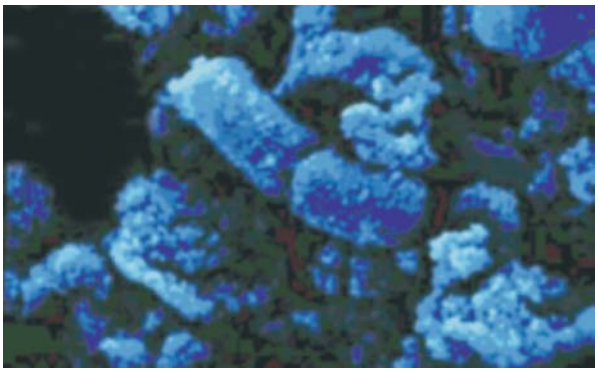


Figure 6.2: *Acidithiobacillus* sp.

Health Biotechnology

Biotechnical methods are now used to produce many proteins for pharmaceutical and other specialized purposes. A harmless strain of *Escherichia coli* bacteria, given a copy of the gene for human insulin, can make insulin. As these genetically modified (GM) bacterial cells grow, they produce human insulin, which can be purified and used to treat diabetes in humans. Microorganisms can also be modified to produce digestive enzymes. In the future, these microorganisms could be colonized in the intestinal tract of persons with digestive enzyme insufficiencies. Products of modern biotechnology include artificial blood vessels from collagen tubes coated with a layer of the anticoagulant heparin.

Direct Effects of Biotechnology on Human Health

Pharmaceuticals and Diagnostics: For centuries, biotechnology has contributed to the discovery of medicines and vaccines used to fight disease (Figure 6.3). Advances in scientific understanding and laboratory techniques during the last half of the twentieth century were significant and led to new medicines for diseases and conditions such as cancer, heart disease, stroke, diabetes, arthritis, obesity, and AIDS.

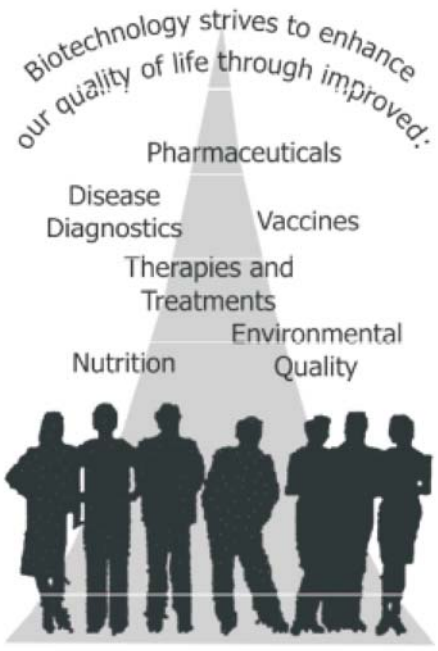


Figure 6.3: Biotechnology and human health

Using basic research and the tools of biotechnology, scientists better understand what causes certain diseases, giving them the ability to identify specific targets in the body for drug treatment. This results in more effective medicines that have fewer side effects (Table 6.1).

Additionally, as the underlying causes of diseases are revealed and diagnostic methods are improved, "personalized" disease diagnosis and treatment will become more prevalent and promises to change the way medicine is practiced. For example,

traditional treatments for breast cancer were surgery, radiation therapy, and chemotherapy. Recently, through basic cancer research, it was discovered that sapproximately 30% of breast cancers are associated with the overproduction of a protein called HER2. Combining this knowledge with modern techniques of biotechnology, scientists developed a drug that specifically targets HER2. Now, through better diagnostic methods, doctors can more specifically determine the type of breast cancer present, and if appropriate, use this new drug for more effective disease treatment.

Table 6.1: Partial List of drugs that are products of modern biotechnology.

Drug	For Treatment of /Use
Activase	Heart attack/stroke/ pulmonary embolism
AZT	HIV infection (one of 14 drugs for HIV)
BeneFix	Hemophilia B
Celebrex	Osteoarthritis and adult rheumatoid arthritis
Enbrel	Rheumatoid arthritis
Epogen	Anemia associated with kidney failure
Gleevec	Chronic myeloid leukemia
Herceptin	Certain type of breast cancer
Humulin	Diabetes
LYMErix	Lyme disease vaccine
Protropin	Growth hormone deficiency
Provigil	Narcolepsy
Recom-bivax-HB	Hepatitis B vaccine
Remicade	Crohn's disease and Rheumatoid arthritis
Rituxan	Certain non-Hodgkin's lymphoma (cancer)
Tamiflu	Flu infection
Xenical	Obesity
Zantac	Heartburn
Zenapax	Prevention of kidney transplant rejection
Zocor	High cholesterol levels

Advances in biotechnology-driven diagnostic methods aid doctors in other ways. For example, it used to take 24 to 48 hours to conduct a throat culture test to

determine if a patient with a sore throat had "Strep throat." Strep throat is caused by a bacterium and is treated with an antibiotic. A sore throat caused by a virus, such as cold or flu, will not respond to antibiotic treatment. Therefore, the doctor must know the cause of the sore throat before initiating treatment. Today, a "quick" Strep test can be performed in about 10 minutes, allowing the doctor to begin antibiotic treatment immediately if the test is positive.

Gene therapy is to alter DNA within cells in an organism to treat or cure a disease, is one of the most promising areas of biotechnology research. New genetic therapies are being developed to treat diseases such as cystic fibrosis, AIDS and cancer. DNA fingerprinting is the process of cross matching two strands of DNA. In criminal investigations, DNA from samples of hair, bodily fluids or skin at a crime scene are compared with those obtained from the suspects. In practice, it has become one of the most powerful and widely known applications of biotechnology today. Another process, polymerase chain reaction (PCR), is also being used to more quickly and accurately identify the presence of infections such as AIDS, Lyme disease and Chlamydia.

DNA testing is also used on human fossils to determine how closely related fossil samples are from different geographic locations and geologic areas. The results shed light on the history of human evolution and the manner in which human ancestors settled different parts of the world.

Paternity and Maternity: Because a person inherits his or her VNTRs (variable number of tandem repeats) from his or her parents, VNTR patterns can be used to establish paternity and maternity. The patterns are so specific that a parental VNTR pattern can be reconstructed even if only the children's VNTR patterns are known (the more children produced, the more reliable the reconstruction). Parent-child VNTR pattern analysis has been used to solve standard father-identification cases as well as more complicated cases of confirming legal nationality and, in instances of adoption, biological parenthood.

Criminal Identification and Forensics: DNA isolated from blood, hair, skin cells, or other genetic evidence left at the scene of a crime can be compared, through VNTR patterns, with the DNA of a criminal suspect to determine guilt or innocence. On some human chromosomes, a short sequence of DNA has been repeated a number of times. In any particular chromosomes the repeat number may vary from one to thirty repeats. Since specific restriction enzyme sites usually bound these repeat regions, it

is possible to cut out the segment of the chromosome containing this variable number of tandem repeats or VNTR's, run the total DNA on a gel, and identify the VNTR's by hybridization with a probe specific for the DNA sequence of the repeat. VNTR patterns are also useful in establishing the identity of a homicide victim, either from DNA found as evidence or from the body itself.

Personal Identification: The notion of using DNA fingerprints as a sort of genetic bar code to identify individuals has been discussed, but this is not likely to happen in the foreseeable future. The technology required to isolate, keep on file, and then analyze millions of very specified VNTR patterns is both expensive and impractical. Social security numbers, picture ID, and other more mundane methods are much more likely to remain the prevalent ways to establish personal identification.

Agricultural Biotechnology

Experts in United States anticipate the world's population in 2050 to be approximately 8.7 billion persons. The world's population is growing, but its surface area is not. Compounding the effects of population growth is the fact that most of the earth's ideal farming land is already being utilized. To avoid damaging environmentally sensitive areas, such as rain forests, we need to increase crop yields for land currently in use. By increasing crop yields, through the use of biotechnology the constant need to clear more land for growing food is reduced. Countries in Asia, Africa, and elsewhere are grappling with how to continue feeding a growing population. They are also trying to benefit more from their existing resources. Biotechnology holds the key to increase the yield of staple crops by allowing farmers to reap bigger harvests from currently cultivated land, while preserving the land's ability to support continued farming.

Malnutrition in underdeveloped countries is also being combated with biotechnology. The Rockefeller Foundation is sponsoring research on “golden rice”, a crop designed to improve nutrition in the developing world. Rice breeders are using biotechnology to build Vitamin A into the rice. Vitamin A deficiency is a common problem in poor countries. A second phase of the project will increase the iron content in rice to combat anaemia, which is widespread problem among women and children in underdeveloped countries. Golden rice, expected to be for sale in Asia in less than five years, will offer dramatic improvements in nutrition and health for millions of people, with little additional costs to consumers.

Biotechnology is essentially an industrial wealth creating activity encompassing the disciplines of microbiology, biochemistry, molecular biology and chemical engineering. Biotechnology either produces specific products or processes to deal with potential environmental problems, thus resulting in cost saving of projects. Therefore, it must be realized that the ultimate success or failure of a biotechnological process is whether or not it makes money or produces a social benefit.

Some technologies and expertise have been developed which are ready for up scaling leading to commercial exploitation, these are:

- Micropropagation of plants (virus free Banana, Potato, Sugarcane).
- Production of biofertilizers for nutrient supply to crops.
- Production of methane from lignocellulosic biomass.
- Production of alcohol from agricultural residues.
- Production of enzymes.
- Recovery of metals from Low-grade ores through microbial leaching.
- Bio-upgradation of fossil fuels (Coals etc.).
- Detoxification of industrial effluents.

Major tasks of modern biotechnology are:

Plant Improvement Through Tissue Culture: Major impact of tissue culture technology is not only in the area of micro propagation, but also in the area of controlled manipulations of plant germplasm at the cellular level. The ability to unorganize, rearrange, and reorganize the constituents of higher plants has been demonstrated with a few model systems to date, but such basic research is already being conducted on ornamental trees and shrubs with the intent of obtaining new and better landscape plants.

Selection of Plants with Enhanced Stress or Pest Resistance: Perhaps the most heavily researched area of tissue culture today is the concept of selecting disease, insect, or stress resistant plants through tissue culture. Just as selecting and propagating superior individuals have obtained significant gains in the adaptability of many species, so the search for these superior individuals can be tremendously accelerated using in vitro systems (Figure 6.4). Such systems can attempt to exploit

the natural variability known to occur in plants or variability can be induced by chemical or physical agents known to cause mutations.

Figure 6.4: Herbicide tolerant plants.

All those are familiar with bud sprouts, variegated foliage and other types of chimeras have an appreciation for the natural variability in the genetic makeup or expression in plants. Chimeras are the altered cellular expressions, which are visible, but for each of these, which are observed many, more differences probably exist but are masked by the overall organization of the plant as a whole. For example, even in frost-tender species, certain cells or groups of cells may be frost hardy. However, because most of the organism is killed by frost, the tolerant cells eventually die because they are unable to support themselves without the remainder of the organized plant. Plant tissues grown in vitro can be released from the organization of the whole plant through callus formation. If these groups of cells are then subjected to a selection agent such as freezing, then those tolerant ones can survive while all those which are susceptible will be killed. This concept can be applied to many types of stress as well as resistance to fungal and bacterial pathogens and various types of phytotoxic chemical agents. Current research in this area extends across many interests including attempts to select salt tolerant lines of tomato, freezing resistant tobacco plants, herbicide resistant agronomic crops, and various species of plants with enhanced pathogen resistance.

Tissue Culture and Pathogen Free Plants: Another purpose for which plant tissue culture is uniquely suited is in the obtaining, maintaining, and mass propagating of specific pathogen-free plants. Pathogens contribute a great loss towards national economy (Figure 6.5). The concept behind indexing plants free of pests is closely allied to the concept of using tissue culture as a selection system. Plant tissues known to be free of the pathogen under consideration (viral, bacterial, or fungal) are physically selected as the explants for tissue culture.

Figure 6.5: Corn pathogen

Cultures which reveal the presence of the pathogen are destroyed (Figure 6.6), while those which are indexed free of pathogen are maintained as a stock of pathogen-free material. Procedures similar to these have been used successfully to obtain virus-free plants of a number of species and bacteria-free plants of species known to have certain leaf spot diseases.

Figure 6.6: Range of damage due to corn rootworm feeding, from severe (left) to no damage (right)

Somatic Hybridization: The ability to fuse plant cells from species, which may be incompatible as sexual crosses, and the ability of plant cells to take up and incorporate foreign genetic codes extend the realm of plant modifications through

tissue culture to the limits of the imagination. Most such manipulations are carried out using plant "protoplasts". Protoplasts are single cells, which have been stripped of their cell walls by enzymatic treatment. A single leaf treated under these conditions may yield tens of millions of single cells, each theoretically capable of eventually producing a whole plant.

Similar initiatives using genetic manipulation are aimed at making crops more productive by reducing their dependence on pesticides, fertilizers and irrigation, or by increasing their resistance to plant diseases. Increased crop yield (Figure 6.7), greater flexibility in growing environments, less use of chemical pesticides and improved nutritional contents make agricultural biotechnology, quite literally, the future of the world's food supply.

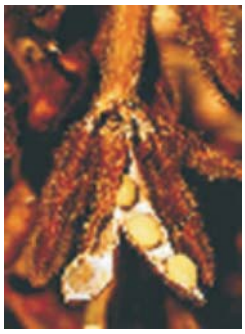


Figure 6.7: Increase in grain numbers induced by genetic modification

Green Plastics: Biotechnology also offers us the prospect of replacing petroleum-derived polymers with biological polymers derived from grain or agricultural biomass. Cotton, genetically modified to contain a bacterial gene, produces a polyester-like substance that is biodegradable and has the texture of cotton, but is warmer. Other biopolymers with the potential to replace synthetic fabrics and fibers are under development in Japan and the United States. Industrial scientists have also genetically modified both plants and microbes to produce polyhydroxybutyrate, a feedstock for producing biodegradable plastics. In place of petroleum-based chemicals to create plastics and polyesters, biotechnology uses sugar from plant material. Almost all the giant chemical companies are building partnerships with biotech companies to develop enzymes that can break down plant sugars.

Nanotechnology: Remember the movie "Fantastic Voyage", in which technology existed to shrink a full-size submarine and its human passengers to microscopic size. Today, industrial biotech companies are embarking on their own fantastic voyage into the submicroscopic worlds of biotechnology and nanotechnology. There, they are exploiting the physiochemical activities of cells to accomplish tasks at nano (10^{-9} meters) scale. Some are taking genomics and proteomics one-step further and exploring how to apply this knowledge gained in the organic world to the inorganic world of carbon and silicon. Such convergence of biotech and nanotech promises to yield many exciting and diverse materials and products. In the area of photonics lies the potential for developing new micro-optical switches and optical micro-processing platforms. In the field of catalysis, the use of inorganic carbon or silicon substrates embedded with biocatalysts has high commercial potential.

Building Nanostructures: One of the more exciting research-stage nano-biotech applications uses knowledge about protein engineering to "build" pre-engineered nanostructures for specific tasks. For instance, we know that certain genes in aquatic microorganisms code for proteins that govern the construction of inorganic exoskeletons. In theory, it should be possible to elucidate these gene functions and re-engineer them to code for nanostructures that could be commercially important, such as specific silicon chips or micro-transistors.

Researchers at the University of Illinois recently discovered a first-of-its-kind carbon-silicon compound in freshwater diatoms. This discovery promises to open the door to understanding the molecular process of biosilicification, or the ways plants and animals build natural structures. This understanding may lead to applications ranging from low-cost synthesis of advanced biomaterials to new treatments for osteoporosis. Protein polymer structures are another area ripe for research and development. Industrial biotech companies have years of experience with genetic platform technologies that can be applied to repeating amino acid sequences. These five to six repeat segments can govern the physical structure of a host of biopolymers.

Foods, Nutritionally Enhanced Through Biotechnology, That may be Used in the Future:

- Cooking oils with healthier fats to lower cholesterol levels
- Tomatoes with more lycopene to help prevent cancer
- Potatoes that absorb less fat during frying to lower cholesterol levels

- Cereals and vegetables with increased protein content
- Foods that can protect people from chronic diseases such as juices and cereals containing extra calcium to reduce osteoporosis
- Peanuts, milk, and wheat with allergenic proteins removed allowing people with allergies to eat them
- Cow's milk that contains extra lysozyme, a natural anti-bacterial compound which could help prevent infection in infants and increase the shelf-life of milk
- Meats with less fat and better flavor

Concerns About Biotechnology

As biotechnology has become widely used, questions and concerns have also been raised. The most vocal opposition has come from European countries. One of the main areas of concern is the safety of genetically engineered food. In assessing the benefits and risks involved in the use of modern biotechnology, there are a series of issues to be addressed so that informed decisions can be made.

The health effects of foods grown from genetically engineered crop depend on the composition of the food itself. Any new product may have either beneficial or occasional harmful effects on human health. For example, a biotech-derived food with a higher content of digestible iron is likely to have a positive effect if consumed by iron-deficient individuals. Alternatively, the transfer of genes from one species to another may also transfer the risk for exposure to allergens. These risks are systematically evaluated by FDA and identified prior to commercialization. Individuals allergic to certain nuts, for example, need to know if genes conveying this trait are transferred to other foods such as soybeans. Labeling would be required if such crops were available to consumers. Among the potential ecological risks identified is increased weediness, due to cross-pollination from genetically modified crops spreads to other plants in nearby fields. This may allow the spread of traits such as herbicide-resistance to non-target plants that could potentially develop into weeds. This ecological risk is assessed when deciding if a plant with a given trait should be released into a particular environment, and if so, under what conditions.

Other potential ecological risks stem from the use of genetically modified corn and cotton with insecticidal genes from *Bacillus thuringiensis* (Bt genes). This may lead to the development of resistance to Bt in insect populations exposed to the biotech-derived crop. There also may be risks to non-target species, such as birds and butterflies, from the plants with Bt genes. The monitoring of these effects of new crops in the environment and implementation of effective risk management approaches is an essential component of further research. It is also important to keep all risks in perspective by comparing the products of biotechnology and conventional agriculture.

The issues surrounding objections to transgenic crops can be broadly grouped into concerns about

Damage to human health

- Allergeni city horizontal transfer and antibiotic resistance
- Eating foreign DNA
- Cauliflower mosaic virus promoter
- Changed nutrient levels

Damage to natural environment

- Monarch butterfly
- Crop-to-weed gene flow
- Antibiotic resistance
- Leakage of GM proteins into soil
- Reductions in pesticide spraying

Disruption of current practices in farming and food production in developed countries crop-to-crop gene flow.

National and International Biotechnology Policy

National governments and international policy making bodies rely on food scientists and others to develop innovations that will create marketable food products and

increase food supplies. Governments also rely on scientific research because they are responsible for setting health and safety standards regarding new developments. International organizations can suggest policy approaches and help develop international treaties that are ratified by national governments. Economic success in the competitive international market demands that food production become more efficient and profitable. National governments and international organizations support food biotechnology as a means to avoid global food shortages. Many policy-making bodies are also trying to balance support of the food biotechnology industry with public calls for their regulation. Such regulations are necessary to protect public health and safety, to promote international trade, conserve natural resources and account for ethical issues.

Biotechnology is at a crossroads in terms of public acceptance. International developments over the next few years will certainly have a major influence on the long-term viability of biotechnology. The future of the world food supply depends upon how well scientists, government, and the food industry is able to communicate with consumers about the benefits and safety of the technology. In summary, modern biotechnology offers opportunities to improve product quality, nutritional content, and economic benefits. The genetic makeup of plants and animals can be modified by either insertion of new useful genes or removal of unwanted ones. Biotechnology is changing the way plants and animals are grown, boosting their value to growers, processors, and consumers.

GLOSSARY

Abiotic Stress: Outside (nonliving) factors, which can cause harmful effects to plants, such as soil conditions, drought, and extreme temperatures.

Acclimatization: Adaptation of an organism to a new environment.

Active Immunity: A type of acquired immunity whereby resistance to a disease is built up by either having the disease or receiving a vaccine to it.

Aerobe: A microorganism that grows in the presence of oxygen.

Aerobic: Needing oxygen for growth.

Aflatoxin: A toxin that can be found on some fungal-contaminated nuts and cereals. Mycotoxins are a type of aflatoxin.

Agarose Gel Electrophoresis: A matrix composed of a highly purified form of agar that is used to separate larger DNA and RNA molecules ranging 20,000 nucleotides.

***Agrobacterium tumefaciens*:** A common soil bacterium causes crown gall disease in dicots and is used as a vector to create transgenic plants. This bacterium can be used by scientists to introduce desirable genes into plants in the process of genetic modification.

Allele: Any of several alternative forms of a gene.

Alleles: Alternate forms of a gene or DNA sequence, which occur on either of two homologous chromosomes in a diploid organism.

Allergy: Adverse overreaction, of the body's self-defense system caused by the production of antibodies against specific substances. Asthma and hay fever and intolerance to milk or egg are familiar examples of allergies.

Allogenic: Of the same species, but with a different genotype.

Alternative mRNA Splicing: The inclusion or exclusion of different exons to form different mRNA transcripts.

Alzheimer's Disease: A disease characterized by, among other things, progressive loss of memory. The development of Alzheimer's disease is thought to be associated, in part, with possessing certain alleles of the gene that encodes apolipoprotein E.

Amino Acid: Any of 20 basic building blocks of proteins composed of a free amino (NH_2) end,

a free carboxyl (COOH) end, and a side group (R).

Ampicillin (beta-lactamase): An antibiotic derived from penicillin that prevents bacterial growth by interfering with cell wall synthesis.

Amplification: The process of increasing the number of copies of a particular gene or chromosomal sequence.

Amplify: To increase the number of copies of a DNA sequence, in vivo by inserting into a cloning vector that replicates within a host cell, or in vitro by polymerase chain reaction (PCR).

Anneal: The pairing of complementary DNA or RNA sequences, via hydrogen bonding, to form a double-stranded polynucleotide. Most often used to describe the binding of a short primer or probe.

Antibiotic Resistance: The ability of a microorganism to produce a protein that disables an antibiotic or prevents transport of the antibiotic into the cell.

Antibiotic: Chemical substance formed as a metabolic byproduct in bacteria or fungi and used to treat bacterial infections. Antibiotics can be produced naturally, using microorganisms, or synthetically.

Antibodies: Proteins produced by the immune system in response to the introduction of foreign molecules called antigens. Antibodies neutralize these molecules to prevent infection or disease.

Anticodon: Triplet of nucleotide bases (codon) in transfer RNA that pairs with (is complementary to) a triplet in messenger RNA. For example, if the codon is UCG, the anticodon is AGC.

Antigen: A substance that, when introduced into the body, induces an immune response by a specific antibody.

Antisense: A piece of DNA producing a mirror image ("antisense") messenger RNA that is opposite in sequence to one directing protein synthesis. Antisense technology is used to selectively turn off production of certain proteins.

Antiserum: Blood serum containing specific antibodies against an antigen. Antisera are used to confer passive immunity to many diseases.

Aquaculture: The cultivation of plants using water as the support medium. It can also mean rearing marine life under controlled conditions in water, such as fish farming.

Assay: Technique for measuring a biological response.

B Lymphocytes (B-cells): A class of lymphocytes, released from the bone marrow, which produce antibodies.

Bacillus subtilis: A bacterium commonly used as a host in recombinant DNA experiments. Important because of its ability to secrete proteins.

Bacillus thuringiensis **(B.t.):** A naturally occurring bacterium present in soil, which has been used successfully by home gardeners and organic farmers to control certain insects for more than 30 years. When ingested by these insects, a protein produced by B.t. interferes with the insect's digestion, and acts as an insecticide.

Bacteriophage: A virus that infects bacteria. In genetic engineering, it is used to introduce genes into bacteria cells.

Bacterium: Any of a large group of microscopic organisms with a very simple cell structure. Some manufacture their own food, some live as parasites on other organisms, and some live on decaying matter.

Base pair: Two nucleotide bases on different strands of the nucleic acid molecule that bond together. The bases can pair in only one-way: adenine with thymine (DNA) or uracil (RNA), and guanine with cytosine.

Base: A key component of DNA and RNA molecules. Four different bases are found in DNA: adenine (A), cytosine (C), guanine (G) and thymine (T). In RNA, uracil (U) substitutes for thymine. Also known as nitrogenous bases. A base, a phosphate molecule and a sugar joined together constitute a nucleotide.

Bioassay: Determination of the effectiveness of a compound by measuring its effect on animals, tissues or organisms in comparison with a standard preparation.

Biocatalyst: In bioprocessing, an enzyme that activates or speeds up a biochemical reaction.

Biochemical: The product of a chemical reaction in a living organism.

Biochip: An electronic device that uses organic molecules to form a semiconductor.

Bioconversion: Chemical restructuring of raw materials by using a biocatalyst.

Biodegradable: Capable of being reduced to water and carbon dioxide by the action of microorganisms.

Bioenrichment: A bioremediation strategy that involves adding nutrients or oxygen, thereby bolstering the activity of microbes as they break down pollutants.

Bioinformatics: The science of informatics as applied to biological research. Informatics is the management and analysis of data using advanced computing techniques. Bioinformatics is particularly important as an adjunct to genomics research, because of the large amount of complex data this research generates.

Biolistic device: A device that shoots microscopic DNA-coated particles into target cells.

Biological Oxygen Demand (BOD): The amount of oxygen used for growth by organisms in water that contains organic matter.

Biomass: The totality of biological matter in a given area. As commonly used in biotechnology, refers to the use of cellulose, a renewable resource, for the production of chemicals that can be used to generate energy or as alternative feedstock for the chemical industry to reduce dependence on nonrenewable fossil fuels or use of microbes as such like inoculants, starter cultures etc.

Biomaterials: Biological molecules, such as proteins and complex sugars, used to make medical devices, including structural elements used in reconstructive surgery.

Bioprocess: A process in which living cells, or components thereof, are used to produce a desired product.

Bioreactor: Vessel used for bioprocessing.

Bioremediation: The use of microorganisms to remedy environmental problems, rendering hazardous wastes non hazardous.

Biosensor: A technology in which enzymes or antibodies are used to detect sugars and proteins in body fluids, contaminants in water and gases in air.

Biosynthesis: Production of a chemical by a living organism.

Biotechnology: The use of biological processes to solve problems or make useful products. The scientific manipulation of living organ- isms, especially at the molecular genetic level, to produce useful products. Gene splicing and use of recombinant DNA (rDNA) are major techniques used.

Biotransformation: The use of enzymes or microbes in biochemical synthesis to produce biochemical compounds of a desired stereochemistry.

Callus: A cluster of undifferentiated plant cells that can, in some species, be induced to form the whole plant.

Carbohydrate: A type of biological molecule composed of simple sugars such as glucose. Common examples include starch and cellulose.

Carcinogen: Cancer-causing agent.

Catalyst: An agent (such as an enzyme or a metallic complex) that facilitates a reaction but is not itself changed during the reaction.

Catalytic RNA (ribozyme): A natural or synthetic RNA molecule that cuts an RNA substrate.

cDNA Library: A library composed of complementary copies of cellular mRNAs.

cDNA: Complementary DNA synthesized from an RNA template using reverse transcriptase.

Cell Culture: Growth of cells under laboratory conditions.

Cell Line: Cells that grow and replicate continuously outside the living organism.

Cell: The smallest structural unit of a living organism that can grow and reproduce independently.

Central Dogma: Francis Crick's seminal concept that in nature genetic information generally flows from DNA to RNA to protein.

Centrifugation: Separating molecules by size or density using centrifugal forces generated by a spinning rotor. G forces of several hundred thousand times gravity are generated in ultracentrifugation.

Centromere: The central portion of the chromosome to which the spindle fibers attach during mitotic and meiotic division.

Chromatid: Each of the two daughter strands of a duplicated chromosome joined at the centromere during mitosis and meiosis.

Chromatography: A technique for separating complex mixtures of chemicals or proteins into their various constituents.

Chromosome: A single DNA molecule, a tightly coiled strand of DNA, condensed into a compact structure in vivo by complexing with accessory histones or histone-like proteins. Chromosomes exist in pairs in higher eukaryotes.

Clinical Studies: Human studies that are designed to measure the efficacy of a new drug or biologic. Clinical studies routinely involve the use of a placebo group that is given an inactive substance that looks like the test product.

Clone: A term that is applied to genes, cells or entire organisms that are derived from-and are genetically identical to-a single common ancestor gene, cell or organism, respectively. Cloning of genes and

cells to create many copies in the laboratory is a common procedure essential for biomedical research. Note that several processes commonly described as cell "cloning" give rise to cells that are almost but not completely genetically identical to the ancestor cell. Cloning of organisms from embryonic cells occurs naturally in nature (e.g., identical twins). Researchers have achieved laboratory cloning using genetic material from adult animals of a number of species including mice, pigs and sheep.

Cloning: A form of asexual reproduction in which the genome from a cell of an individual is used to form another genetically identical individual.

Code: The sequence (order) of DNA bases in a gene, which make up the instructions for a particular characteristic.

Codon: A sequence of three nucleotide bases that specifies an amino acid or represents a signal to stop or start a function.

Coenzyme (cofactor): An organic molecule, such as a vitamin, that binds to an enzyme and is required for its catalytic activity.

Co-Factor: A nonprotein substance required for certain enzymes to function. Co-factors can be co-enzymes or metallic icons.

Colony: A group of identical cells (clones) derived from a single progenitor cell.

Complement: The complement of a nucleic acid sequence replaces each base by its complementary base: adenine (A) by thymidine (T), cytosine (C) by guanine (G), and vice versa. In RNA, adenine is paired not with thymidine but with uracil (U). By convention, DNA and RNA molecules have a consistent orientation (5' to 3'), which is used in writing their sequences. To preserve this orientation, the complement of a sequence is written backwards compared to the original. For example, an RNA sequence ACGGUACU has the DNA complement AGTACCGT.

Complementarity: The relationship of the nucleotide bases on two different strands of DNA or RNA. When the bases are paired properly (adenine with thymine [DNA] or uracil [RNA]; guanine with cytosine), the strands are complementary.

Complementary DNA (cDNA): DNA synthesized from a messenger RNA rather than from a DNA template. This type of DNA is used for cloning or as a DNA probe for locating specific genes in DNA hybridization studies.

Concatemer: A DNA segment composed of repeated sequences linked end to end.

Conjugation: The joining of two bacteria cells when genetic material is transferred from one bacterium to another.

Copy Gene: Genetic material that incorporates the genetic code for a desirable trait that has been copied from the DNA of the donor organism to the host organism.

Crossing Over: Exchange of genes between two paired chromosomes.

Culture Medium: Any nutrient system for the artificial cultivation of bacteria or other cells; usually a complex mixture of organic and inorganic materials.

Culture: As a noun, cultivation of living organisms in prepared medium; as a verb, to grow in prepared medium.

Cyclic AMP (cyclic adenosine monophosphate): A second messenger that regulates many intracellular reactions by transducing signals from extracellular growth factors to cellular metabolic pathways.

Cyto-: Referring to cell or cell plasm.

Cytogenetics: Study of the cell and its heredity-related components, especially chromosomes.

Cytoplasm: Cellular material that is within the cell membrane and surrounds the nucleus.

Dalton: A unit of measurement equal to the mass of a hydrogen atom, 1.67 x 10E-24 gram/L (Avogadro's number).

Death Phase: The final growth phase, during which nutrients have been depleted and cell number decreases.

Denature: To induce structural alterations that disrupts the biological activity of a molecule. Often refers to breaking hydrogen bonds between base pairs in double-stranded nucleic acid molecules to produce in single-stranded polynucleotides or altering the secondary and tertiary structure of a protein, destroying its activity.

Deoxyribonucleic Acid (DNA): The substance from which genes are made. The DNA molecule, which is found in all cells, consists of four bases: adenine, cytosine, guanine, and thymine. The sequence in which these four appear determines heredity.

Diabetes: A disease associated with the absence or reduced levels of insulin, a hormone produced by the pancreas that is essential for the transport of glucose to cells.

Diagnostic: A product used for the diagnosis of disease or medical

condition. Both monoclonal antibodies and DNA probes are useful diagnostic products.

Differentiation: The process of biochemical and structural changes by which cells become specialized in form and function.

Digestion: The breakdown of food by the digestive enzymes: proteins are broken down to amino acids, starch to glucose, fats to glycerol and fatty acids. These breakdown products are then absorbed into the bloodstream.

Diploid Cell: A cell, which contains two copies of each chromosome.

DNA Chip: A small piece of glass or silicon that has small pieces of DNA arrayed on its surface.

DNA Fingerprint: The unique pattern of DNA fragments identified by Southern hybridization (using a probe that binds to a polymorphic region of DNA) or by polymerase chain reaction (using primers flanking the polymorphic region).

DNA Hybridization: The formation of a double-stranded nucleic acid molecule from two separate strands. The term also applies to a molecular technique that uses one nucleic acid strand to locate another.

DNA Library: A collection of cloned DNA fragments that collectively represent the genome of an organism.

DNA Polymerase: An enzyme that replicates DNA. DNA polymerase is the basis of PCR-the polymerase chain reaction.

DNA Probe: A small piece of nucleic acid that has been labeled with a radioactive isotope, dye or enzyme and is used to locate a particular nucleotide sequence or gene on a DNA molecule.

DNA Repair Enzymes: Proteins that recognize and repair certain abnormalities in DNA.

DNA Replication: The duplication of a DNA molecule.

DNA Sequence: The order of nucleotide bases in the DNA molecule.

DNA Sequencing: Procedures for determining the nucleotide sequence of a DNA fragment.

DNA Vaccines: Pieces of foreign DNA that are injected into an organism to trigger an immune response.

Dominant Gene: A gene whose phenotype is when it is present in a single copy.

Dormancy: A period in which a plant does not grow, awaiting necessary environmental conditions such as temperature, moisture, and

nutrient availability.

Double Helix: A term often used to describe the configuration of the DNA molecule. The helix consists of two spiraling strands of nucleotides (a sugar, phosphate and base) joined crosswise by specific paring of the bases.

Downstream: The region extending in a 3' direction from a gene.

Drug Delivery: The process by which a formulated drug is administered to the patient. Traditional routes have been oral or intravenous perfusion. New methods deliver through the skin with a transdermal patch or across the nasal membrane with an aerosol spray.

Ecology: The study of the interactions of organisms with their environment and with each other.

Ecosystem: The organisms in a plant population and the biotic and abiotic factors which impact on them.

Electrophoresis: A technique for separating different types of molecules based on their patterns of movement in an electrical field.

Embryonic Stem Cells: Cells that can give rise to any type of differentiated cell. They can be derived from two sources: the inner cell mass from a blastocyt or the primordial germ cells (eggs and sperm) of an older embryo.

Environmental Protection Agency (EPA): The U.S. regulatory agency for biotechnology of microbes. The major laws under which the agency has regulatory powers are the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA); and the Toxic Substances Control Act (TSCA).

Enzyme: A protein catalyst that facilitates specific chemical or metabolic reactions necessary for cell growth and reproduction.

Escherichia coli (E. coli): A bacterium that inhabits the intestinal tract of most vertebrates. Much of the work using recombinant DNA techniques has been carried out with this organism because it has been genetically well characterized.

Eukaryote: A cell or organism containing a true nucleus, with a well-defined membrane surrounding the nucleus. All organisms except bacteria, viruses and cyanobacteria) are eukaryotic. An organism whose cells possess a nucleus and other membrane-bound vesicles, including all members of the protist, fungi, plant and animal kingdoms; and excluding viruses, bacteria, and blue-green algae.

Exon: In eukaryotic cells, that part of the gene that is transcribed into

messenger RNA and encodes a protein.

Expression: In genetics, manifestation of a characteristic that is specified by a gene. With hereditary disease, for example, a person can carry the gene for the disease but not actually have the disease. In this case, the gene is present but not expressed. In industrial biotechnology, the term is often used to mean the production of a protein by a gene that has been inserted into a new host organism.

FDA: Food and Drug Administration.

Feedstock: The raw material used for chemical or biological processes.

Fermentation: The process of growing microorganisms for the production of various chemical or pharmaceutical compounds. Microbes are normally incubated under specific conditions in the presence of nutrients in large tanks called fermentors.

Fungus: A microorganism that lacks chlorophyll.

Fusion: Joining of the membrane of two cells, thus creating a daughter cell that contains some of the same properties from each parent cells. Used in making hybridomas.

Gamete: A haploid sex cell, egg or sperm that contains a single copy of each chromosome.

Gene Amplification: The presence of multiple genes. Amplification is one mechanism through which proto-oncogenes are activated in malignant cells.

Gene Expression: The process in which a cell produces the protein, and thus the characteristic, which is specified by a gene's nucleotide sequence.

Gene Insertion: The addition of one or more copies of a normal gene into a defective chromosome.

Gene Linkage: The hereditary association of genes located on the same chromosome.

Gene Mapping: Determination of the relative locations of genes on a chromosome.

Gene Modification: The chemical repair of a gene's defective DNA sequence.

Gene Splicing: Combining genes from different organisms into one organism.

Gene Therapy: The replacement of a defective gene in an organism suffering from a genetic disease. Recombinant DNA techniques are used to isolate the functioning gene and insert it into cells. More than 300 single-gene genetic disorders have been identified in humans. A significant percentage of these may be amenable to gene therapy.

Gene Translocation: The movement of a gene fragment from one chromosomal location to another, which often alters or abolishes expression.

Gene: A locus on a chromosome that encodes a specific protein or several related proteins. It is considered the functional unit of heredity.

Genetic Code: The code by which genetic information in DNA is translated into biological function. A set of three nucleotides (codons), the building blocks of DNA, signifies one amino acid, the building blocks of proteins.

Genetic Engineering: The manipulation of an organism's genetic endowment by introducing or eliminating specific genes through modern molecular biology techniques. A broad definition of genetic engineering also includes selective breeding and other means of artificial selection.

Genetic Linkage Map: A linear map of the relative positions of genes along a chromosome. Distances are established by linkage analysis, which determines the frequency at which two gene loci become separated during chromosomal recombination.

Genetic Mapping: The process of locating the positions of specific genes on a chromosome.

Genetic Marker: A gene or group of genes used to "mark" or track the action of microbes.

Genetic Modification: A number of techniques, such as selective breeding, mutagenesis, transposon insertions and recombinant DNA technology, that are used to alter the genetic material of cells in order to make them capable of producing new substances, performing new functions or blocking the production of substances.

Genetic Screening: The use of a specific biological test to screen for inherited diseases or medical conditions. Testing can be conducted prenatally to check for metabolic defects and congenital disorders in the developing fetus as well as postnatally to screen for carriers of heritable diseases.

Genetic Testing: The analysis of an individual's genetic material. Genetic testing can be used to gather information on an individual's genetic predisposition to a particular health condition, or to confirm a diagnosis of genetic disease.

Genome: The total hereditary material of a cell, comprising the entire chromosomal set found in each nucleus of a given species. The genetic complement contained in the chromosomes of a given organism, usually the haploid chromosome state.

Genomics: The study of genes and their function. Recent advances in genomics are bringing about a revolution in our understanding of the molecular mechanisms of disease, including the complex interplay of genetic and environmental factors. Genomics is also stimulating the discovery of breakthrough health-care products by revealing thousands of new biological targets for the development of drugs and by giving scientists innovative ways to design new drugs, vaccines and DNA diagnostics. Genomic-based therapeutics may include "traditional" small chemical drugs, protein drugs and gene therapy.

Genotype: Genetic makeup of an individual or group.

Germ cell (germ line) Gene Therapy: The repair or replacement of a defective gene within the gamete-forming tissues, which produces a heritable change in an organism's genetic constitution.

Germplasm: The total genetic variability, represented by germ cells or seeds, available to a particular population of organisms.

Glycoprotein: A protein conjugated with a carbohydrate group.

Granulocyte: One of three types of white blood cells. Granulocytes digest bacteria and other parasites.

Green Revolution: Advances in genetics, petrochemicals, and machinery that culminated in a dramatic increase in crop productivity during the third quarter of the 20th century.

Growth Factor: A serum protein that stimulates cell division when it binds to its cell-surface receptor.

Growth Factors: Naturally occurring proteins that stimulate the growth and reproduction of specific cell types. Growth factors are essential to regenerative medicine and tissue engineering.

Growth Hormone: A protein produced by the pituitary gland that is involved in cell growth. Human growth hormone is used clinically to treat dwarfism. Various animal growth hormones can be used to improve milk production as well as produce a leaner variety of meat. Also called somatotropin.

Growth Phase (Curve): The characteristic periods in the growth of a bacterial culture, as indicated by the shape of a graph of viable cell number versus time.

Haploid Cell: A cell containing only one set, or half the usual (diploid) number, of chromosomes.

Herbicide: Any substance that is toxic to plants; usually used to kill specific unwanted plants.

Heredity: Transfer of genetic information from parent cells to progeny.

Homologous: Corresponding or alike in structure, position or origin.

Hormone: A chemical or protein that acts as a messenger or stimulatory signal, relaying instructions to stop or start certain physiological activities. Hormones are synthesized in one type of cell and then released to direct the function of other cell types.

Host: A cell or organism used for growth of a virus, plasmid or other form of foreign DNA, or for the production of cloned substances.

Host-Vector System: Combination of DNA-receiving cells (host) and DNA-transporting substance (vector) used for introducing foreign DNA into a cell.

Human Genome Project: A project coordinated by the National Institutes of Health (NIH) and the Department of Energy (DOE) to determine the entire nucleotide sequence of the human chromosomes.

Human Genome Project: An international research effort aimed at discovering the full sequence of bases in the human genome. Led in the United States by the National Institutes of Health and the Department of Energy.

Human Immunodeficiency Virus (HIV): The virus that causes acquired immune deficiency syndrome (AIDS).

Hybridization: Production of offspring, or hybrids, from genetically dissimilar parents. The process can be used to produce hybrid plants (by crossbreeding two different varieties) or hybridomas (hybrid cells formed by fusing two unlike cells, used in producing monoclonal antibodies).

Immune Response: The response of the immune system to challenge by a foreign antigen.

Immune System: The combination of cells, biological substances (such as antibodies) and cellular activities that work together to provide resistance to disease.

Immunity: Nonsusceptibility to a disease or to the toxic effects of antigenic material.

In Situ: In its original or natural place or position.

In Vitro: Literally, "in glass." Performed in a test tube or other laboratory apparatus.

In vivo: In the living organism.

Inducer: A molecule or substance that increases the rate of enzyme synthesis, usually by blocking the action of the corresponding repressor.

Insecticide: A chemical used to kill or control certain populations of insect pests. In agriculture, insecticides are used to control insect pests that feed on crops or carry plant disease.

Insulin: A peptide hormone secreted by the islets of Langerhans of the pancreas that regulates the level of sugar in the blood.

Integrated Pest Management (IPM): An approach to pest control that includes biological, mechanical and chemical means. The goal of IPM is to produce a healthy crop in an economically efficient and environmentally sound manner.

Interferon: A class of lymphokine proteins important in the immune response. There are three major types of interferon: alpha (leukocyte), beta (fibroblast) and gamma (immune). Interferons inhibit viral infections and may have anticancer properties.

Intron: In eukaryotic cells, a sequence of DNA that is contained in the gene but does not encode for protein. The presence of introns "splits" the coding region of the gene into segments called exons.

Isoenzyme: One of the several forms that a given enzyme can take. The forms may differ in certain physical properties, but function similarly as biocatalysts.

Lactobacillus: A bacterium used in the production of a wide variety of dairy foods, such as cheese and yogurt

Lag Phase: The initial growth phase, during which cell number remains relatively constant prior to rapid growth.

Leukocyte: A colorless cell in the blood, lymph and tissues that is an important component of the body's immune system. Also called white blood cells.

Library: A set of cloned DNA fragments that taken collectively contain the entire genome of an organism. Also called a DNA library.

Ligase (DNA Ligase): An enzyme that catalyzes a condensation reaction that links two DNA molecules via the formation of a phosphodiester bond between the 3' hydroxyl and 5' phosphate of adjacent nucleotides.

Linker: A fragment of DNA with a restriction site that can be used to join DNA strands.

Medium: A substance containing nutrients needed for cell growth.

Meiosis: Process of cell reproduction whereby the daughter cells has half the chromosome number of the parent cells. Sex cells are formed by meiosis. The reduction division process by which

haploid gametes and spores are formed, consisting of a single duplication of the genetic material followed by two mitotic divisions.

Messenger RNA (mRNA): Nucleic acid that carries instructions to a ribosome for the synthesis of a particular protein.

Metabolism: All biochemical activities carried out by an organism to maintain life.

Microbial herbicides and pesticides: Microorganisms that is toxic to specific plants or insects. Because of their narrow host range and limited toxicity, these microorganisms may be preferable to their chemical counterparts for certain pest control applications.

Microbiology: Study of living organisms that can be seen only under a microscope.

Microinjection: The injection of DNA using a very fine needle into a cell.

Microorganism: Any organism that can be seen only with the aid of a microscope. Also called microbe.

Mitosis: Process of cell reproduction whereby the daughter cells are identical in chromosome number to the parent cells.

Molecular Biology: The study of the biochemical and molecular interactions within living cells.

Molecular Cloning: The biological amplification of a specific DNA sequence through mitotic division of a host cell into which it has been transformed or transfected.

Monoclonal Antibodies: Antibodies that are derived from only one cell and recognize only one portion of a molecule. Because monoclonal antibodies are highly specific, they can be used to diagnose disease or to protect against disease-causing organisms.

Moulds: Fungi that grow as a mesh of fine branched filaments (mycelium): include mushrooms and smaller fungi. Some can cause food spoilage, and produce toxins (or mycotoxins, such as aflatoxin).

Mutagen: A substance that induces mutations.

Mutant: A cell that manifests new characteristics due to a change in its DNA.

Mutation: A change in the genetic material of a cell.

Nanotechnology: A precise molecule by molecule control of products and byproducts in the development of functional structures.

Nitrogen Fixation: A biological process (usually associated with plants) whereby certain bacteria convert nitrogen in the air to

ammonia, thus forming a nutrient essential for growth.

Nodule: The enlargement or swelling on roots of nitrogen- fixing plants. The nodules contain symbiotic nitrogen- fixing bacteria.

Noncoding DNA: DNA that does not encode any product (RNA or protein). The majority of the DNA in plants and animals is noncoding.

Northern Hybridization: (Northern blotting). A procedure in which RNA fragments is transferred from an agarose gel to a nitrocellulose filter, where the RNA is then hybridized to a radioactive probe.

Nuclease: An enzyme that, by cleaving chemical bonds, breaks down nucleic acids into their constituent nucleotides.

Nucleic Acids: Large molecules, generally found in the cell's nucleus and/or cytoplasm, which are made up of nucleotides. The two most common nucleic acids are DNA and RNA.

Nucleotide: A building block of DNA and RNA, consisting of a nitrogenous base, a five-carbon sugar, and a phosphate group. Groups of three nucleotides form codons (each codon codes for particular amino acid) which when strung together form genes. In turn, genes are linked together to form chromosomes.

Nucleus: The structure within eukaryotic cells that contains chromosomal DNA.

Oligonucleotide: A polymer consisting of a small number (about two to 10) of nucleotides.

Operon: Sequence of genes responsible for synthesizing the enzymes needed for biosynthesis of a molecule. An operon is controlled by an operator gene and a repressor gene.

Organic Compound: A compound containing carbon.

Palindromic Sequence: A DNA locus whose 5'-to-3' sequence is identical on each DNA strand. The sequence is the same when one strand is read left to right and the other strand is read right to left. Recognition sites of many restriction enzymes are palindromic.

Pathogen: Disease-causing organism.

Peptide: Two or more amino acids joined by a linkage called a peptide bond.

Photosynthesis: Conversion by plants of light energy into chemical energy, which is then used to support the plants' biological processes.

Plaque: A clear spot on a lawn of

bacteria or cultured cells where cells have been Iysed by viral infection.

Plasma: The fluid (noncellular) fraction of blood.

Plasmid (p): A circular DNA molecule, capable of autonomous replication, which typically carries one or more genes encoding antibiotic resistance proteins. Plasmids can transfer genes between bacteria and are important tools of transformation for genetic engineers. **Polyacrylamide Gel Electrophoresis:** Electrophoresis through a matrix composed of a synthetic polymer, used to separate proteins, small DNA, or RNA molecules of up to 1000 nucleotides. Used in DNA sequencing.

Polymerase (DNA): Synthesizes a double-stranded DNA molecule using a primer and DNA as a template.

Polymerase Chain Reaction (PCR): A technique to amplify a target DNA sequence of nucleotides by several hundred thousandfold.

Primer: A short DNA or RNA fragment annealed to single-stranded DNA, from which DNA polymerase extends a new DNA strand to produce a duplex molecule.

Processing (Food): Any and all processes to which food is subjected after harvesting to improve its appearance, texture, palatability, nutritional value, keeping properties, ease of preparation, and for eliminating microorganisms, toxins and other undesirable constituents.

Prokaryote: An organism (e.g., bacterium, virus, cyrobacterium) whose DNA is not enclosed within a nuclear membrane.

Promoter: A DNA sequence that is located in front of a gene and controls gene expression. Promoters are required for binding of RNA polymerase to initiate transcription.

Prophage: Phage nucleic acid that is incorporated into the host's chromosome but does not cause cell lysis.

Protease: An enzyme that cleaves peptide bonds that link amino acids together in protein molecules.

Protein: A molecule composed of amino acids. There are many types of proteins, all carrying out a number of different functions essential for cell growth.

Proteomics: Each cell produces thousands of proteins, each with a specific function. This collection of proteins in a cell is known as the proteome, and, unlike the genome, which is constant irrespective of cell type, the proteome varies from one cell type to the next. The science of

proteomics attempts to identify the protein profile of each cell type, assess protein differences between healthy and diseased cells, and uncover not only a protein's specific function but also how it interacts with other proteins.

Protoplast: The cellular material that remains after the cell wall has been removed from plant and fungal cells.

Pure Culture: *In vitro* growth of only one type of microorganism.

Recombinant DNA (rDNA): The DNA formed by combining segments of DNA from two different sources.

Regeneration: Laboratory technique for forming a new plant from a clump of plant cells.

Replication: Reproduction or duplication, as of an exact copy of a strand of DNA.

Replicon: A chromosomal region containing the DNA sequences necessary to initiate DNA replication processes.

Repressor: A protein that binds to an operator adjacent to a structural gene, inhibiting transcription of that gene.

Restriction Endonuclease (Enzyme): A class of endonucleases that cleaves DNA after recognizing a specific sequence, such as BamH1 (GGATCC), EcoRI (GAATTC), and HindIII (AAGCTT). Type I. Cuts nonspecifically a distance greater than 1000 bp from its recognition sequence and contains both restriction and methylation activities. Type II. Cuts at or near a short, and often symmetrical, recognition sequence. A separate enzyme methylates the same recognition sequence. Type III. Cuts 24-26 bp downstream from a short, asymmetrical recognition sequence. Requires ATP and contains both restriction and methylation activities.

Restriction Enzyme: An enzyme that cuts DNA at a particular base sequence to provide gaps for the insertion of new genes.

Retrovirus: A virus that contains the enzyme reverse transcriptase. This enzyme converts the viral RNA into DNA, which can combine with the DNA of the host cell and produce more viral particles.

Rhizobium: A class of microorganisms that converts atmospheric nitrogen into a form that plants can utilize for growth. Species of this microorganism grow symbiotically on the roots of certain legumes, such as peas, beans and alfalfa.

Ribonucleic Acid (RNA): A molecule similar to DNA that

delivers DNA's genetic message to the cytoplasm of a cell where proteins are made.

Ribosome: A cellular component, containing protein and RNA that is involved in protein synthesis.

Scale-Up: Transition from small-scale production to production of large industrial quantities.

Selectable Marker: A gene whose expression allows one to identify cells that have been transformed or transfected with a vector containing the marker gene.

Selective Medium: Nutrient material constituted such that it will support the growth of specific organisms while inhibiting the growth of others.

Semiconservative Replication: During DNA duplication, each strand of a parent DNA molecule is a template for the synthesis of its new complementary strand. Thus, one half of a preexisting DNA molecule is conserved during each round of replication.

Signal Transduction: The biochemical events that conduct the signal of a hormone or growth factor from the cell exterior, through the cell membrane, and into the cytoplasm. This involves a number of molecules, including receptors, proteins, and messengers.

Single-Cell Protein: Cells or protein extracts from microorganisms, grown in large quantities for use as protein supplements.

Site-Directed Mutagenesis: The process of introducing specific base-pair mutations into a gene.

Somatic Cell Gene Therapy: Somatic cell gene therapy involves the insertion of genes into cells for therapeutic purposes; for example, to induce the treated cells to produce a protein that the body is missing. It does not affect genetic makeup of a patient's offspring and generally does not change all, or even most, cells in the recipient. Somatic cell gene therapy is only one way of applying the science of genomics to improve health care.

Somatic Cells: Cells other than sex or germ cells.

Southern Hybridization (Southern Blotting): A procedure in which DNA restriction fragments are transferred from an agarose gel to a nitrocellulose filter, where the denatured DNA is then hybridized to a radioactive probe (blotting).

Species: A classification of related organisms that can freely interbreed to produce fertile offspring, and usually resemble each other most closely.

Splicing: The removal of introns and joining of exons to form a continuous coding sequence in RNA.

Stationary Phase: The plateau of the growth curve after log growth, during which cell number remains constant. New cells are produced at the same rate as older cells die.

Stop Codon: One of three codons in messenger RNA that signal the end of the amino acid chain in protein synthesis.

Structural Gene: A gene that codes for a protein, such as an enzyme.

Substrate: Material acted on by an enzyme.

T Lymphocytes (T-cells): White blood cells that are produced in the bone marrow but mature in the thymus. They are important in the body's defense against certain bacteria and fungi, help B lymphocytes make antibodies and help in the recognition and rejection of foreign tissues. T lymphocytes may also be important in the body's defense against cancers.

Taq Polymerase: A heat-stable DNA polymerase isolated from the bacterium *Thermus aquaticus*, used in PCR.

TATA Box: An adenine- and thymine-rich promoter sequence located 25-30 bp upstream of a gene, which is the binding site of RNA polymerase.

Technology Transfer: The process of transferring discoveries made by basic research institutions, such as universities and government laboratories, to the commercial sector for development into useful products and services.

Template: A molecule that serves as the pattern for synthesizing another molecule.

Termination Codon: Any of three mRNA sequences (UGA, UAG, UAA) that do not code for an amino acid and thus signal the end of protein synthesis. Also known as stop codon.

Terminator: Sequence of DNA bases that tells the RNA polymerase to stop synthsizing RNA.

Tertiary Structure: The total three-dimensional shape of a protein that is essential to protein function.

Tetracycline: An antibiotic that interferes with protein synthesis in prokaryotes.

Tissue Culture: In vitro growth in nutrient medium of cells isolated from tissue.

Toxin: A poisonous substance produced by certain microorganisms or plants.

Transcription: The process of changing genetic information coded

in a piece of DNA into messenger RNA.

Transduction: Transfer of genetic material from one cell to another by means of a virus or phage vector.

Transfection: Infection of a cell with nucleic acid from a virus, resulting in replication of the complete virus.

Transfer RNA (tRNA): RNA molecules that carry amino acids to sites on ribosomes where proteins are synthesized.

Transformation: In prokaryotes, the natural or induced uptake and expression of a foreign DNA sequence--typically a recombinant plasmid in experimental systems. In higher eukaryotes, the conversion of cultured cells to a malignant phenotype--typically through infection by a tumor virus or transfection with an oncogene.

Transgenic Organism: An organism formed by the insertion of foreign genetic material into the germ line cells of organisms. Recombinant DNA techniques are commonly used to produce transgenic organisms.

Transgenic Plant: Genetically engineered plant or offspring of genetically engineered plants. The transgenic plant usually contains material from at least one unrelated organisms, such as from a virus, animal, or other plant.

Translation: Process by which the information on a messenger RNA molecule is used to direct the synthesis of a protein.

Translocation: The movement or reciprocal exchange of large-chromosomal segments, typically between two different chromosomes.

Transposon (Transposable, or Movable Genetic Element): A relatively small DNA segment that has the ability to move from one chromosomal position to another.

U.S. Department of Agriculture: The U.S. agency responsible for regulation of biotechnology products in plants and animals. The major laws under which the agency has regulatory powers include the Federal Plant Pest Act (PPA), the Federal Seed Act, and the Plant Variety Act (PVA). In addition, the Science and Education (S&E) division has nonregulatory oversight of research activities that the agency funds.

Upstream: The region extending in a 5' direction from a gene.

Vaccine: A preparation that contains an antigen, consisting of whole disease-causing organisms (killed or weakened) or parts of such

organisms that is used to confer immunity against the disease that the organisms cause. Vaccine preparations can be natural, synthetic or derived by recombinant DNA technology.

Variation: Differences in the frequency of genes and traits among individual organisms within a population.

Vector: An autonomously replicating DNA molecule into which foreign DNA fragments are inserted and then propagated in a host cell. Also living carriers of genetic material (such as pollen) from plant to plant, such as insects.

Virion: An elementary viral particle consisting of genetic material and a protein covering.

Virology: Study of viruses.

Virulence: Ability to infect or cause disease.

Virus: A submicroscopic organism that contains genetic information but cannot reproduce itself. To replicate, it must invade another cell and use parts of that cell's reproductive machinery.

VNTR: Variable number of tandem repeats on human DNA.

Weed: A plant that is seen as undesirable because it is growing either in the wrong place or at the wrong time. Weeds in crops reduce the value of the crop for the farmer: if there are too many weeds, he may not be able to sell the harvest.

White Blood Cells: Leukocytes.

Wild Type: An organism as found in nature; the organism before it is genetically engineered.

Yeast: A general term for single-celled fungi that reproduce by budding. Some yeast can ferment carbohydrates (starches and sugars) and thus are important in brewing and baking.

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