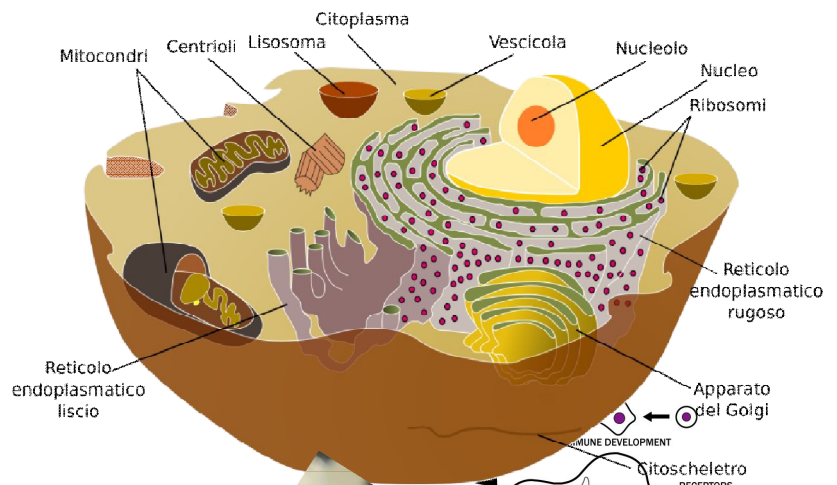


APPLIED Molecular Cell Biology

An Academic and Research Guidelines for
Genetic Engineering, Cell Biology and
Molecular Studies

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Applied

Molecular Cell Biology

1st Edition (2014)

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NOTICE

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PREFACE

The molecular and cellular level investigation provides us accurate guidelines to reach an appropriate decision regarding the research/ diagnosis. The scientists may then be more confident to design an accurate therapy plan. That also potentially helps to offer quality clinical and pharmaceutical care. Moreover; the microbial infectious diseases demand an integrated and valid collection of simple and conceptual informations, which support the researchers to elaborate the molecular cell pathology. The molecular biology explores the morphology, function, genome organization, regulation of genetic expression, morphogenesis, and somatic cell genetics. Thus, we have carefully tried to present the latest and authenticated informations which help to improve the scientific skills and minimize the complications of polypharmacy/ multi disease therapies.

Additionally, it is also vindictive reality that, currently we are offering substandard clinical facilities and poor pharmaceutical care, especially in developing part of the world. That potentially needs attention to save the innocent live (including Pakistan). There are so many avoidable causalities; that can be saved by offering correct medication on behalf of accurate differential microbiological examination. But; unluckily, a considerable proportion of seriously ill patients are passed away because of the current health care system. Therefore, the Biochemist, Chemical Pathologists and Molecular Biologists have to perform their role to assure appropriate therapeutical monitoring.

The "Applied Molecular Cell Biology" is therefore compiled to present the most current scientific learning's. The material contain herein facilitates an in-depth level of understanding of essential molecualre cell biological research information.

The valuable feedback and constructive suggestions will warmly be welcomed

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April 25, 2015

MOLECULAR CELL BIOLOGY, MICROBIOLOGY AND IMMUNOLOGY

Chapter 1.

Introduction and History

In some society, it was not until the late eighteenth century that a rational approach to the origin of disease developed. Prior to the discovery that disease was the result of pathogenic organisms, it was commonly accepted that disease was a punishment from God (or the Gods), or even a witches curse. Eastern cultures perceived disease as an imbalance in the energy channels within the body. Later, the great plagues of Europe were assumed the result of virulent or noxious vapors. Nevertheless, there were intimations as early as 430 B.C. that if one survived a disease, the person thereafter became “immune” to any subsequent exposures. However, this was never recognized as evidence of some type of internal defense system until the later part of the seventeenth century.

Although most historical accounts credit Edward Jenner for the development of the first immunization process, a previous similar procedure had become established in China by 1700. The technique was called variolation. This was derived from the name of the infective agent—the variola virus. The basic principal of variolation was to deliberately cause a mild infection with unmodified pathogen. The risk of death from variolation was around two to three percent. Although still a risk, variolation was a considerable improvement on the death rate for uncontrolled infection. Immunity to smallpox was conferred by inserting the dried exudate of smallpox pustules into the nose. This technique for the transfer of smallpox, as a form of limited infection, traveled to the west from China along the traditional trade routes to Constantinople where it spread throughout Europe. Hearing of this practice, the Royal family of England had

their children inoculated against the disease in 1721, but the practice aroused severe opposition as physicians felt it was far too risky. In 1798, Edward Jenner, noticed that milkmaids were protected from smallpox if they had been first infected with cowpox. It was not his intention to make medical history, as his interests were mostly scholarly and involved the transfer of infections from one species to another, especially from animals to humans. However, Jenner's work led him to the conclusion, that inoculation with cowpox (a bovine analogue of smallpox) could confer immunity to smallpox.

Thus, the concept of vaccination was initiated. (Incidentally, the Latin word for cow is vacca). Jenner's ideas first made him a medical as well as a social pariah, as they were in opposition to both the church and popular beliefs. Because his method was much safer than variolation, however, the use of vaccinations gradually became widely accepted and most European countries had some form of compulsory program within fifty years of Jenner's discovery. The idea that a pathogenic organism caused disease was not fully realized until certain technological advances had occurred. Initially, **Antoni van Leeuwenhoek's** development of the **microscope** and the subsequent realization that entities existed that were not visible to the human eye, allowed the concept of germs to be appreciated. That these organisms were the causative agent of disease was not recognized until **Louis Pasteur** developed his **germ theory of disease**. His original interests were in **fermentation** in wine and beer, and he was the first to isolate the organisms that caused the fermentation process. Pasteur's work eventually led him to the development of **pasteurization**

(heating) as a means of halting fermentation. While working with silk worms and **anthrax**, he was able to demonstrate that the same method for transferring the fermentation process also worked in transmitting disease from infected animals to unaffected animals.

Finally, in 1878, Pasteur accidentally used an attenuated (weakened) chicken cholera **culture** and realized, when he repeated the experiment using a fresh culture, that the weakened form protected the chickens from the virulent form of the disease. Pasteur went on to develop an attenuated **vaccine** against **rabies** and swine erysipelas. Pasteur was not the only proponent of the germ theory of disease. His chief competitor was **Robert Koch**. Koch was the first to isolate the anthrax microbe and, unaware of Pasteur's work, he was able to show that it caused the disease. Then in 1882, Koch was able to demonstrate that the germ theory of disease applied to human ailments as well as animals, when he isolated the microbe that caused **tuberculosis**. His "Koch's postulates" are still used to identify infective organisms. Much of the basis for modern medicine, as well as the field of **immunology**, can be traced back to these two scientists, but the two major questions still to be answered were how did infection cause the degradation of tissue, and how did vaccines work? The first question was addressed in 1881 by **Emile Roux** and Alexander Yersin when they isolated a soluble toxin from **diphtheria** cultures. Later, **Emil von Behring** and **Shibasaburo Kitasato** were able to demonstrate passive immunity when they took serum from animals infected with diphtheria and injected into healthy animals. These same animals were found to be resistant to the disease. Eventually these serum factors were recognized in 1930 as antibodies.

However, thirty years before antibodies were finally isolated and identified, **Paul Ehrlich** and others, recognized that a specific antigen elicited the production of a specific **antibody**. Ehrlich hypothesized that these antibodies were specialized molecular structures with specific receptor sites that fit each pathogen like a lock

and key. Thus, the first realization that the body had a specific defense system was introduced. In addition, sometime later, he realized that this powerful effector mechanism, used in host defense would, if turned against the host, cause severe tissue damage. Ehrlich termed this *horror autotoxicus*. Although extremely valuable, his work still left a large gap in understanding how the **immune system** fights a pathogenic challenge. The idea that specific cells could be directly involved with defending the body was first suggested in 1884 by **Élie Metchnikoff**. His field was zoology and he studied **phagocytosis** in single cell organisms. Metchnikoff postulated that vertebrates could operate in a similar manner to remove pathogens. However, it was not until the 1940s that his theories were accepted and the cell mediated, as opposed to the humoral, immune response was recognized. The clarification of the immune response and the science of immunology did not progress in a systematic or chronological order. Nonetheless, once scientists had a basic understanding of the cellular and humoral branches of the immune system, what remained was the identification of the various components of this intricate system, and the mechanisms of their interactions. This could not have been accomplished without the concomitant development of **molecular biology** and genetics. Milestones in the history of immunology include:

- 1798 Edward Jenner initiates smallpox vaccination.
- 1877 Paul Erlich recognizes mast cells.
- 1879 Louis Pasteur develops an attenuated chicken cholera vaccine.
- 1883 Elie Metchnikoff develops cellular theory of vaccination.
- 1885 Louis Pasteur develops rabies vaccine.
- 1891 Robert Koch explored delayed type hypersensitivity.
- 1900 Paul Erlich theorizes specific antibody formation.
- 1906 Clemens von Pirquet coined the word allergy.

- 1938 John Marrack formulates antigen-antibody binding hypothesis.
- 1942 Jules Freund and Katherine McDermott research adjuvants.
- 1949 Macfarlane Burnet & Frank Fenner formulate immunological tolerance hypothesis.
- 1959 Niels Jerne, David Talmage, Macfarlane Burnet develop clonal **selection** theory.
- 1957 Alick Isaacs & Jean Lindemann discover interferon (cytokine).
- 1962 Rodney Porter and team discovery the structure of antibodies.
- 1962 Jaques Miller and team discover thymus involvement in cellular immunity.
- 1962 Noel Warner and team distinguish between cellular and humoral immune responses.
- 1968 Anthony Davis and team discover T cell and B cell cooperation in immune response.
- 1974 Rolf Zinkernagel and Peter Doherty explore **major histocompatibility complex** restriction.
- 1985 Susumu Tonegawa, Leroy Hood, and team identify immunoglobulin genes.
- 1987 Leroy Hood and team identify genes for the T cell receptor.
- 1985 Scientists begin the rapid identification of genes for immune cells that continues to the present.

See also Antibody and antigen; B cells or B lymphocytes; Germ theory of disease; History of the development of antibiotics; History of public health; Immunity, active, passive and delayed; Immunity, cell mediated; Immunity, humoral regulation; Infection and resistance; T cells or T-lymphocytes.

HISTORY OF MICROBIOLOGY

Microbiology was born in 1674 when **Antoni van Leeuwenhoek** (1632–1723), a Dutch drapery merchant, peered at a drop of lake water through a carefully ground glass lens. Through this he

beheld the first glimpse of the microbial world. Perhaps more than any other science, the development of microbiology depended on the invention and improvement of a tool, the **microscope**. Since **bacteria** cannot be seen individually with the unaided eye, their existence as individuals can only be known through microscopic observations. Indeed, it is interesting to speculate on how microbiology might have developed if the limits of resolution of the microscope were poorer. The practical and scientific aspects of microbiology have been closely woven from the very beginning. Perhaps it is for this reason that microbiology as a field of study did not really develop until the twentieth century. Nineteenth century “microbiologists” were chemists and physicians and a few were botanists. At that stage, the science of microbes was developing to solve very practical problems in two clear scientific fields, the science of **fermentation** and in medicine. Although medicine and fermentation presented the practical problems that stimulated the development of microbiology, the first studies that put the subject on a scientific basis arose from a problem of pure science. This was the controversy over spontaneous generation. Although the crude ideas of spontaneous generation (e.g., maggots from meat) were dispelled by Francesco Redi (1626?–1698?) in the seventeenth century, more subtle ideas such as that **protozoa** and bacteria can arise from vegetable and animal infusions, were still accepted in the nineteenth century. The controversy also involved fermentations, since it was considered that the **yeast** fermentation was of spontaneous origin. Many workers became involved in the study of fermentation and spontaneous generation, but **Louis Pasteur** (1822–1895) stands out as a giant. He came into biology from the field of chemistry and was apparently able to remove all the philosophical hurdles that blocked the thinking of others. Within a period of four years after he began his studies, he had clarified the problems of spontaneous generation so well that the controversy died a natural death. Pasteur was also able to go easily

from fermentation into the field of medical microbiology, which occupied the later part of his life. His contributions in that field were numerous, and his work in fields such as microbial attenuation and **vaccination** has been the basis of many modern medical practices. It should be emphasized that the development of **sterilization** methods by researchers such as Pasteur and John Tyndall (1820–1893), so necessary to the solution of the spontaneous generation controversy, were essential to put the science of microbiology on a firm foundation. The workers did not set out to develop these methods, but they evolved as a bonus that was received for solving the spontaneous generation question.

Other important developments were in medicine. The microbiological aspects of medicine arose out of considerations of the nature of contagious disease. Although the phenomenon of contagion, especially with respect to diseases such as **smallpox**, was recognized far back in antiquity, its nature and relationship to **microorganisms** was not understood. It was probably the introduction of **syphilis** into Europe, which served to crystallize thinking as here was a disease that could only be transmitted by contact and helped to formulate the question, what is being transmitted? Gerolamo Fracastoro (1478–1553) gave syphilis its name in the sixteenth century and came close to devising a **germ theory of disease**, an idea that later attracted a number of workers all the way down to the nineteenth century. By the late 1830s, Schwann and Cagniard-Latour had shown that alcoholic fermentation and putrefaction were due to living, organized beings. If one accepted the fact that the decomposition of organic materials was due to living organisms, it was only a step further to reason that disease, which in many ways appears as the decomposition of body tissues, was due to living agents. Jacob Henle, in 1840, further commented on this similarity and with the newfound knowledge on the nature of fermentation, he proceeded to draw rather clear conclusions also saying that experimental proof

would be required to clinch this hypothesis. That evidence came later from **Robert Koch** provided, in 1867, the final evidence proving the germ theory. He established the etiologic role of bacteria in **anthrax** and as a result proposed a set of rules to be followed in the establishment of etiology. The key to Koch's observation was the isolation of the organism in pure **culture**. While limiting dilutions could have been used (as described previously by **Joseph Lister**, 1827–1912), Koch promoted the use of solid media, giving rise to separate colonies and the use of stains. In 1882, Koch identified the tubercle bacillus and so formalized the criteria of Henle for distinguishing causative pathogenic microbes. This set of criteria is known as **Koch's postulates**. One of the most important applied developments in microbiology was in understanding the nature of specific acquired **immunity** to disease. That such immunity was possible was known for a long time, and the knowledge finally crystallized with the prophylactic treatment for smallpox introduced by **Edward Jenner** (1749–1823). Using **cowpox**, Jenner introduced the first vaccination procedures in 1796. This occurred long before the germ theory of disease had been established. Later workers developed additional methods of increasing the immunity of an individual to disease, but the most dramatic triumph was the discovery of the **diphtheria** and **tetanus** antitoxins by von Behring and Kitasato in the 1890s. This work later developed into a practical tool by **Paul Ehrlich** (1854–1915) and it was now possible to cure a person suffering from these diseases by injecting some antitoxic serum prepared by earlier **immunization** of a horse or other large animal. This led for the first time to rational cures for infectious diseases, and was responsible for Ehrlich's later conception of **chemotherapy**. The **antibiotics** era, which followed the groundbreaking work of **Alexander Fleming** (1881–1955) with **penicillin**, was another important step in the understanding of microbiology.

Most of the most recent work in the development of microbiology has been in the field of **microbial genetics** and how it evolved into a separate discipline known as **molecular biology**. This work really began in the 1940s, when **Oswald Avery, Colin MacLeod** and **Maclyn McCarty** demonstrated that the transforming principle in bacteria, previously observed by Frederick Griffiths in 1928, was **DNA**. **Joshua Lederberg** and **Edward Tatum** demonstrated that DNA could be transferred from one bacterium to another in 1944. With the determination of the structure of DNA in 1953, a new and practical aspect of microbiology suddenly became realised, and the foundations of genetic

engineering were laid. It is perhaps important to realize that if it were not for bacteria and their characteristics, genetic engineering would not be possible. The concept of DNA transfer was essentially born in the 1940s. Later on, in the late 1960s bacterial **restriction enzymes** were discovered and the possibilities of splicing and rearranging DNA emerged. The advances in molecular biology following these major breakthroughs have been immense but it is important to realize that the field of microbiology lies at their root.

See also Antibiotics; Fermentation; Microscope and microscopy; Vaccine.

BIOTECHNOLOGY, BIOTERRORISM AND GENETIC ENGINEERING

Chapter 2.

Fundamentals of Biotechnology and its application

The word biotechnology was coined in 1919 by Karl Ereky to apply to the interaction of biology with human technology. Today, it comes to mean a broad range of technologies from genetic engineering (recombinant **DNA** techniques), to animal breeding and industrial **fermentation**. Accurately, biotechnology is defined as the integrated use of **biochemistry**, microbiology, and engineering sciences in order to achieve technological (industrial) application of the capabilities of **microorganisms**, cultured tissue cells, and parts thereof. The nature of biotechnology has undergone a dramatic change in the last half century. Modern biotechnology is greatly based on recent developments in **molecular biology**, especially those in genetic engineering. Organisms from **bacteria** to cows are being genetically modified to produce pharmaceuticals and foods. Also, new methods of disease **gene** isolation, analysis, and detection, as well as gene therapy,

promise to revolutionize medicine. In theory, the steps involved in genetic engineering are relatively simple. First, scientists decide the changes to be made in a specific DNA molecule. It is desirable in some cases to alter a human DNA molecule to correct errors that result in a disease such as diabetes. In other cases, researchers might add instructions to a DNA molecule that it does not normally carry: instructions for the manufacture of a chemical such as insulin, for example, in the DNA of bacteria that normally lack the ability to make insulin. Scientists also modify existing DNA to correct errors or add new information. Such methods are now well developed. Finally, scientists look for a way to put the recombinant DNA molecule into the organisms in which it is to

function. Once inside the organism, the new DNA molecule gives correct instructions to cells in humans to correct genetic disorders, in bacteria (resulting in the production of new chemicals), or in other types of cells for other purposes. Genetic engineering has resulted in a number of impressive accomplishments. Dozens of products that were once available only from natural sources and in limited amounts are now manufactured in abundance by genetically engineered microorganisms at relatively low cost. Insulin, human growth hormone, tissue plasminogen activator, and alpha interferon are examples. In addition, the first trials with the alteration of human DNA to cure a genetic disorder began in 1991.

Molecular geneticists use molecular **cloning** techniques on a daily basis to replicate various genetic materials such as gene segments and cells. The process of molecular cloning involves isolating a DNA sequence of interest and obtaining multiple copies of it in an organism that is capable of growth over extended periods. Large quantities of the DNA molecule can then be isolated in pure form for detailed molecular analysis. The ability to generate virtually endless copies (clones) of a particular sequence is the basis of recombinant DNA technology and its application to human and medical genetics. A technique called positional cloning is used to map the location of a human disease gene. Positional cloning is a relatively new approach to finding genes. A particular DNA marker is linked to the disease if, in general, family members with certain nucleotides at the marker always have the disease, and family members with other nucleotides at the marker do not have the disease. Once a suspected linkage result is

confirmed, researchers can then test other markers known to map close to the one found, in an attempt to move closer and closer to the disease gene of interest. The gene can then be cloned if the DNA sequence has the characteristics of a gene and it can be shown that particular **mutations** in the gene confer disease.

Embryo cloning is another example of genetic engineering. Agricultural scientists are experimenting with embryo cloning processes with animal embryos to improve upon and increase the production of livestock. The first successful attempt at producing live animals by embryo cloning was reported by a research group in Scotland on March 6, 1997. Although genetic engineering is a very important component of biotechnology, it is not alone. Biotechnology has been used by humans for thousands of years. Some of the oldest

manufacturing processes known to humankind make use of biotechnology. Beer, wine, and bread making, for example, all occur because of the process of fermentation. As early as the seventeenth century, bacteria were used to remove copper from its ores. Around 1910, scientists found that bacteria could be used to decompose organic matter in sewage. A method that uses microorganisms to produce glycerol synthetically proved very important in the World War I since glycerol is essential to the manufacture of explosives.

See also Fermentation; Immune complex test; Immunoelectrophoresis; Immunofluorescence; Immunogenetics; Immunologic therapies; Immunological analysis techniques; Immunosuppressant drugs; *In vitro* and *in vivo* research.

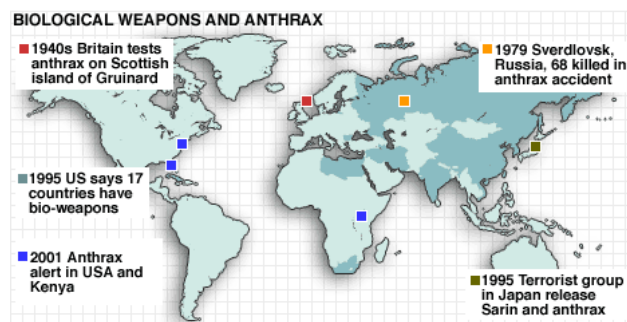
BIOTERRORISM

Bioterrorism is would be divided to one of terrorism by international attack or dissemination of biological agents such as toxins, viruses or bacteria. Because of its strong destroying ability and potential fatal consequences, it is flowing up as a serious social issue through the world.



As it brings out tremendous result in short time, it is quiet attractive weapon. A bioterrorism attack is the release of biological agents (bacteria, viruses, or toxins) and causes illness or death to human, animals and even plants. The biological agents used for the bioterrorism are found in nature, but it is developed by human-modified

form and gets higher ability. They would be spread through air, water, and food.



Bioterrorism is the use of a biological weapon against a civilian population. As with any form of terrorism, its purposes include the undermining of morale, creating chaos, or achieving political goals. Biological weapons use **microorganisms** and toxins to produce disease and death in humans, livestock, and crops. Biological, chemical, and nuclear weapons can all be used to achieve similar destructive goals, but unlike chemical and nuclear technologies that are expensive to create, biological weapons are

relatively inexpensive. They are easy to transport and resist detection by standard security systems. In general, chemical weapons act acutely, causing illness in minutes to hours at the scene of release. For example, the release of sarin gas by the religious sect Aum Shinrikyo in the Tokyo subway in 1995 killed 12 and hospitalized 5,000 people. In contrast, the damage from biological weapons may not become evident until weeks after an attack. If the pathogenic (disease-causing) agent is transmissible, a bioterrorist attack could eventually kill thousands over a much larger area than the initial area of attack. Bioterrorism can

also be enigmatic, destructive, and costly even when targeted at a relatively few number of individuals. Starting in September 2001, bioterrorist attacks with anthrax-causing **bacteria** distributed through the mail targeted only a few U.S. government leaders, media representatives, and seemingly random private citizens. As of June 2002, these attacks remain unsolved. Regardless, in addition to the tragic deaths of five people, the terrorist attacks cost the United States millions of dollars and caused widespread concern. These attacks also exemplified the fact that bioterrorism can strike at the political and economic infrastructure of a targeted country.

Category	A	B	C
Definition	Pose the highest risk to national security because they • Can be easily disseminated or transmitted from person to person • Result in high mortality rates • Have potential to cause public panic and social disruption • Require special preparedness actions	Pose the second highest risk because they • Are moderately easy to disseminate • Result in low mortality rates • Require enhancement of diagnostic and surveillance capability	Emerging pathogens that could be engineered for mass dissemination because they: • Are available • Are easily produced and disseminated • Have potential for high mortality rates
Examples	• Anthrax • Botulism • Plague • Smallpox • Tularemia • Viral hemorrhagic fevers (e.g. Ebola, Marburg)	• West Nile Virus • Caliciviruses • Hepatitis A • Ricin toxin • Salmonella • Diarrheagenic E. coli	• Influenza • SARS • Rabies • Multi-drug resistant tuberculosis • Yellow fever • Tickborne hemorrhagic fever

Although the deliberate production and stockpiling of biological weapons is prohibited by the 1972 **Biological Weapons Convention** (BWC)—the United States stopped forwomimal bioweapons programs in 1969—unintended byproducts or deliberate misuse of emerging

technologies offer potential bioterrorists opportunities to prepare or refine biogenic weapons. Genetic engineering technologies can be used to produce a wide variety of bioweapons, including organisms that produce toxins or that are more weaponizable because they are easier to aerosolize (suspend as droplets in the air). More conventional laboratory

technologies can also produce organisms resistant to **antibiotics**, routine vaccines, and therapeutics. Both technologies can produce organisms that cannot be detected by antibody-based sensor systems. Among the most serious of potential bioterrorist weapons are those that use **smallpox** (caused by the **Variola virus**), **anthrax** (caused by *Bacillus anthracis*), and plague (caused by *Yersinia pestis*). During naturally occurring **epidemics** throughout the ages, these organisms have killed significant portions of afflicted populations. With the advent of vaccines and antibiotics, few U.S. physicians now have the experience to readily recognize these diseases, any of which could cause catastrophic numbers of deaths.

Although the last case of smallpox was reported in Somalia in 1977, experts suspect that smallpox **viruses** may be in the biowarfare laboratories of many nations around the world. At present, only two facilities—one in the United States and one in Russia—are authorized to store the virus. As recently as 1992, United States intelligence agencies learned that Russia had the ability to launch missiles containing weapons-grade smallpox at major cities in the U.S. A number of terrorist organizations—including the radical Islamist Al Qaeda terrorist organization—actively seek the acquisition of state-sponsored research into weapons technology and pathogens.

There are many reasons behind the spread of biowarfare technology. Prominent among them are economic incentives; some governments may resort to selling bits of scientific information that can be pieced together by the buyer to create biological weapons. In addition, scientists in politically repressive or unstable countries may be forced to participate in research that eventually ends up in the hands of terrorists.

Abiological weapon may ultimately prove more powerful than a conventional weapon because its effects can be farreaching and uncontrollable. In 1979, after an accident involving *B. anthracis* in the Soviet Union, doctors reported civilians dying

of anthrax **pneumonia** (i.e., inhalation anthrax). Death from anthrax pneumonia is usually swift. The bacilli multiply rapidly and produce a toxin that causes breathing to stop. While antibiotics can combat this bacillus, supplies adequate to meet the treatment needs following an attack on a large urban population would need to be delivered and distributed within 24 to 48 hours of exposure. The National Pharmaceutical Stockpile Program (NPS) is designed to enable such a response to a bioterrorist attack. Preparing a strategy to defend against these types of organisms, whether in a natural or genetically modified state, is difficult. Some of the strategies include the use of bacterial **RNA** based on structural templates to identify pathogens; increased abilities for rapid **genetic identification of microorganisms**; developing a database of virtual pathogenic molecules; and development of antibacterial molecules that attach to pathogens but do not harm humans or animals. Each of these is an attempt to increase—and make more flexible—identification capabilities. Researchers are also working to counter potential attacks using several innovative technological strategies. For example, promising research is being done with biorobots or microchip-mechanized insects, which have computerized artificial systems that mimic biological processes such as neural networks, can test responses to substances of biological or

chemical origin. These insects can, in a single operation, process **DNA**, screen blood samples, scan for disease genes, and monitor genetic cell activity. The robotics program of the Defense Advanced Research Project (DARPA) works to rapidly identify bio-responses to pathogens, and to design effective

and rapid treatment methods.

Biosensor technology is the driving force in the development of biochips for detection of biological and chemical contaminants. Bees, beetles, and other insects outfitted with sensors are used to collect real-time information about the presence of toxins or similar threats. Using

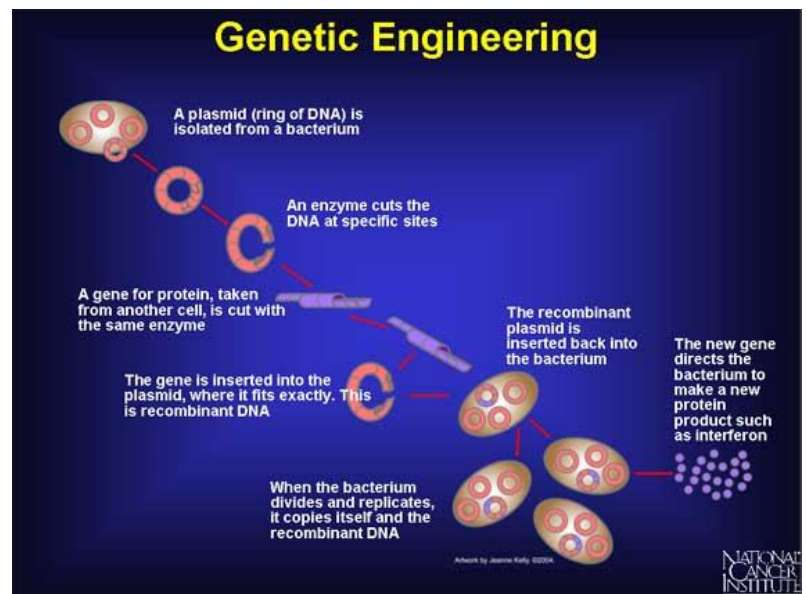
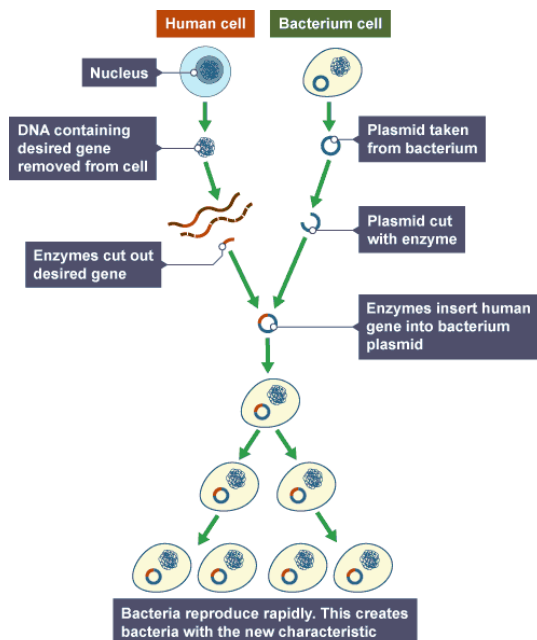
fiber optics or electrochemical devices, biosensors have detected microorganisms in chemicals and foods, and they offer the promise of rapid identification of biogenic agents following a bioterrorist attack. The early accurate identification of biogenic agents is critical to implementing effective response and treatment protocols. To combat biological agents, bioindustries are developing a wide range of antibiotics and vaccines. In addition, advances in **bioinformatics** (i.e., the computerization of information acquired during, for example, genetic screening) also increases flexibility in the development of effective counters to biogenic weapons.

In addition to detecting and neutralizing attempts to weaponize biogenic agents (i.e., attempts to develop bombs or other instruments that could effectively disburse a bacterium or virus), the

major problem in developing effective counter strategies to bioterrorist attacks involves the breadth of organisms used in **biological warfare**. For example, researchers are analyzing many pathogens in an effort to identify common genetic and cellular components. One strategy is to look for common areas or vulnerabilities in specific sites of DNA, RNA, or proteins. Regardless of whether the pathogens evolve naturally or are engineered, the identification of common traits will assist in developing counter measures (i.e., specific vaccines or antibiotics).

See also Anthrax, terrorist use of as a biological weapon; Biological warfare; Contamination, bacterial and viral; Genetic identification of microorganisms; Public health, current issues

GENETIC ENGINEERING



Genetic engineering allows scientists to pluck genes--segments of DNA--from one type of organism and to combine them with genes of a second organism.

It can be defined as the group of applied techniques of genetics and biotechnology used to

cut up and join together genetic material especially DNA from one or more species of

organism and to introduce into an organism in order to change one or more of its characteristics.

Genetics: the branch of biology that deals with the principles and mechanisms of heredity and with the genetic contribution to similarities and differences among related organisms.

Biotechnology: the technique of using microorganisms, such as bacteria, to perform chemical processing, such as waste recycling, or to produce other materials, such as beer and wine, cheese, antibiotics, and (using genetic engineering) hormones, vaccines, etc.

So in easy words we can define **genetic engineering as making changes to DNA in order to change the way living things work.**

BASIC / FUNDAMENTAL TOOLS USED IN GENETIC ENGINEERING

There are four major components of Genetic Engineering

- A. Complementary DNA
- B. Enzymes:
 - 1. Nucleases
 - 2. Restriction enzymes
 - 3. Dna ligase
 - 4. Kinase
 - 5. Phosphatase
 - 6. Reverse transcriptase
- C. Vectors:
 - 1. Plasmid
 - 2. Phage vectors
 - 3. Cosmid
 - 4. Viral vectors
- D. Genetic engineering technologies
 - 1. Gene therapy
 - a. Viral approaches:
 - b. Non-viral approaches:
 - 2. Monoclonal antibody
 - 3. Vaccine
 - 4. Nanotechnology
 - 5. Biosensor
 - 6. Cell culture
 - 7. Bioinformatics
 - 8. Polymerase chain reaction

Complementary DNA:

Complementary DNA (cDNA) is [DNA](#) synthesized from a messenger RNA ([mRNA](#)) template in a reaction catalysed by the enzymes [reverse transcriptase](#) and [DNA polymerase](#). cDNA is often used to clone [eukaryotic genes](#) in [prokaryotes](#). When scientists want to express a specific [protein](#) in a cell that does not normally [express](#) that protein they will transfer the cDNA that codes for the protein to the recipient cell. cDNA is also produced naturally by [retroviruses](#).

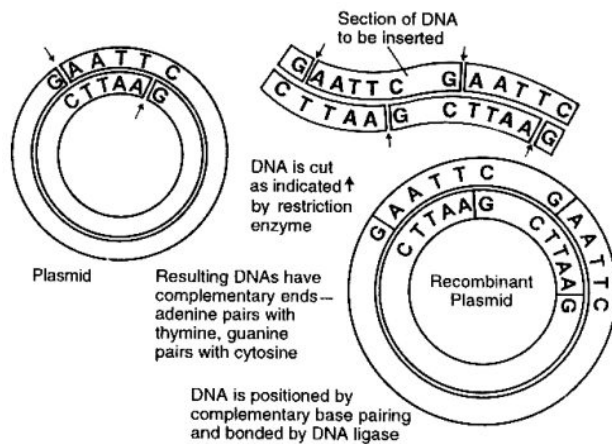
Enzymes:

The various enzymes used in genetic engineering are as follows:

- c. Nucleases
- d. Restriction enzymes
- e. DNA ligase
- f. Kinase
- g. Phosphatase
- h. Reverse transcriptase

1.Nucleases :

Nucleases are a group of enzymes which cleave or cut the genetic material (DNA or RNA). Nucleases are further classified into two types based upon the substrate on which they act. Nucleases which act on or cut the DNA are classified as DNases, whereas those which act on the RNA are called as RNases. DNases that act on the ends or terminal regions of DNA are called as exonucleases and those that act at a non-specific region in the centre of the DNA are called as endonucleases.



2. Restriction Enzymes :

DNases which act on specific positions or sequences on the DNA are called as restriction endonucleases. The sequences which are recognized by the restriction endonucleases or restriction enzymes (RE) are called as recognition sequences or restriction sites.

Types

- Type I enzymes cleave the DNA at random site.
- Type II restriction modification system possess separate enzymes for endonucleases
- Type III cleave at a specific site

3. DNA Ligase :

Recombinant DNA experiments require the joining of two different DNA segments or fragments in vitro. The cohesive ends generated by some RE will anneal themselves by forming hydrogen bonds. But the segments annealed thus are weak and do not withstand experimental conditions. To get a stable joining, the DNA should be joined by using an enzyme called ligase. DNA ligase joins the DNA molecule covalently.

4. Kinase :

Kinase is the group of enzymes, which adds a free pyrophosphate (PO_4) to a wide variety of substrates like proteins, DNA and RNA. It is widely used in molecular biology and genetic engineering to add radiolabelled phosphates.

5. Alkaline phosphatases:

Phosphatases are a group of enzymes which remove a phosphate from a variety of substrates like DNA, RNA and proteins. Phosphatases which act in basic buffers with pH 8 or 9 are called as alkaline phosphatases.

6. Reverse Transcriptase:

This enzyme uses an RNA molecule as template and synthesizes a DNA strand complementary to the RNA molecule. These enzymes are used to synthesize the DNA from RNA.

Vectors:

A vector is a DNA molecule used as a vehicle to artificially carry foreign genetic material into another cell, where it can be [replicated](#) and/or [expressed](#).

Types of vectors:

- Plasmid
- Phage vectors
- Cosmid
- Viral vectors

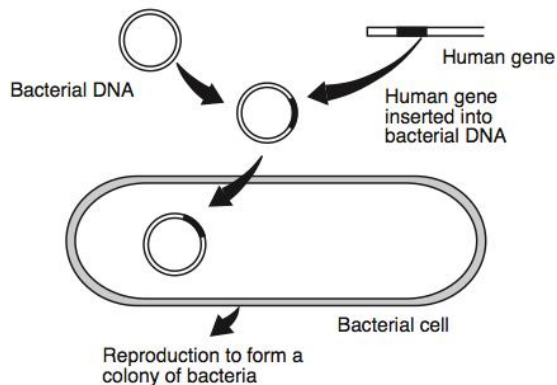
1.Plasmid :

Plasmids are circular DNA molecules that lead an independent existence in the bacterial cells. They are naturally occurring, extra chromosomal DNA fragments that are stably inherited from one generation to another generation in an extra chromosomal state. The incorporation of DNA fragments into plasmid vectors not only allow foreign DNA to be replicated in cloned cells for later isolation and identification, but can also be designed so that cells transcribe and translate this DNA into protein.

2.Phage as vectors:

Bacteriophages are natural vectors that transduce bacterial DNA from one cell to another. Phage vectors have a natural advantage over plasmids that is they infect cells much more efficiently than plasmids transformed cells, so the

yield of clones with phage vectors is usually higher.

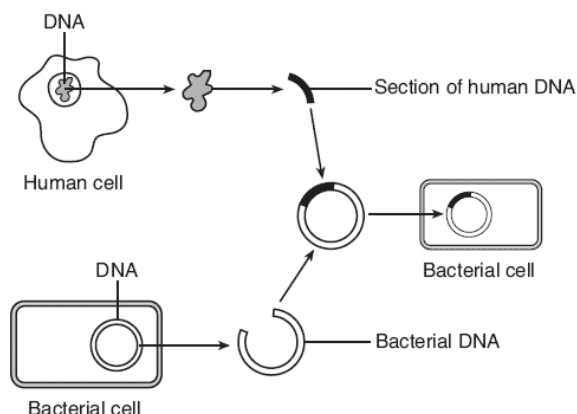


3.Cosmid:

The segment of the phage DNA is inserted into the double stranded plasmid which replicates in *E.Coli*. The points at which the segment of bacteriophage lambda is cut are called *cos* (cohesive) sites. The resulting vector is called a cosmid. It is a hybrid vector, part phage, part bacteria, that can be used to clone larger genes or fragments of DNA.

4.Viral vectors :

Viral vectors are generally genetically engineered viruses carrying modified viral DNA or RNA that has been rendered non-infectious.



D- TECHNOLOGIES USED IN GENETIC ENGINEERING:

GENE THERAPY

When genes are altered so that the encoded proteins are unable to carry out their normal functions, genetic disorders can result.

Gene therapy applies several **approaches for correcting faulty genes**:

- A normal gene may be inserted into a nonspecific location within the genome to replace a nonfunctional gene.
- An abnormal gene could be swapped for a normal gene through homologous recombination.
- The abnormal gene could be repaired through selective reverse mutation.
- The regulation (the degree to which a gene is turned on or off) of a particular gene could be altered.

How does gene therapy work?

In most gene therapy studies, a "normal" gene is inserted into the genome to replace an "abnormal," disease-causing gene.

Viral approaches:

The vector (mostly virus) unloads its genetic material containing the therapeutic human gene into the target cell. The generation of a functional protein product from the therapeutic gene restores the target cell to a normal state.

Some of the different types of viruses used as gene therapy vectors:

- o Retroviruses
- o Adeno-associated viruses
- o Herpes simplex viruses

non-viral approaches:

- the direct introduction of therapeutic DNA into target cells.
- Liposome, which carries the therapeutic DNA which is capable of passing the DNA through the target cell's membrane.
- by chemically linking the DNA to a molecule that will bind to special cell receptors.

MONOCLONAL ANTIBODY

“**Monoclonal antibodies** are monospecific antibodies that are the same because they are made by identical immune cells that are all clones of a unique parent cell and they bind to the same epitope”.

VACCINE

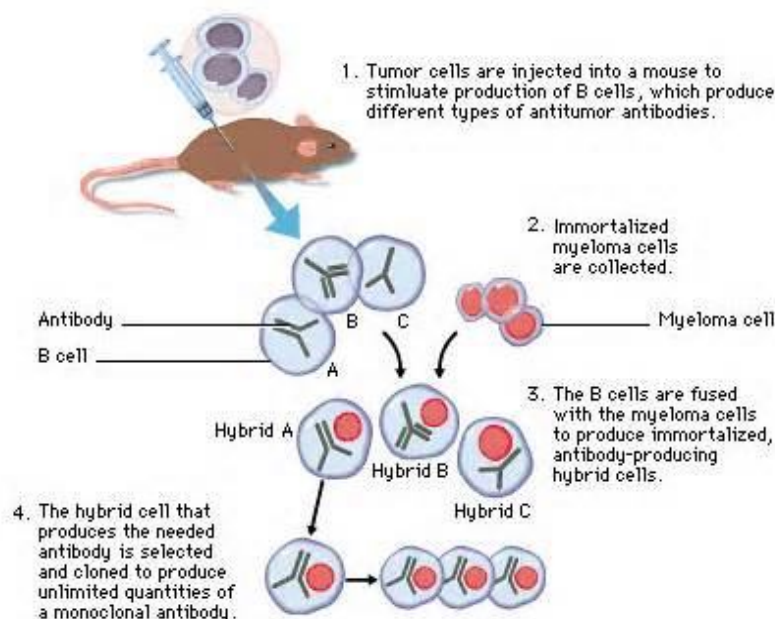
A **vaccine** is a biological preparation that improves immunity to a particular disease.

Vaccines can be:-

- Prophylactic: to prevent the effects of a future infection by any natural pathogen.
- Therapeutic: vaccines against some diseased state e.g cancer

Genetically engineered vaccines are as follows:

- **Subunit vaccines**: single viral or bacterial protein, such as HBsAg
- **DNA vaccines**: They employ genes encoding proteins of pathogens rather than using the proteins themselves, a live replicating vector i.e. the plasmid which is grown in bacteria (e. coli), purified, dissolved in a saline solution, and then simply injected into the host.
- **Recombinant (DNA) vaccines**: Made by isolation of DNA fragment(s) coding for the immunogen(s) of an infectious agent/cancer cell, followed by the insertion of the fragment(s) into vector DNA molecules (i.e. plasmids or viruses) which can replicate and conduct protein-expression within bacterial, yeast, insect or mammalian cells.



- **Naked DNA vaccines**: They may persist much longer in the environment than commonly believed.
- **Live vector vaccines**: These are produced by the insertion of the DNA fragment(s) coding for an immunogen(s) intended for vaccination into the genome of a 'non-dangerous' virus or bacterium, the vector.

The insertion is performed in such a way that the vector is still infectious 'live'.

- **RNA vaccines**: This involves the use of in vitro synthesised
- **Edible vaccines**: These are produced by making transgenic, edible crop plants as the production and delivery systems for subunit vaccines.

NANOTECHNOLOGY

- Nanotechnology refers to "a field of applied science and technology whose theme is the control of matter on the atomic and molecular scale, and the fabrication of devices or materials that lies within that size range.
- Molecular Nanotechnology would involve combining physical principles demonstrated by chemistry, other nanotechnologies, and the molecular machinery of life.
- Application of nanotechnology in genetic engineering: developing a new method of delivering DNA(NANOINJECTION) to fertilized mouse eggs using a nanochip (MEMS CHIP) with a moveable, nanometer-sized lance that is capable of holding DNA and injecting it in the zygote's pronucleus.

BIOSENSOR

A **biosensor** is an analytical device which converts a biological response into an electrical signal. Their application include:

- Glucose sensors
- Tumor cells can be used to monitor chemotherapeutic drug susceptibility.
- In organ replacement
- Environmental monitoring

CELL CULTURE

"Growing of cells outside living body". The cells are grown on a culture and must be subcultured.

Applications:

It has led to discoveries in fields such as:

- Stem cell self-renewal
- Cancer cell phenotype
- Fibrosis
- Hepatocyte function
- drug discovery, cancer biology, regenerative medicine

- **Tissue culture and engineering:** The major application of human cell culture is in stem cell industry
- **Vaccines:** Vaccines for polio, measles, mumps, rubella, and chickenpox .

BIOINFORMATICS

1. It is a interdisciplinary scientific field that develops methods for storing, retrieving, organizing and analyzing scientific data.
2. A major activity in bioinformatics is to develop software tools to generate useful biological knowledge i.e.
 - mapping and analyzing DNA and protein sequences,
 - aligning DNA and protein sequences to compare them
 - Creating and viewing 3-D models of protein structures.
 - Gene and protein expression
 - Genetics of Disease

POLYMERASE CHAIN REACTION

This technique allows the generation of large amount of copies of a specified DNA sequence from a single DNA molecule without the need for cloning. PCR uses single stranded DNA as a template for the synthesis of complementary new strands in a 5' to 3' direction.

Denaturation step:

This step is the first regular cycling event and consists of heating the reaction. It causes melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single-stranded DNA molecules.

Annealing step:

The reaction temperature is lowered allowing annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. DNA polymerase require small fragments of double stranded DNA to initiate DNA synthesis. The polymerase binds to the primer-template hybrid and begins DNA formation.

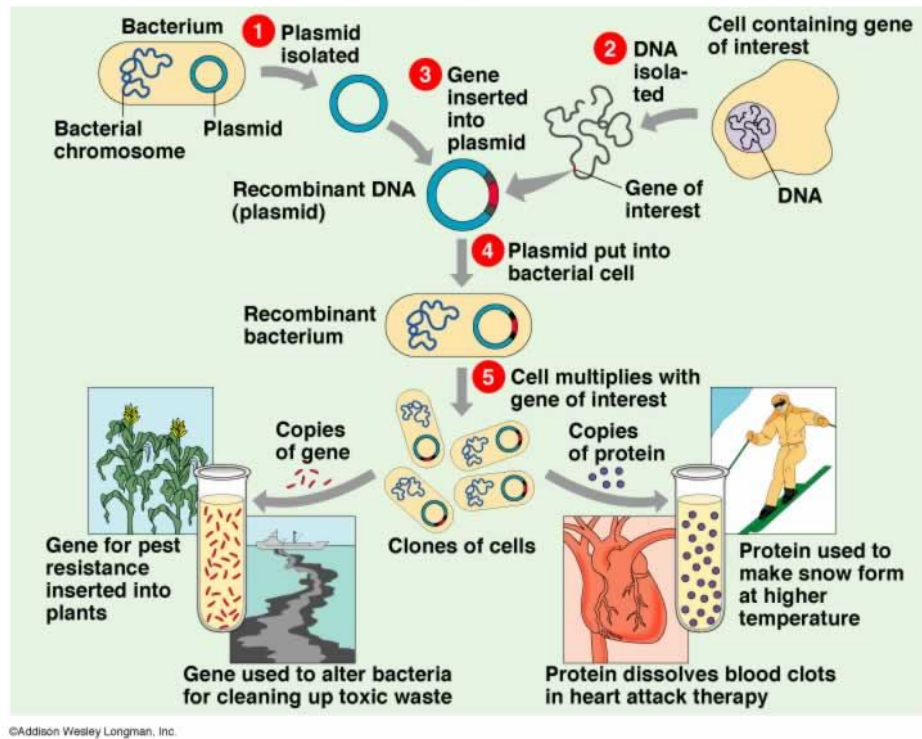


Figure. Use of biotechnology in different fields of life.

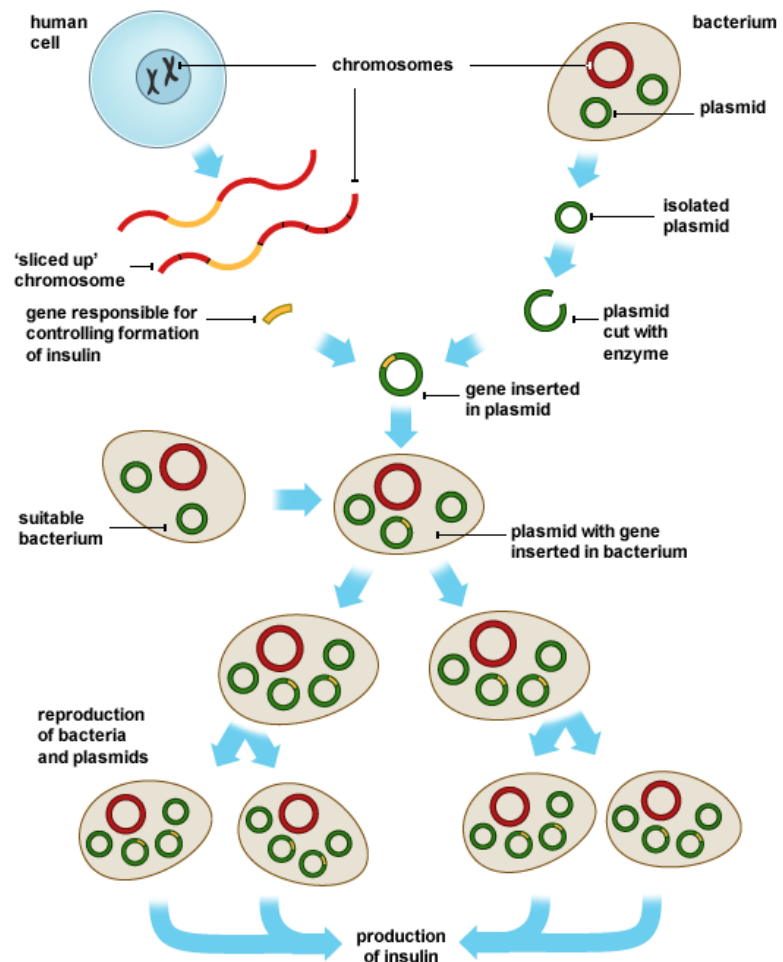


Figure. Production of insulin.

Extension/elongation step:

The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75–80 °C, and commonly a temperature of 72 °C is used with this enzyme which reduces the risk of mismatches that occasionally occur at lower temperatures. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand.

Final elongation:

This single step is occasionally performed after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended. **Final hold:**

This step at 4–15 °C for an indefinite time may be employed for short-term storage of the reaction.

In PCR both strands of DNA serve as template upon the addition of a pair of primers, one for each strand of DNA. Every PCR cycle is normally repeated up to 30 times. The net result of a PCR at the end of n cycles, will generate maximum of 2ⁿ double stranded DNA copies of the DNA fragment located between the two primers

ADVANTAGES OF PCR:

1. It is highly specific.
2. It is rapid technique.
3. It is versatile,
4. The equipment is inexpensive and allows the analysis of a large number of sequences at one time.
5. PCR does not require pure template DNA and can amplify genes from whole cells or tissue samples.

LIMITATIONS OF PCR:

1. The designing of primers for this technique requires partial knowledge of the DNA sequence to be amplified.
2. The slightest sample contamination can lead to false positive results, which can have detrimental effects when this technique is used in diagnostics.

There is a risk of non specific amplification when the primers bind to closely related sequences, leading to the amplification of wrong sequence.

APPLICATIONS OF GENETIC ENGINEERING:

There are three main application of genetic engineering;

1. Medicine
2. Industrial
3. Bio Art and entertainment

CLINICAL APPLICATIONS OF PCR:

Application	Example
Diagnostics of inherited diseases	Cystic fibrosis Haemophilia B
Infectious disease screening	HIV Measles virus
Forensic examination	Identification of suspect criminals from samples of blood, tissue, etc
Prenatal screening	Haemophilia Sickle cell anaemia
HLA subtyping	Prevention of insulin dependent diabetes mellitus.
Susceptibility to cardiovascular disease	Mutations in gene coding for ACE Mutation in the angiotensinogen gene
Susceptibility to cancer	Neoplastic disease Thyroid cancer

Medicine

1. Recombinant human insulin (**humulin R** by Eli Lilly and Company)
2. Recombinant somatostatin (antigrowth hormone, used to treat acromegaly)
3. Recombinant somatotropin (human growth hormone, used in children with dwarfism) (**Genotropin** by Pfizer)
4. Recombinant hepatitis B vaccine (HBsAg)(**HepaGam B**)
5. Production of recombinant antibiotics
6. Factor VIII for males suffering from hemophilia A(**Hemofil M**)
7. Factor IX for hemophilia (**Benefix**)
8. Erythropoietin (EPO) for treating anemia
9. Three types of interferon's(**Multiferon**)

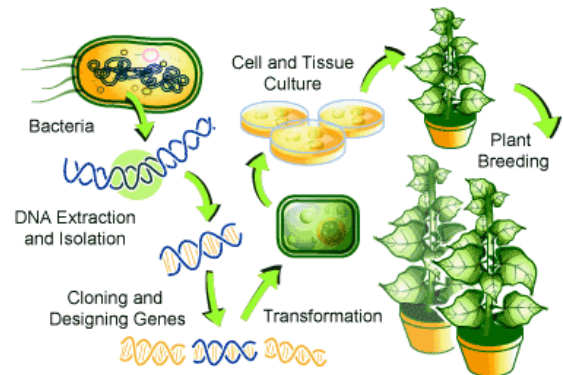
10. Several interleukins(**Proleukin**)
11. Granulocyte-macrophage colony stimulating factor (GM-CSF) for stimulating a bone marrow after bone marrow transplant(**Leucotropin**)
12. Tissue plasminogen activator (TPA) for dissolving blood clots(**TNKase**)
13. Adenosine deaminase (ADA) for treating some forms of severe combined immunodeficiency (SCID)(**Nipent**)
14. Angiostatin and endostatin for trial as anti-cancer drugs
15. Parathyroid hormone
16. Monoclonal antibodies as immunosuppressive drugs(**GVAX**)

Industrial

1. bacteria or yeast, or insect mammalian cells are transformed with a desired gene e.g. protein, such as an enzyme, and transformed organism will over express the desired protein. mass quantities of the protein can be manufactured by growing the transformed organism in bioreactor equipment
2. genetically engineered bacteria is used for making biofuels, cleaning up oil spills, carbon

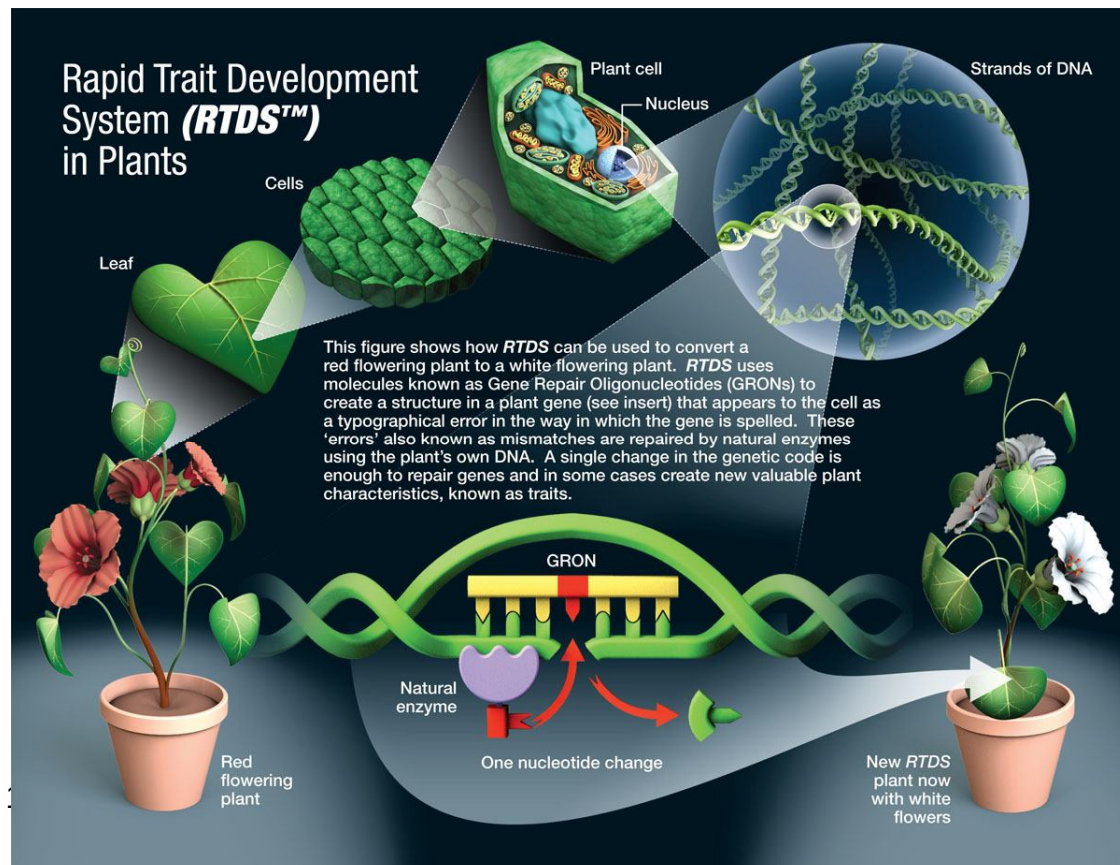
and other toxic waste and detecting arsenic in drinking water.

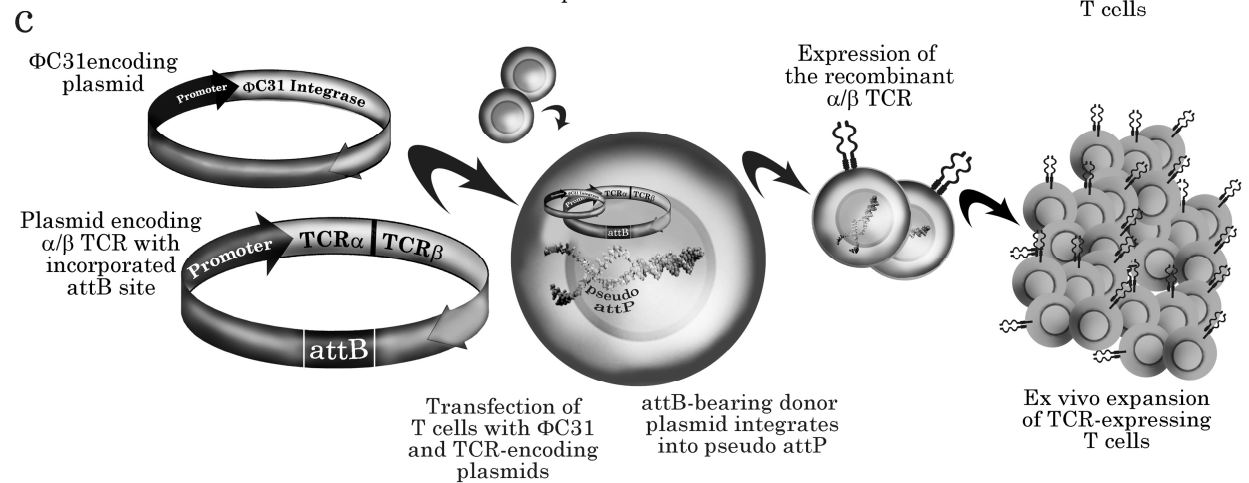
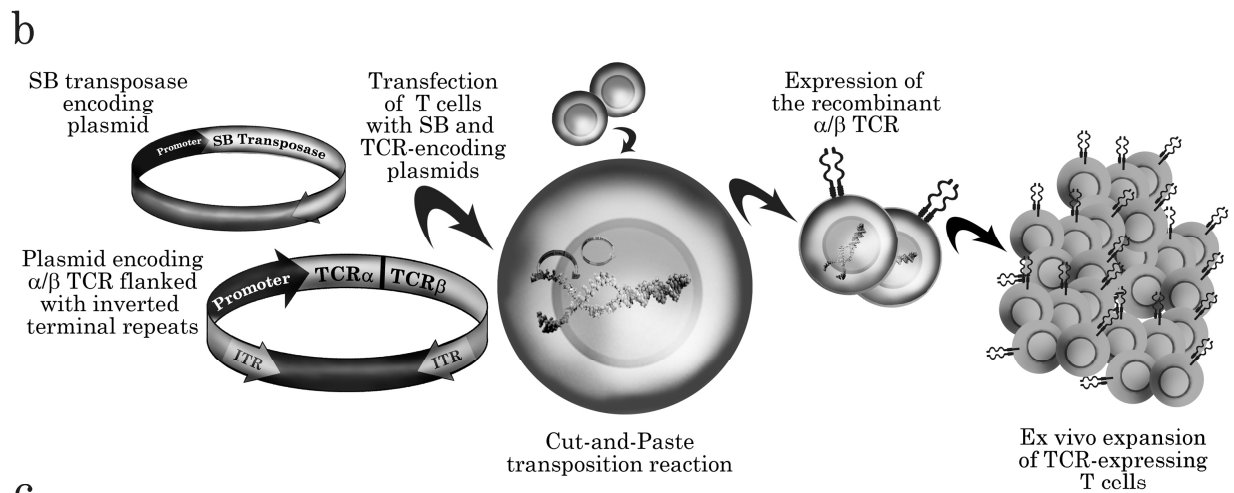
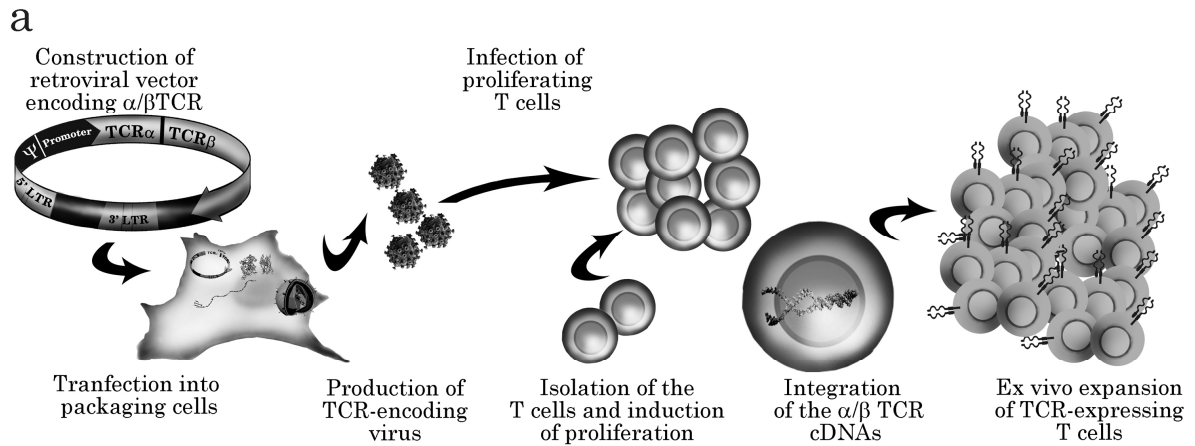
3. genetically engineered bacteria is used as sensors by expressing a fluorescent protein.



Bio Art and entertainment

1. Genetic engineering is also being used to create Bio Art. Some bacteria have been genetically engineered to create black and white photographs
2. Genetic engineering has also been used to create novelty items such as lavender-colored carnations, blue roses, and glowing fish.





MICROBIAL ENZYMES

Chapter 3.

Classification, Mechanism of Action, Commercial Production and application and Immobilized Enzymes.

Enzymes are molecules that act as critical catalysts in biological systems. Catalysts are substances that increase the rate of chemical reactions without being consumed in the reaction. Without enzymes, many reactions would require higher levels of energy and higher temperatures than exist in biological systems. Enzymes are proteins that possess specific binding sites for other molecules (substrates). A series of weak binding interactions allow enzymes to accelerate reaction rates. Enzyme kinetics is the study of enzymatic reactions and mechanisms. Enzyme inhibitor studies have allowed researchers to develop therapies for the treatment of diseases, including **AIDS**.

French chemist **Louis Pasteur** (1822–1895) was an early investigator of enzyme action. Pasteur hypothesized that the conversion of sugar into alcohol by **yeast** was catalyzed by “ferments,” which he thought could not be separated from living cells. In 1897, German biochemist Eduard Buchner (1860–1917) isolated the enzymes that catalyze the **fermentation** of alcohol from living yeast cells. In 1909, English physician Sir Archibald Garrod (1857–1936) first characterized enzymes genetically through the one gene-one enzyme hypothesis. Garrod studied the human disease alkaptonuria, a hereditary disease characterized by the darkening of excreted urine after exposure to air. He hypothesized that alkaptonurics lack an enzyme that breaks down alkaptans to normal excretion products, that alkaptonurics inherit this inability to produce a specific enzyme, and that they inherit a mutant form of a **gene** from each of their parents and have two mutant forms of the same gene. Thus, he hypothesized, some genes contain information to specify particular enzymes.

The early twentieth century saw dramatic advancement in enzyme studies. German chemist Emil Fischer (1852–1919) recognized the importance of substrate shape for binding by enzymes. German-American biochemist Leonor Michaelis (1875–1949) and Canadian biologist Maud Menten (1879–1960) introduced a mathematical approach for quantifying enzyme-catalyzed reactions. American chemists James Sumner (1887–1955) and John Northrop (1891–1987) were among the first to produce highly ordered enzyme crystals and firmly establish the proteinaceous nature of these biological catalysts. In 1937, German-born British biochemist **Hans Krebs** (1900–1981) postulated how a series of enzymatic reactions were coordinated in the citric acid cycle for the production of ATP from glucose metabolites. Today, enzymology is a central part of biochemical study, and the fields of industrial microbiology and genetics employ enzymes in numerous ways, from food production to gene **cloning**, to advanced therapeutic techniques.

Enzymes are proteins that encompass a large range of molecular size and mass. They may be composed of more than one polypeptide chain. Each polypeptide chain is called a subunit and may have a separate catalytic function. Some enzymes require non-protein groups for enzymatic activity. These components include metal ions and organic molecules called coenzymes. Coenzymes that are tightly or covalently attached to enzymes are termed prosthetic groups. Prosthetic groups contain critical chemical groups which allow the overall catalytic event to occur. Enzymes bind their substrates at special folds and clefts

in their structures called active sites. Because active sites have chemical groups precisely located and orientated for binding the substrate, they generally display a high degree of substrate specificity. The active site of an enzyme consists of two key regions, the catalytic site, which interacts with the substrate during the reaction, and the binding site, the chemical groups of the enzyme that bind the substrate, allowing the interactions at the catalytic site to occur. The crevice of the active site creates a microenvironment whose properties are critical for catalysis. Environmental factors influencing enzyme activity include **pH**, polarity and hydrophobicity of amino acids in the active site, and a precise arrangement of the chemical groups of the enzyme and its substrate.

Enzymes have high catalytic power, high substrate specificity, and are generally most active in aqueous solvents at mild temperature and physiological pH. Most enzymes catalyze the transfer of electrons, atoms, or groups of atoms. There are thousands of known enzymes, but most can be categorized according to their biological activities into six major classes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases.

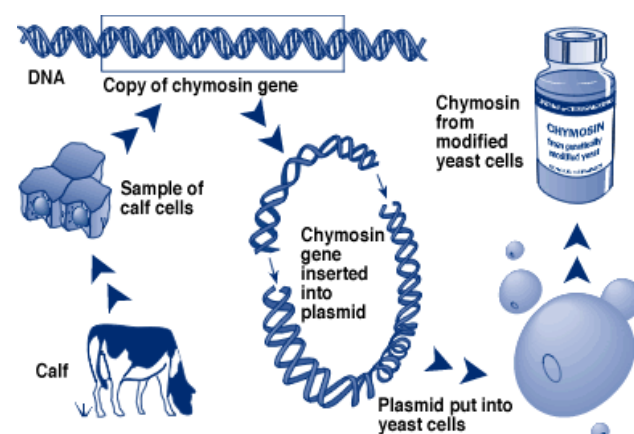
Enzymes generally have an optimum pH range in which they are most active. The pH of the environment will affect the ionization state of catalytic groups at the active site and the ionization of the substrate. Electrostatic interactions are therefore controlled by pH. The pH of a reaction may also control the conformation of the enzyme by influencing amino acids critical for the three-dimensional shape of the macromolecule. Inhibitors can diminish the activity of an enzyme by altering the binding of substrates. Inhibitors may resemble the structure of the substrate, thereby binding the enzyme and competing for the correct substrate. Inhibitors may be large organic molecules, small

molecules, or ions. They can be used for chemotherapeutic treatment of diseases.

Regulatory enzymes are characterized by increased or decreased activity in response to chemical signals. Metabolic pathways are regulated by controlling the activity of one or more enzymatic steps along that path. Regulatory control allows cells to meet changing demands for energy and metabolites.

See also Biochemical analysis techniques; Biotechnology; Bioremediation; Cloning, application of cloning to biological problems; Enzyme induction and repression; Enzyme-linked immunosorbent assay (ELISA); Food preservation; Food safety; Immunologic therapies; Immunological analysis techniques.

MICROBIAL ENZYMES



Restriction Enzyme

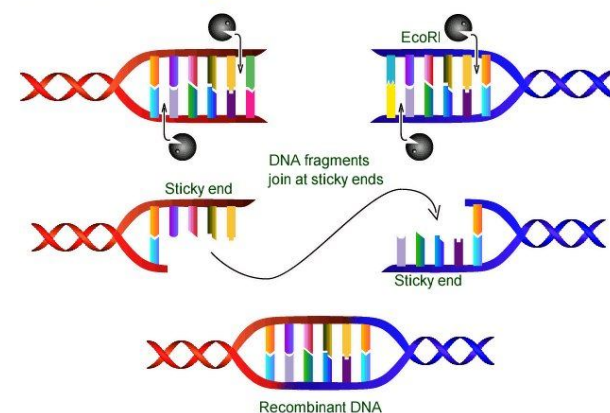


Table 1
Technological Properties of Immobilized Enzyme Systems (3)

<i>Advantages</i>	<i>Disadvantages</i>
Catalyst reuse	Loss or reduction in activity
Easier reactor operation	Diffusional limitation
Easier product separation	Additional cost
Wider choice of reactor	

Table 2
Major Products Obtained Using Immobilized Enzymes (3)

Enzyme	Product
Glucose isomerase	High-fructose corn syrup
Amino acid acylase	Amino acid production
Penicillin acylase	Semi-synthetic penicillins
Nitrile hydratase	Acrylamide
β -Galactosidase	Hydrolyzed lactose (whey)

Table 3
Steps in the Development of Immobilized Enzymes (11)

Step	Date	Use
First	1815	Empirical use in processes such as acetic acid and waste water treatment.
Second	1960s	Single enzyme immobilization: production of L-aminoacids, isomerization of glucose.
Third	1985–1995	Multiple-enzyme immobilization including co-factor re-generation and cell immobilization. Example: production of L-aminoacids from keto-acids in membrane reactors.

Microbial enzymes

Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells.

Enzymes are highly specific in their action on substrates and often many different enzymes are required to bring about, by concerted action, the sequence of metabolic reactions performed by the living cell. All enzymes which have been purified are protein in nature, and may or may not possess a non protein prosthetic group.

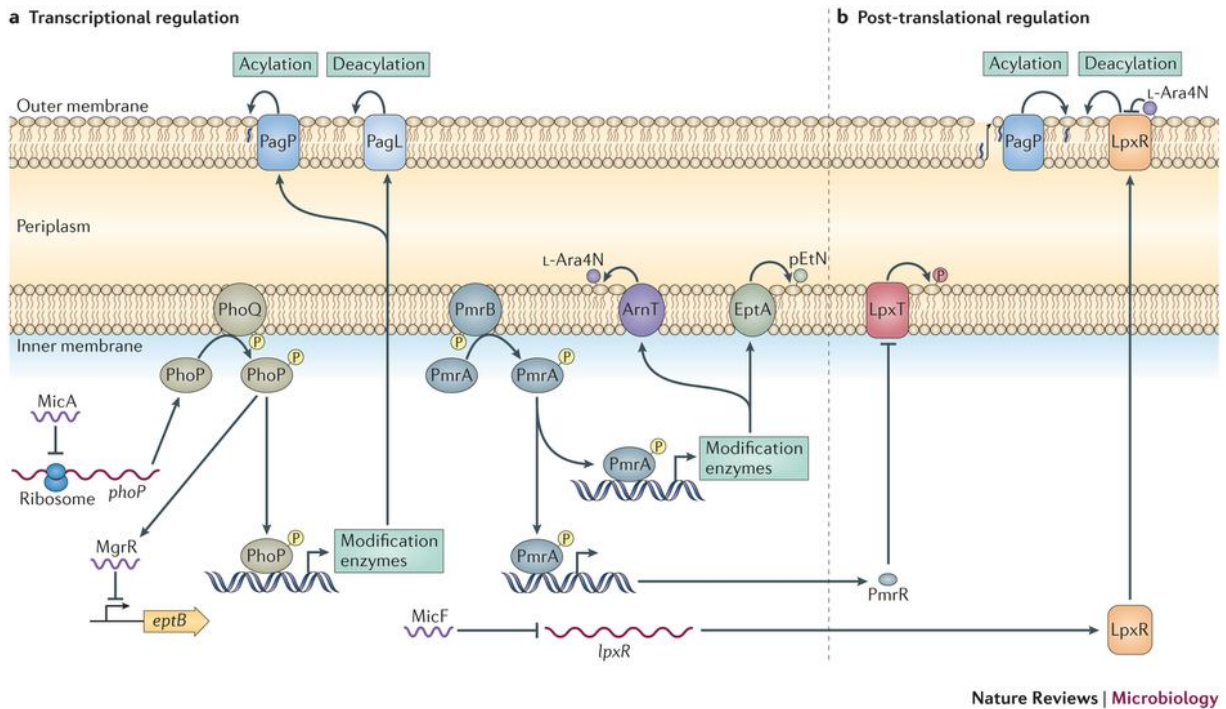


Figure. Transcriptional and post-translational regulation of lipid A modification enzymes.

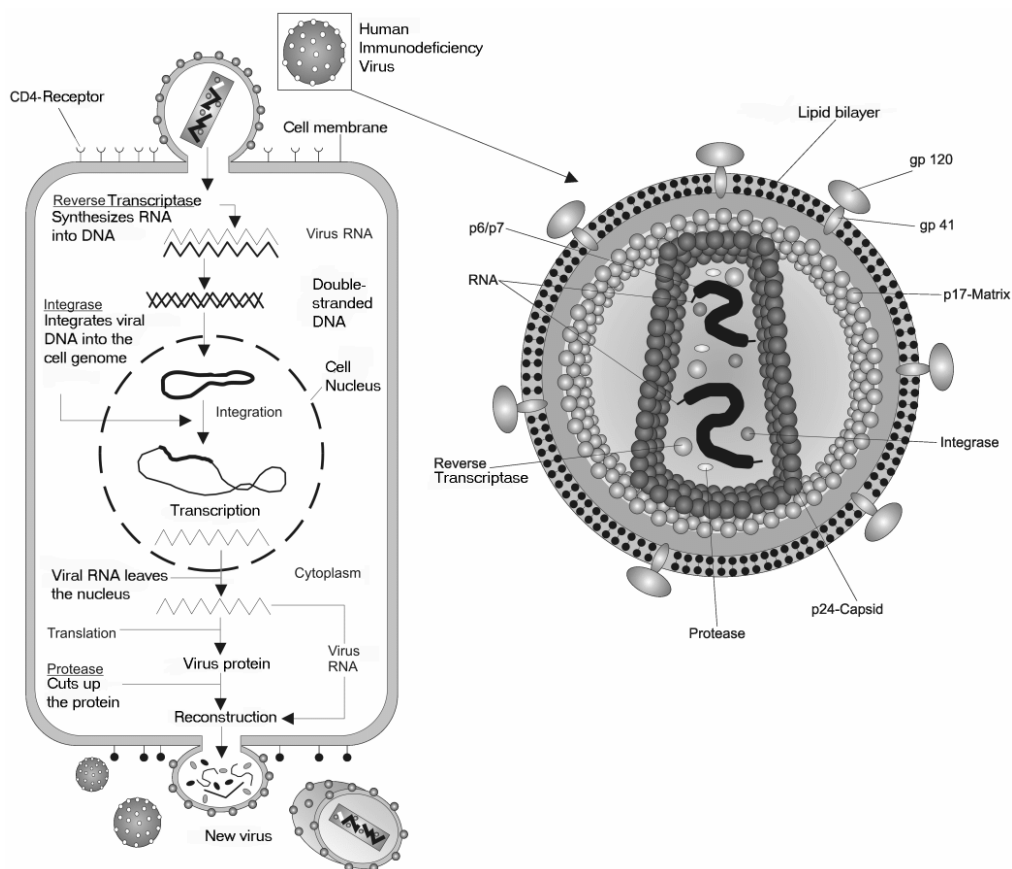


Figure. The components, enzymes and key events at the replication fork during DNA replication

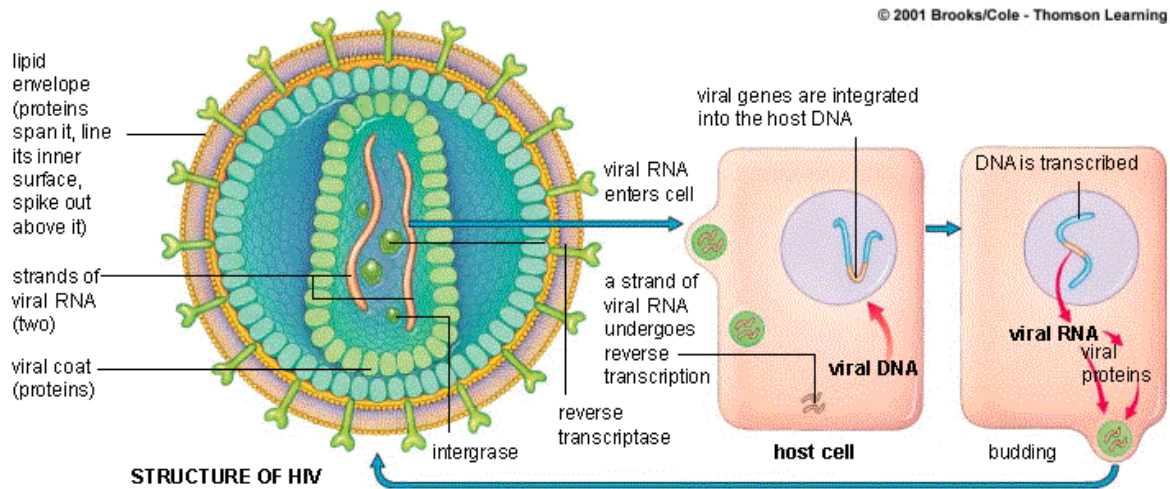
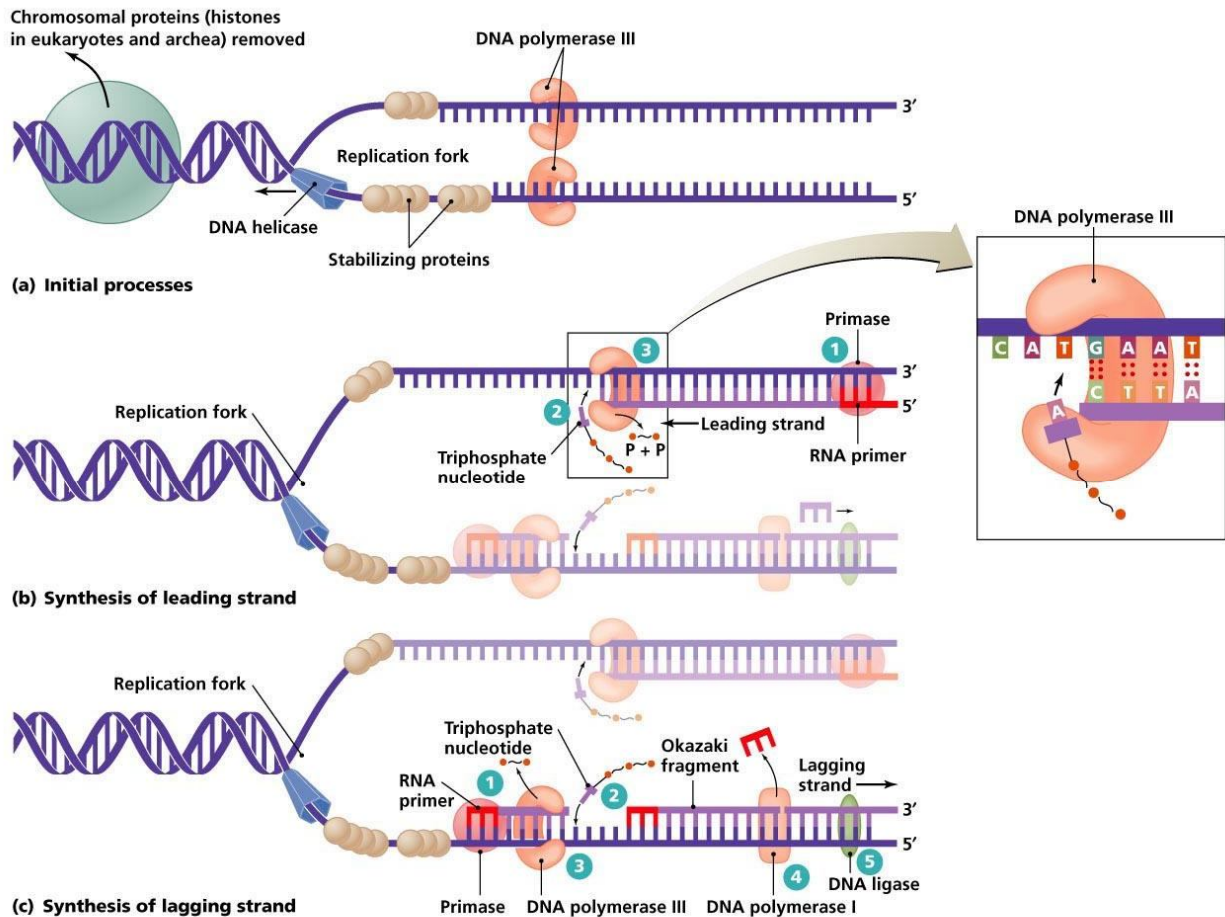
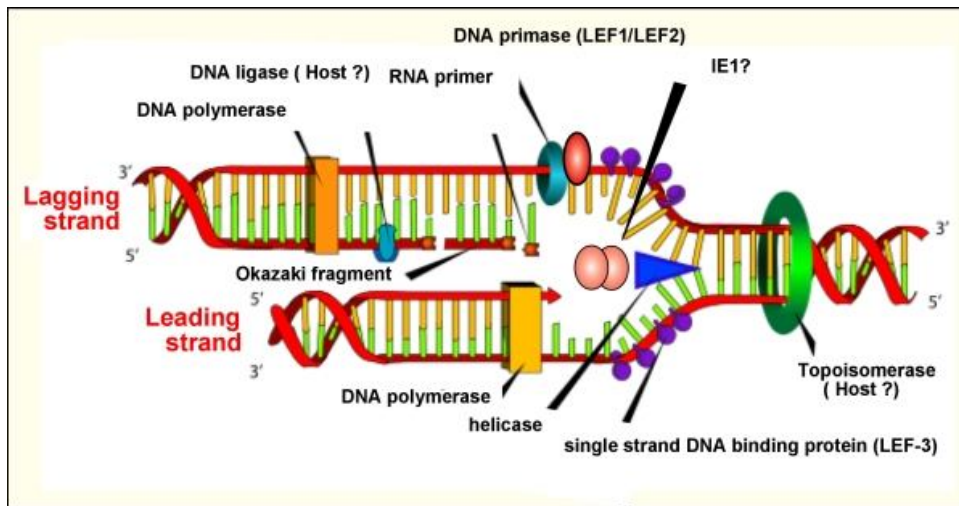


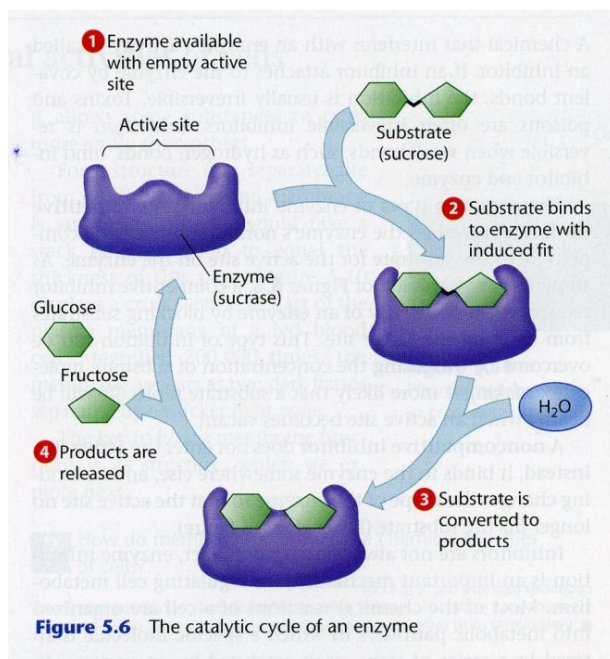
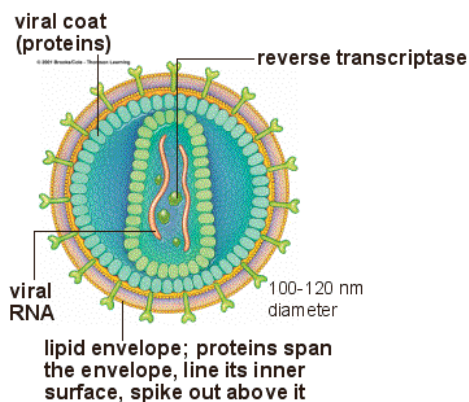
Figure. Restriction Endonucleases



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Enveloped Virus (HIV)



History:

1. The practical application and industrial use of enzymes to accomplish certain reactions apart from the cell dates back many centuries and was practiced long before the nature or function of enzymes was understood.
2. Use of barley malt for starch conversion in brewing, and of dung for bating of hides in leather making, are examples of ancient use of enzymes.
3. It was found that certain microorganisms produce enzymes similar in action to the amylases of malt and pancreas, or to the proteases of the pancreas and papaya fruit. This led to the development of processes for producing such microbial enzymes on a commercial scale.
4. Dr. Jokichi Takamine (1894, 1914) was the first person to realize the technical possibility of cultivated enzymes and to introduce them to industry. He was mainly concerned with fungal enzymes, whereas Boidin and Effront (1917) in France pioneered in the production of bacterial enzymes about 20 years later.

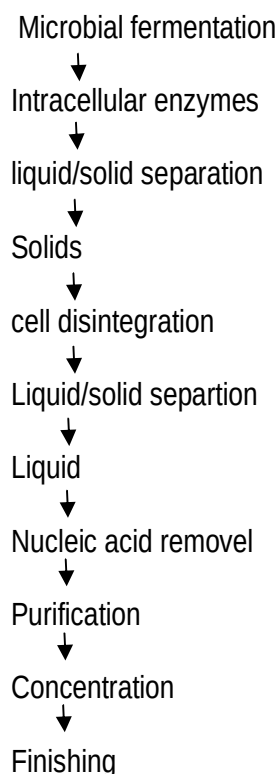
Production of microbial enzymes:

Enzymes occur in every living cell, hence in all microorganisms. Each single strain of organism produces a large number of enzymes, hydrolyzing, oxidizing or reducing, and metabolic in nature. But the absolute and relative amounts of the various individual enzymes produced vary

markedly between species and even between strains of the same species. Hence,

it is customary to select strains for the commercial production of specific enzymes which have the capacity for producing highest amounts of the particular enzymes desired. Commercial enzymes are produced from strains of molds, bacteria, and yeasts as shown in table:

The general plan for the production of microbial enzymes is as follow:



WHY MICROORGANISM ARE USED?

Microorganisms are preferred over plants and animals sources because:

- 1) They are usually cheaper to produce.
- 2) Their enzyme contents are predictable and controllable.
- 3) These can be modified genetically with less efforts.
- 4) Microbial enzymes are active at pH range of 3.0 to 9.0 whereas animal enzymes are active at pH 5.0 to work effectively.

- 5) Plant and animal tissues contain more harmful materials than microbes.

Some commercial enzymes and source microorganisms:

Source	Enzyme	Microorganism
Fungal	Amylases	<i>Aspergillus oryzae</i>
	Glucosidases	<i>Aspergillus flavus</i>
	Proteases	<i>Aspergillus niger</i>
	Pectinases	<i>Aspergillus niger</i>
	Glucose oxidases	<i>Penicillium notatum</i>
	Catalases	<i>Aspergillus niger</i>
Bacterial	Proteases	<i>Bacillus subtilis</i>
	Penicillanases	
Yeast	Invertases	<i>Saccharomyces cerevisiae</i>
	Lactases	<i>Saccharomyces fragilis</i>

Applications of microbial enzymes:

1) In bakery industry:

- o Bread making
- o Beer industry
- o Carbonated beverages industry
- o Chocolate, coffee, candies preparation
- o Fruit juices industry
- o Animal feed preparation
- o Other bakery products

2) Dairy industry:

- o Milk sterilization
- o Cheese production
- o Ice cream production

3) Leather industry

4) Pharmaceuticals:

- o Digestive aids like amylases, proteases, lipases
- o Wound debriment like streptokinase

Important microbial enzymes:

1) Lactase:

- Produced by *Saccharomyces fragilis*

- Enzyme used to degrade lactose into glucose and galactose
- Given to lactose intolerant patients
- Used in the production of ice cream and frozen desserts

2) **Amylase:**

- Found in a variety of organisms, including the saliva of humans
- *Aspergillus oryzae* (fungal) and *Bacillus subtilis* (bacterial)
- Sugar-digesting enzyme
- One of several enzymes used to manufacture food
- Used to create various syrups
- Degrades starch in order to produce corn syrup (fungal)

3) **Cellulase:**

- Enzyme produced by *E. coli* that degrades cellulose
- Widely used in the biotech industry, including:
 - Making animal food more easily digested
 - Makes faded jeans by digesting cellulose fibers in cotton
 - Processing of coffee beans
 - Fermentation processes to create biofuels

4) **Invertase:**

- Derived from *Saccharomyces cerevisiae*
- Also known as sucrase
- Enzyme digests sucrose into glucose and fructose
- Used to create candies with a soft center
- Most commonly used to make chocolate covered cherries

5) **Streptokinase and streptodornase:**

- Streptokinase is administered by intravenous or intra-arterial infusion in the treatment of thromboembolic disorders, e.g. pulmonary embolism, deep vein thrombosis and arterial occlusions. It is also used in acute myocardial infarction.

- A second enzyme, streptodornase, present in streptococcal culture filtrates, was observed to liquefy pus.
- Streptokinase and streptodornase are isolated following growth of non pathogenic streptococcal producer strains in media containing excess glucose.

6) **L-Asparaginase**

L-Asparaginase, an enzyme derived from *E. coli* or *Erwinia chrysanthemi*, has been employed in cancer chemotherapy where its selectivity depends upon the essential requirement of some tumours for the amino acid l-asparagine. Normal tissues do not require this amino acid and thus the enzyme is administered with the intention of depleting tumour cells of asparagine by converting it to aspartic acid and ammonia.

7) **Neuraminidase**

Neuraminidase derived from *Vibrio cholerae* has been used experimentally to increase the immunogenicity of tumour cells.

Future of microbial enzymes:

Industrial uses of enzymes have increased greatly during the past few years. Prospects are excellent for continuing increased usage of presently available enzymes in present applications, and in new uses, and of new enzymes for many purposes.

Enzymes have several distinct advantages for use in industrial processes:

1. They are of natural origin and are nontoxic.
2. They have great specificity of action; hence can bring about reactions not otherwise easily carried out.
3. They work best under mild conditions of moderate temperature and near neutral pH, thus not requiring drastic conditions of high temperature, high pressure, high acidity, and the like, which necessitate special expensive equipment.

4. They act rapidly at relatively low concentrations, and the rate of reaction can be readily controlled by adjusting temperature, pH, and amount of enzyme employed.
5. They are easily inactivated when reaction has gone as far as desired.

Because of these inherent advantages, many industries are keenly interested in adapting enzymatic methods to the requirements of their processes.

Clinical application of enzymes has been developing also. Proteolytic enzymes are used for debridement of wounds, and promising clinical results have been reported by injection of certain enzymes such as streptokinase, crystalline trypsin, and chymotrypsin. Since many physical ailments result from derangement of metabolic enzyme systems, increased therapeutic use of enzymes, presently unpredictable, may be expected.

For clinical and therapeutic uses, highly purified and perhaps crystalline enzymes will be necessary. Availability of high purity enzymes on an industrial scale is just beginning, and rapid advances in this field may be expected.

Currently much enzyme research is underway by various industries.

IMMOBILIZED ENZYMES

An immobilized enzyme is an enzyme that is attached to an inert, insoluble material such as calcium alginate (produced by reacting a mixture of sodium alginate solution and enzyme solution with calcium chloride). This can provide increased resistance to changes in conditions such as pH or temperature. It also allows enzymes to be held in place throughout the reaction, following which they are easily separated from the products and may be used again - a far more efficient process and so is widely used in industry for enzyme catalysed reactions. An alternative to enzyme immobilization is whole cell immobilization.

Commercial use

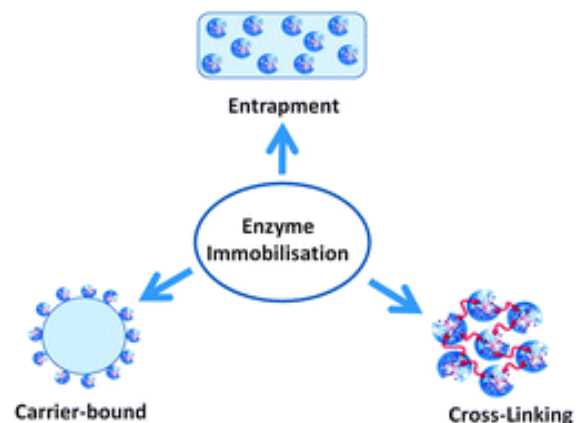
Immobilized enzymes are very important for commercial uses as they possess many benefits to the expenses and processes of the reaction of which include:

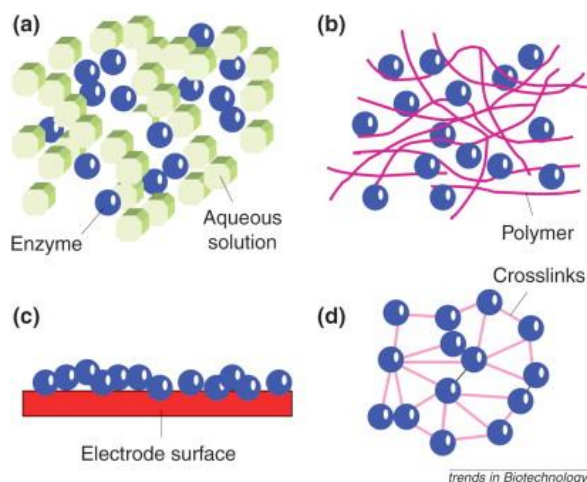
Convenience: Minuscule amounts of protein dissolve in the reaction, so workup can be much easier. Upon completion, reaction mixtures typically contain only solvent and reaction products.

Economy: The immobilized enzyme is easily removed from the reaction making it easy to recycle the biocatalyst. This is particularly useful in processes such as the production of Lactose Free Milk, as the milk can be drained from a container leaving the enzyme (Lactase) inside ready for the next batch.

Stability: Immobilized enzymes typically have greater thermal and operational stability than the soluble form of the enzyme.

In the past, biological washing powders and detergents would contain many proteases and lipases which would break down dirt. However, when the cleaning products would come into contact with the skin, it would create allergic reactions. This is why immobilization of enzymes are important, not just economically.



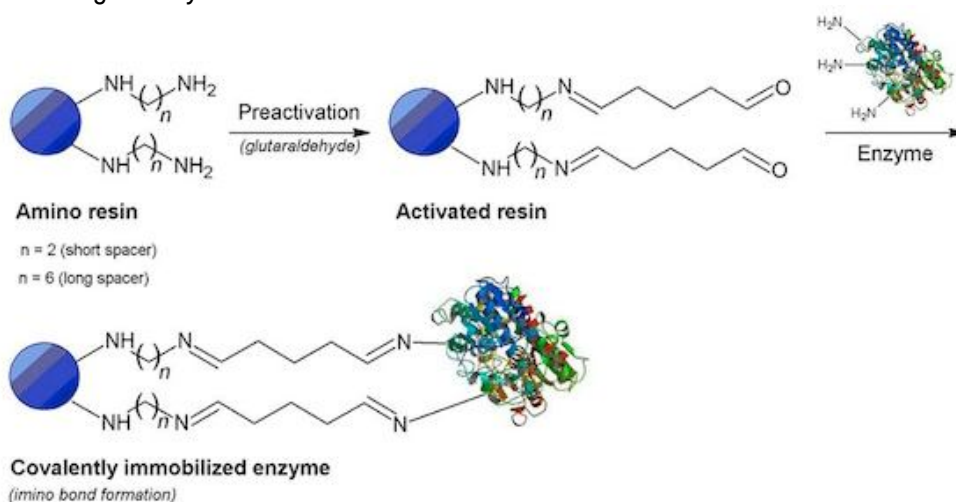


However, in most of the processes, enzymes are mixed in a solution with substrates and cannot be economically recovered after the reaction and are generally wasted. Thus, there is an incentive to use enzymes in an immobilized or insolubilized form so that they may be retained in a biochemical reactor for further catalysis. This is done by Enzyme immobilization which may be defined as "The process whereby the movement of enzymes, cells, organelles, etc. in space is completely or severely restricted usually resulting in a water-insoluble form of the enzyme."

Immobilized enzymes are also sometimes referred to as sound, insolubilized, supported or matrix-linked enzymes.

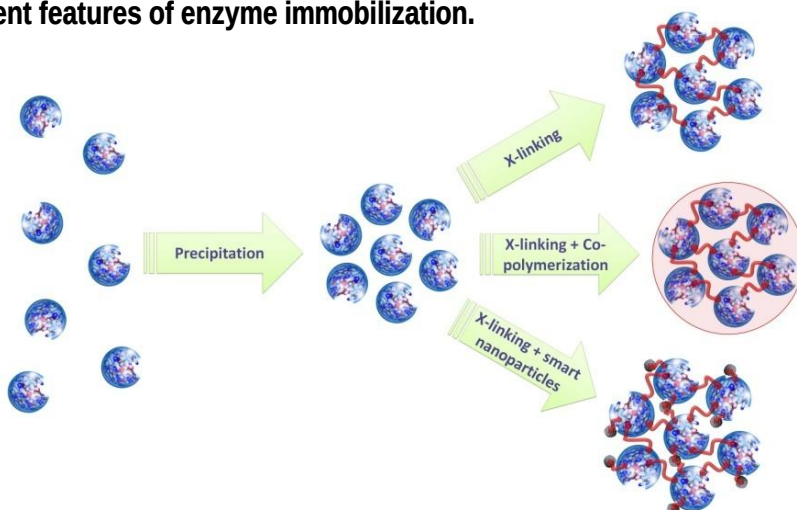
Enzyme immobilization

As enzymes are biological catalysts that promote the rate of reactions but are not themselves consumed in the reactions; they may be used repeatedly for as long as they remain active.



PolyLink Amine Linker Kit with Columns and Reagents for Enzyme Immobilization

Salient features of enzyme immobilization.



The enzyme phase is called as carrier phase which is water insoluble but hydrophilic porous polymeric matrix, e.g. agarose, cellulose, etc.

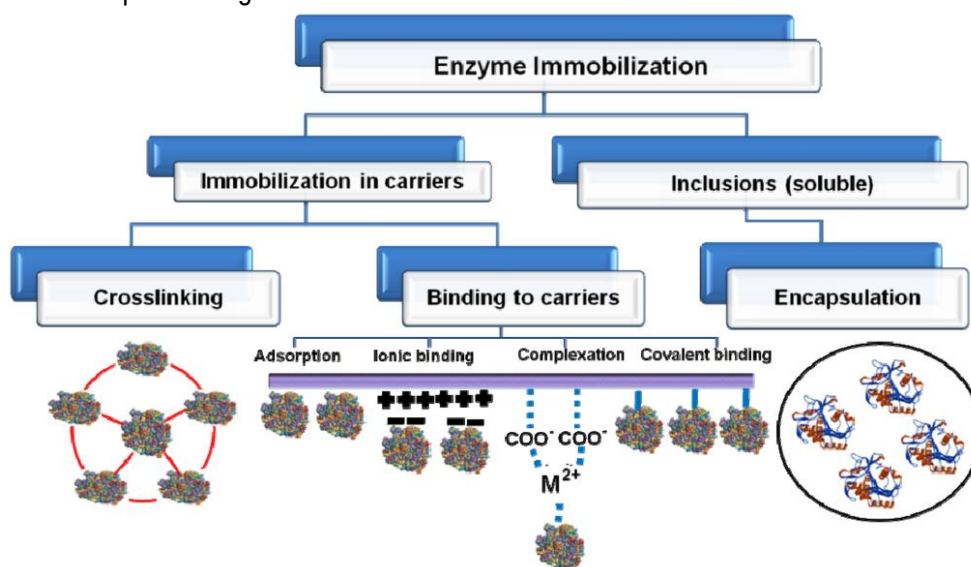
The enzyme phase may be in the form of fine particulate, membranous, or microcapsule.

The enzyme in turn may be bound to another enzyme via cross linking.

A special module is produced employing immobilization techniques through which fluid

can pass easily, transforming substrate into product and at the same time facilitating the easy removal of catalyst from the product as it leaves the reactor.

The support or carrier utilized in immobilization technique is not stable at particular pH, ionic strength, or solvent conditions. Hence, may be disrupted or dissolved releasing the enzyme component after the reaction.



Advantages of enzyme immobilization:-

- Multiple or repetitive use of a single batch of enzymes.
- Immobilized enzymes are usually more stable.
- Ability to stop the reaction rapidly by removing the enzyme from the reaction solution.
- Product is not contaminated with the enzyme.
- Easy separation of the enzyme from the product.
- Allows development of a multienzyme reaction system.
- Reduces effluent disposal problems.

Disadvantages of enzyme immobilization:-

It gives rise to an additional bearing on cost.

It invariably affects the stability and activity of enzymes.

The technique may not prove to be of any advantage when one of the substrate is found to be insoluble.

Certain immobilization protocols offer serious problems with respect to the diffusion of the substrate to have an access to the enzyme.

Technique of enzyme immobilization

1. Carrier binding.
 - i. Physical adsorption.
 - ii. Covalent bonding.
 - iii. Ionic bonding.
2. Cross linking.
3. Entrapment.
 - iv. Occlusion within a cross linked gel.
 - v. Microencapsulation.

Physical adsorption

This method is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers. Examples of suitable adsorbents are ion-exchange matrices, porous carbon, clay, hydrous metal oxides, glasses and polymeric aromatic resins.

Covalent bonding

Covalent binding is the most widely used method for immobilizing enzymes. The covalent bond between enzyme and a support matrix forms a stable complex. The functional group present on enzyme, through which a covalent bond with support could be established, should be non essential for enzymatic activity.

The most common technique is to activate a cellulose-based support with cyanogen bromide, which is then mixed with the enzyme.

The protein functional groups which could be utilized in covalent coupling include:

Amino group

Carboxylic group

Phenol ring

Indole group

Imidazole group

On the other hand examples of the polymeric supports include:

- Amino and related groups of polysaccharides and silica gel etc.
- Carboxylic acid and related groups of polyglutamic acid, carboxy methyl cellulose.
- Aldehyde and acetal groups of polymers.
- Amide gr. Of polypeptide.

The polymers may be engaged in direct coupling as well as could be modified by other coupling groups or activating groups. The most commonly used polymers are polysaccharides, polyvinyl alcohol, silica and porous glasses.

Cross linking

This method is based on the formation of covalent bonds between the enzyme molecules, by means of multifunctional reagents, leading to three dimensional cross linked aggregates.

The most common reagent used for cross-linking is glutaraldehyde.

Entrapment

In entrapment, the enzymes or cells are not directly attached to the support surface, but simply trapped inside the polymer matrix. Entrapment is carried out by mixing the biocatalyst into a monomer solution, followed by polymerization initiated by a change in temperature or by a chemical reaction.

Polymers like polyacrylamide, collagen, cellulose acetate, calcium alginate or carrageenan etc are used as the matrices.

1. Occlusion within a cross linked gel

In this entrapment method, a highly cross-linked gel is formed as a result of the polymerization which has a fine "wire mesh" structure and can more effectively hold smaller enzymes in its cages.

Amounts in excess of 1 g of enzyme per gram of gel or fibre may be entrapped.

Some synthetic polymers such as polyarylamide, polyvinylalcohol, etc... and natural polymer (starch) have been used to immobilize enzymes using this technique.

2. Microencapsulation

This entrapment involves the formation of spherical particle called as "microcapsule" in which a liquid or suspension of biocatalyst is enclosed within a semi permeable polymeric membrane.

MONOCLONAL ANTIBODIES

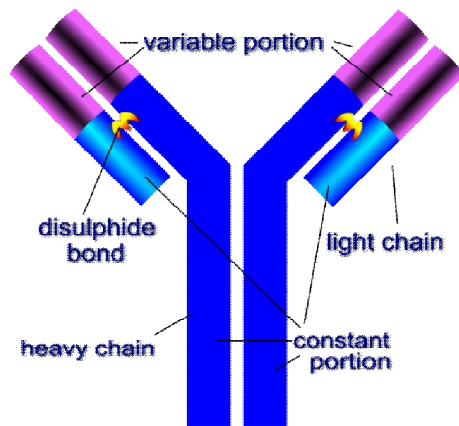
Chapter 4.

Principles, synthesis and applications

Antibodies are highly specific, extremely sensitive molecules of human immune system.

or

Soluble proteins that help to defend against foreign entities such as microorganisms.



Important Terms

Antibody – immunoglobulin secreted by B cells

Antigen (*antibody generator*) – any substance capable of eliciting an immune response

Monoclonal antibodies (mAbs) – antibodies secreted from a single B cell line, have identical paratopes

Epitope – region of the antigen recognized by an antibody

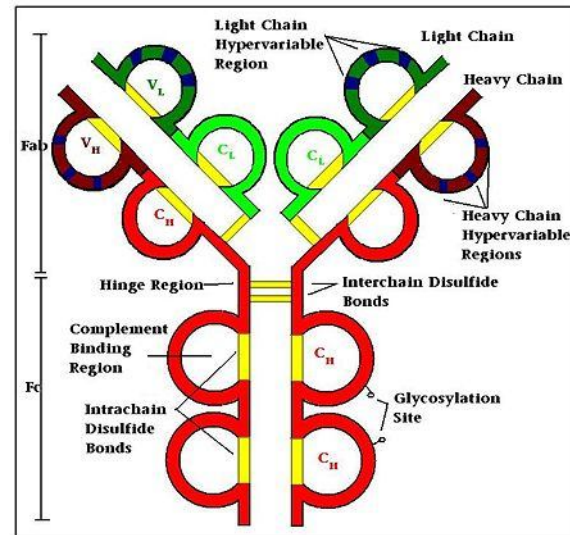
Paratope – region of the antibody that binds the epitope.

The Structure of an Antibody

- o identical light chains (~220 amino acids long)
- o Variable domain: V_L
- o Constant domain: C_L
- o identical heavy chains (~440 amino acids long)
- o Variable domain: V_H
- o 3 Constant domains: C_H1 , C_H2 , C_H3

Covalent, disulfide bonds between cysteine residues

Flexible “hinge region”



Major Components of Immune System

Specific Immunity

- Cell mediated immunity
- Antibody mediated immunity

Non-specific Immunity

- Complement system
- Phagocytosis

Type of Response of Immune System

Two types of response occur:

Humoral Immunity:

(antibody mediated immunity) It is mediated through B lymphocyte through the production of antibodies.

Cell mediated Immunity:

It is mediated by T lymphocytes through the production of lymphokine (directly react with foreign particles).

Origin of t and b cells

T and B cells are derived from lymphocytes, which are one of the classes of WBCs.

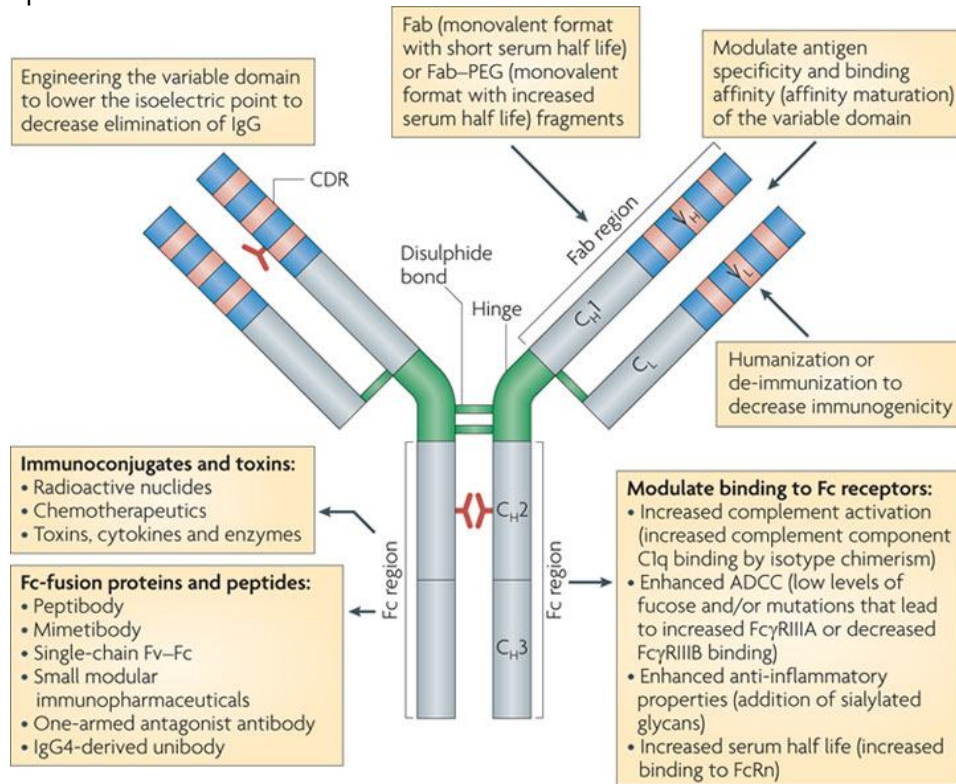
Thymus derived lymphocytes are called as T cells.

Bone marrow derived lymphocytes are called as B cells.

Normal immune response

A normal immune response usually involves the response and proliferation of numerous B cell

clones, bearing Antibodies with varying degrees of specificity for the different epitopes contained within the antigen. Thus the immune response is normally **polyclonal**.



Nature Reviews | Immunology

Why Polyclonal response?

Because antigens have multiple epitopes which stimulate a series of B cells resulting in multiple clones of plasma cells that synthesize the antibody of different specificities. That is why response is polyclonal.

Definition of mAb

Monoclonal antibodies are antibodies that are identical and derived from one type of immune cell, each a clone of a single parent cell. **or**

Antibodies that are produced by a single antigenic determinant molecule, are called monoclonal antibodies.

History of mAb

Monoclonal antibody technique was devised by Köhler and Milstein (for which they shared a Nobel Prize in 1984).

They developed the methods by which large amounts of a single monoclonal antibody specific for one epitope can be obtained.

General consideration

Greater than 20 monoclonal antibodies have been approved by the FDA and are being increasingly used to treat autoimmune and neoplastic disorders.

With recent improvements in techniques involved in the production of mAb, the stage is set for science to take yet another leap forward. Greater

than 200 monoclonal antibodies are currently in development or are awaiting FDA approval.

Origin of mAb

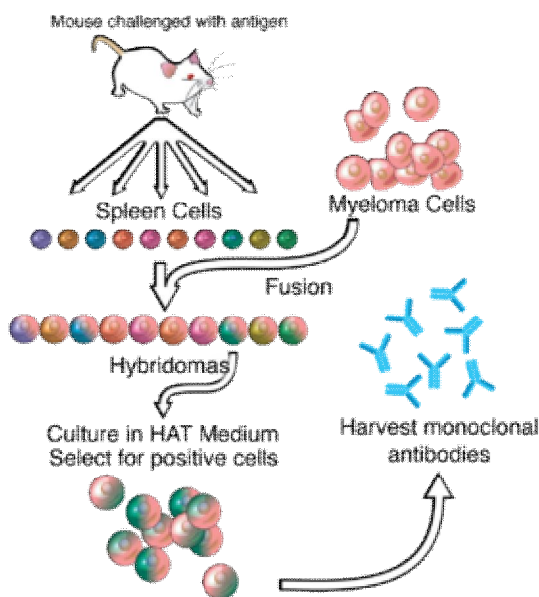
- mAb of Murine Origin
- mAb of Human Origin

mAb of murine origin

Murine mAbs are obtained from murine hybridomas produced by fusion of B-lymphocytes from immunized mice or rats with murine myeloma cells.

A general problem with the therapeutic use of murine monoclonal antibodies in man is the possible induction of antibodies in the recipient against murine immunoglobulin (human anti murine antibody or HAMA response).

This may result in adverse reactions and limit the duration of effective antibody therapy. In addition the in vivo half life of murine monoclonal antibodies is relatively short.



Human mAb

The advantages of human monoclonal antibodies over murine monoclonal antibodies are that human recipients are less likely to develop antibodies against them and that

human antibodies are likely to have the full range of biological functions.

Production of Monoclonal Antibodies

Now-a-days mab are produced by following techniques:-

- A) HYBRIDOMA TECHNOLOGY
- B) EBV Immortalization technique
- C) EBV Hybrid technique

A) HYBRIDOMA TECHNOLOGY

Hybridoma technology is a technology of forming hybrid cell lines (called hybridomas) by fusing a specific antibody-producing B cell with a myeloma (B cell cancer) cell that is selected for its ability to grow in tissue culture.

The antibodies produced by the hybridoma are all of a single specificity and are therefore monoclonal antibodies (in contrast to polyclonal antibodies).

INVENTION OF HYBRIDOMA TECHNOLOGY

The production of monoclonal antibodies was invented by Cesar Milstein and Georges J. F. Köhler in 1975. They shared the Nobel Prize of 1984 for Medicine and Physiology with Niels Kaj Jerne, who made other contributions to immunology. The term hybridoma was coined by Leonard Herzenberg during his sabbatical in Cesar Milstein's laboratory in 1976/1977.

STEPS OF HYBRIDOMA TECHNOLOGY

1. Immunisation of a mouse
2. Isolation of B cells from the spleen
3. Cultivation of myeloma cells
4. Fusion of myeloma and B cells
5. Separation of cell lines
6. Screening of suitable cell lines
7. in vitro (a) or in vivo (b) multiplication
8. Harvesting

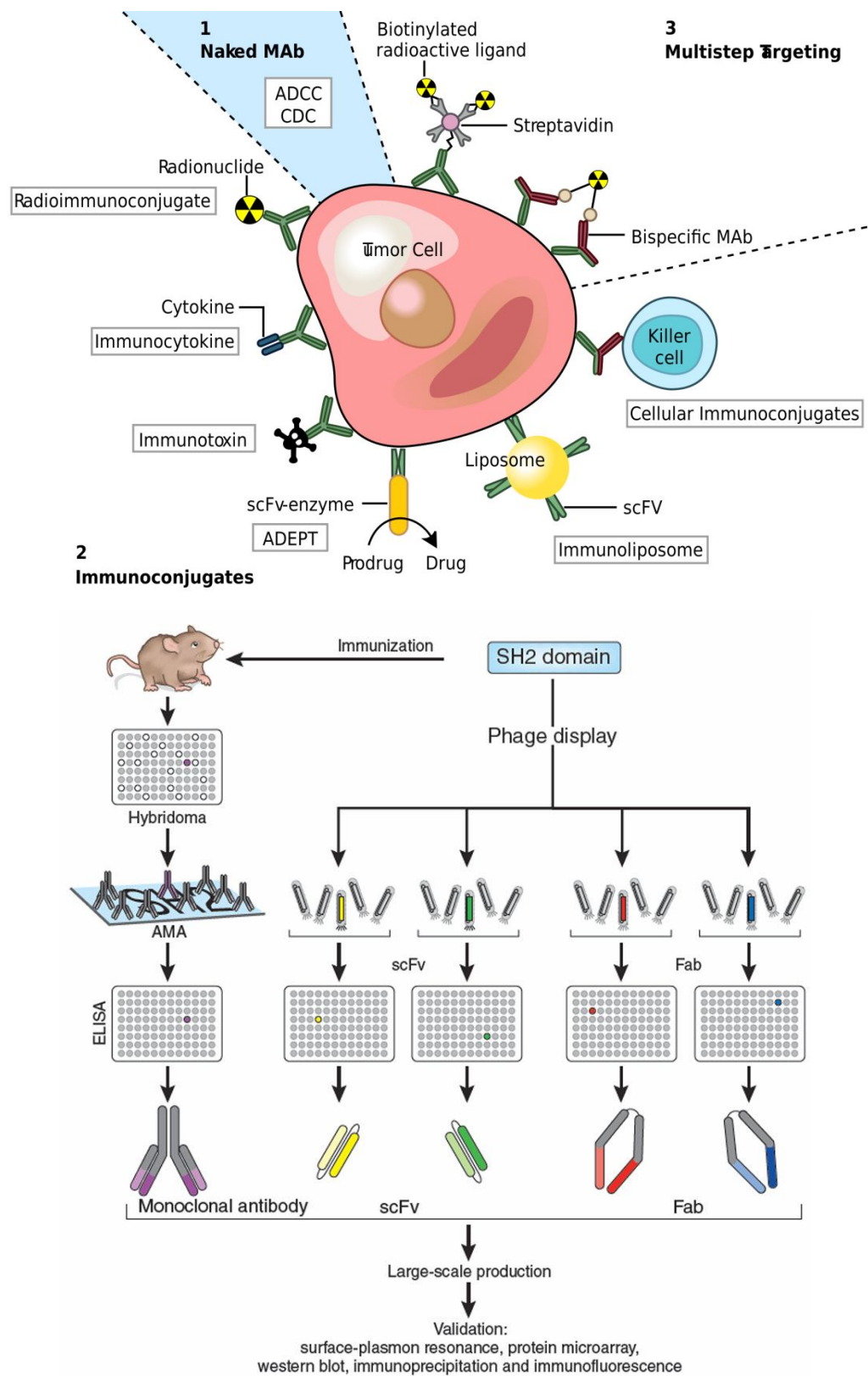


Figure. Monoclonal antibodies were generated by hybridoma technology; the production of monoclonal antibodies was controlled by an antigen microarray assay (AMA)

1. IMMUNIZATION OF A MOUSE

Immunization of animals with immunogens is performed by injecting microgram or milligram quantities of immunogen mixed with an adjuvant (aluminum salts, Freund's complete or incomplete adjuvant), intradermally or subcutaneously at multiple sites repeatedly at different times

2) Isolation of B cells from the spleen .

After the several week of immunization, blood sample are obtained from the mice for the measurement of the serum antibodies. Serum anti body titer is determined by Enzyme Linked immunosorbant Assay (ELISA). If the titer is high the cell fusion can be performed. If the titer is too low, mice can be boosted until the an adequate response is achieved. When the antibody titer is high enough, the mice is commonly boosted b y injecting antigen without adjuvant 3 days before the fusion but 2 weeks after the previous immunization. When the concentration of antibodies is found to be nearly optimal , the animal is sacrificed and the spleen , which contains a large number of plasma cells , is dissociated into single splenocytes by mechanical and/or enzymatic method.

3) Cultivation of myeloma cells

The myeloma cells are selected beforehand to ensure they are not secreting antibody themselves and that they lack the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene. Genetic machinery of myeloma cells are modified as Ig⁺, HGPRT⁻ .

(4) Fusion of myeloma and B cells

The fusion is accomplished using polyethylene glycol or the Sendai virus. It is performed by making the cell membranes more permeable.

5) Separation of cell lines

Removal of the unfused myeloma cells is necessary because they have the potential to outgrow other cells, especially weakly established hybridomas. After fusing the cells , they are placed in a selection medium of HAT (hypoxanthine-aminopterin-thymidine medium)

for roughly 10 to 14 days. . Aminopterin inhibits de novo nucleotide biosynthesis. When de novo synthesis inhibited cells can use salvage pathway to produce nucleotides for that they require HGPRT and TK. Since one of this enzyme is absent in B cells(TK⁻) and one is absent in myeloma cells(HGPRT⁻) , they are unable to survive in the selection medium. Those hybrid cells which are having functional HGPRT and TK are found to be capable of growing in selection medium.

6. Screening of suitable cells

The surviving hybridoma cells would have formed against different epitopes. The next step is to select the hybridoma produced against the desired antigen. The cultures are diluted to such an extent that only a single cell gets transferred to the wells of microtitre plate. The cells are allowed to multiply. These cells produce antibodies that can be readily detected in their supernatant fluids. Supernatant fluids from all the wells are tested for antibodies against the antigen of choice. Finally, a hybridoma cell producing antibodies against the epitope of choice is available. screening is done by ELISA or RIA.

(7) in vitro (a) or in vivo (b) multiplication

It is nothing but culturing or allowing selected clone cells to grow in invitro or invivo conditions respectively.

a) In in vitro technique clone cells are cultured in tissue culture flask with suitable medium. The production rate is found to be 10 – 100micrograms per ml.

b) In in vivo method, selected clones are injected into the peritoneal cavity of histocompatible mice and allowed to multiply and produce antibody. The rate of antibody production is 25 milligram per ml.

Damon Biotech Company encapsulates hybridomas in alginate gels, which allow nutrients to flow in and waste products and antibodies to flow out. In these capsules , a much higher concentration of hybridoma cells can be achieved than in tissue culture, as a result 100 fold greater

yield of antibody production has been obtained. Another approach has been used by Cell Tech in UK. In this method hybridoma are grown in 100 liter fermenters, which yield 100gm of monoclonal antibodies in a week period.

(8) Harvesting

In either of above methods, samples are collected and from these monoclonal antibodies are separated by affinity chromatography and they are used for different purposes.

Long Term Maintenance And Cryopreservation of Mabs:-

The selected hybridomas are cryo-preserved in liquid nitrogen in ampoules which can be thawed for use in future use. the cryo-preserved hybridomas are thawed and recultured when fresh antibodies are required. For long term maintenance. It is always desirable to evaluate the quality of product because there may be some deterioration.

A) EBV Immortalization technique

EBV Immortalization technique is based on use of Epstein-Barr virus to immortalize antigen specific human B lymphocytes. However, EBV transformed cells can't grow indefinitely because they are not malignant cells, so difficult to clone and produce low yield of immunoglobins.

B) EBV Hybrid technique

It is the new technique, that is the combination hybridoma technique and EBV immortalization technique.

Other types of mabs

The immune system of patients react against the foreign mouse proteins, leading to rash, swelling, and even occasional kidney failure, plus the structure of the Mabs. In recognition of this problem researchers are developing new generation of Mabs that are less likely to cause side effects due to their foreignness. Researchers are exploring several approaches such as chimeric Mabs and humanized Mabs.

Chimeric monoclonal antibodies use genetically manipulated mice to make human murine hybrid.

The variable part of the antibody molecule, including the antigen-binding sites is murine. The remainder of the antibody molecules, the constant region, has been derived from a human source. These Mabs are 66% human. An example is Rituximab

Humanized monoclonal antibodies are constructed so that the murine portion is limited to the antigen-binding sites. The balance of the variable region and all of the constant region are derived from human sources. Such Mabs are about 90% human. An example is Demtuzumab.

The eventual goal is to develop fully human antibodies. One approach is to genetically engineer mice to contain human antibody genes. Other approach is to entirely avoid the use of mice by using bacteriophages, to insert desired genes into bacteria, which would be able to produce the desired mabs. Milk of genetically engineered animal and genetically altered plants are also the source of Mabs on large scale production.

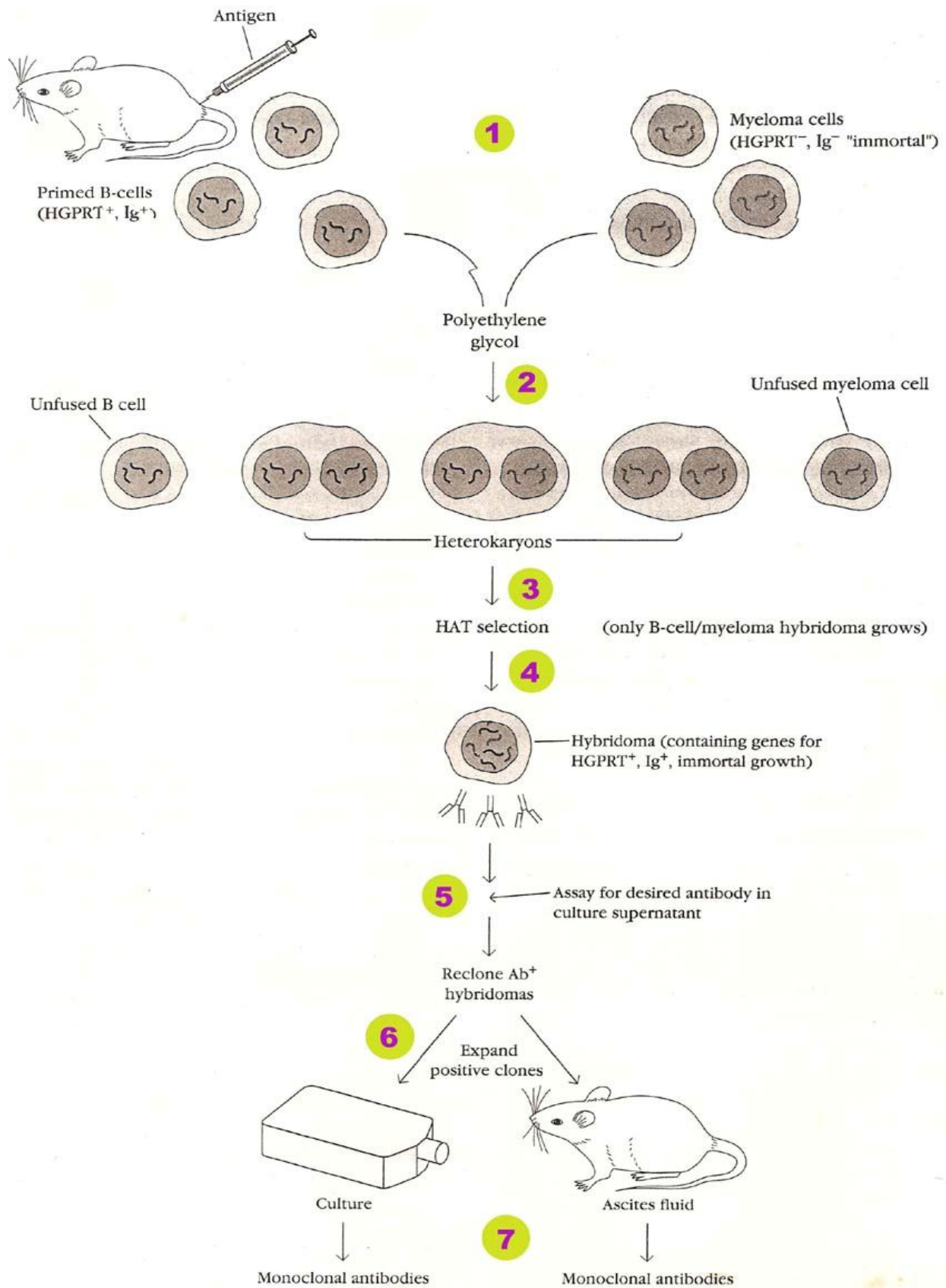
APPLICATION OF MONOCLONAL ANTIBODIES

The application of monoclonal has been evaluated for more than 10 years. The use of monoclonal antibodies falls mainly under diagnostic, therapeutic and catalytic categories. The earliest studies were focused on cancer therapy, but now a day's use of monoclonal antibodies to also another human disease. Some monoclonal antibodies are now commercially products under development and many others are still undergoing evaluation.

Clinical application of monoclonal antibodies

- Diagnostic applications
- Therapeutic application

For both of these applications localization of monoclonal antibodies is very important.



Many factors involve in localization of monoclonal antibody. For effective localization, the monoclonal antibodies should have a right affinity for the target antigen. The cross reactivity should be minimized with other non targeted tissue. The class and sub class of antibody effect the biodistribution blood clearance and interaction with host immune system. Human MAB have a lower rate of blood clearance. The blood clearance is depending upon the molecular size.

If the molecular weight is lower the clearance is faster. The fragment MAB have a lesser half life than the intact body. Due to smaller size the fragments can diffuse into tissue. But for a reaction with immune effectors function the MAB should be intact.

The dose of antibody also has an effect on clearance rate. If the dose is high (more than 10mg) saturation of non specific sites in live and other organ occur, there by reducing the clearance rate.

Routes of administration, other than the IV route also alter the biodistribution of MAB of the antibody.

Diagnostic application of MAB

MAbs are utilized in diagnostic kits for the diagnosis of various infectious diseases, monitoring drug levels, Detecting pregnancy matching histocompatibility antigen detecting diabetes, cancer and in immunoscintigraphy.

Radiolabelled MAB are used in diagnostic purpose. The technique is called the immunoscintigraphy. In this technique a planer gamma camera is used to detect the distribution of gamma emitting radioisotopes conjugated with MAB in a two dimensional manner.

The imaging has been applied successfully for cardiovascular disease infection disease and cancer disease.

FDA licensed a new diagnostic imaging agent that can determine the extent of disease in patients diagnosed with small cell lung cancer (SCLC).

Because these agents can detect tumor in different part of the body at one time, it can help physician to advice certain patients with advanced forms of the disease about treatment option without requiring further diagnostic tests.

The new agent, NofetumomAb, is a fragment of a monoclonal antibody that when tagged with the radioisotope technique, can detect a protein found on the surface of most small lung cancer cells.

1. Cardiovascular disease

MAB is used in myocardial infraction disease Myoscint is the first MAB based imaging agent in market in much European country. The product consists of a kit containing 0.5 mg of antimyocin fab fragment conjugate with chelator DTPA (diethyl triamine penta acetic acid) this is labeled by mixing with (indium chloride) after incubation of 10 minute. In labeled MAB is ready for IV injection. Imaging is done after 24-28 hours. About 600 patients have been tested without adverse effect. The product has high degree of sensitivity and can detect location and extent of necrotic heart function.

2- Infectious Disease

MABs are being tried to image the sites of infection. Antibodies directed against bacterial antigen have proven successfully. Inflammatory leukocytes which accumulate at the site of infection have shown high sensitivity and specificity for detection of localized infection.

3- Cancer

MABs are being evaluated for detection of different type of cancer like breast carcinoma, ovarian carcinoma, and lung carcinoma. These MABs can be targeted against many type of tumor.

Monoclonal antibodies for cancer treatment

- Possible treatment for **cancer** involves monoclonal antibodies that bind only to cancer cells specific **antigen** and induce immunological response on the target cancer cell (naked antibodies). mAb can be modified for delivery of [toxin], radioisotope, cytokine.

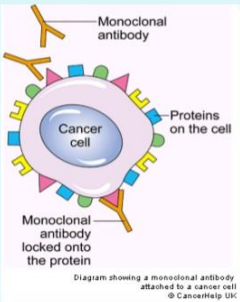


Diagram showing a monoclonal antibody attached to a cancer cell
© Cancerhelp, UK

Therapeutic applications

Improving the out come of bone marrow transplantation by using CD52 MAbs to prevent Graft-Versus-Host disease and Graft rejection.

AlemtuzumAb is the monoclonal antibody used for this purpose.

Graft-versus-Host Disease (GVHD) is a major cause of mortality and morbidity after allergenic bone marrow transplantation, but can be avoided by removing T-lymphocytes from the donor bone marrow.

However, T-cell depletion increases the risk of graft rejection. This study examined the use of CD52 MAb to eliminate T- cells from both donor marrow and recipient to prevent both GVHD and rejection.

Catalytic applications

The antibodies are extremely efficient at binding ground states of the target molecule while enzymes obtained their catalytic efficiency from tight binding of the transition state for the reaction. Thus antibodies can be made efficient catalysts if they are made for reaction transition state. Lemer and his co-workers explored the probability of enzyme like action of antibodies by producing hapten-carrier complex where the hapten structurally resembled transition state and anti-hapten MAbs generated, gave catalytic activity.

- The hydrolysis of substrate increased thousand fold after incubation.
- ABZYMES are MAb used for this purpose.

CULTURE MEDIA

Chapter 5.

Classification, preparation and uses

A laboratory procedure in which a sample from a wound, the blood or other body fluid is taken from an infected person. The sample is placed in conditions under which bacteria can grow. If bacteria grow, identification tests are done to determine the bacteria species causing the infection.



Mentioned in: Animal Bite Infections, Legionnaires' Disease, Mycobacterial Infections, Atypical, Pleural Effusion, Prostatitis, Sexually Transmitted Diseases Cultures, Sputum Culture

Culture medium or growth medium is a liquid or gel designed to support the growth of microorganisms. There are different types of media suitable for growing different types of cells. Here, we will discuss microbiological cultures used for growing microbes, such as bacteria or yeast.

Culture

The culture is an extension of microorganisms or of living tissue cells in media conducive to their growth.

Cell culture - a growth of cells in vitro; although the cells proliferate they do not organize into tissue.

Continuous flow culture - the cultivation of bacteria in a continuous flow of fresh medium to maintain bacterial growth in logarithmic phase.

Hanging-drop culture - a culture in which the material to be cultivated is inoculated into a drop of fluid attached to a coverglass inverted over a hollow slide.

Plate culture - one grown on a medium, usually agar or gelatin, on a Petri dish.

Primary culture - a cell or tissue culture started from material taken directly from an organism, as opposed to that from an explant from an organism.

Pure culture - a culture of a single cell species, without presence of any contaminants.

Slant culture - one made on the surface of solidified medium in a tube which has been tilted to provide a greater surface area for growth.

Stab culture - one in which the medium is inoculated by thrusting a needle deep into its substance.

Streak culture - one in which the medium is inoculated by drawing an infected wire across it.

Suspension culture - a culture in which cells multiply while suspended in a suitable medium.

Tissue culture - maintenance or growth of tissue, organ primordia, or the whole or part of an organ in vitro so as to preserve its architecture and function.

Type culture - a culture of a species of microorganism usually maintained in a central collection of type or standard cultures.

Classification

The bases for classifications of culture media are as under;

- A. Consistence
 - a. Solid
 - b. Semi solid/ agar
 - c. Liquid/ broth
- B. Chemistry
 - a. Lowenstein Jensen
 - b. Beta galactosidase substrate
 - c. Peptone
- C. Micro-organisms
 - a. Bacterial
 - b. Fungal
 - c. Algae
- D. Anti bacterial activities
- E. Characteristic/ pattern of microbial growth
 - a. Selective
 - b. Non selective
 - c. Non-differential
- F. Objective of experiments
 - a. Drug resistance
 - b. Research
 - c. Commercial i.e. MIC, MBC etc
 - d. Academic

Types of culture media:

- 1) Simple media
- 2) Enriched media
- 3) Selective media
- 4) Enrichment media
- 5) Indicator media
- 6) Selective and indicator media

Three Categories of Media Classification		
Physical State (Medium's Normal Consistency)	Chemical Composition (Type of Chemicals Medium Contains)	Functional Type (Purpose of Medium)*
1. Liquid	1. Synthetic	1. General purpose
2. Semisolid	(chemically defined)	2. Enriched
3. Solid (can be converted to liquid)	2. Nonsynthetic	3. Selective
4. Solid (cannot be liquefied)	(not chemically defined)	4. Differential
		5. Anaerobic growth
		6. Specimen transport
		7. Assay
		8. Enumeration

*Some media can serve more than one function. For example, a medium such as brain-heart infusion is general purpose and enriched; mannitol salt agar is both selective and differential; and blood agar is both enriched and differential.

Types of Culture Media

Type of Media	Purpose
Chemically Defined	Growth of chemoheterotrophs and photoautotrophs: microbiological assays
Complex	Growth of most chemoheterotrophic organisms
Reducing	Growth of obligate anO ₂
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.

e.

Major Culture Media

Most aerobic species of bacteria (those that can grow in the ambient 20% O₂ found in earth's atmosphere), will grow on one or more of the media types below.

Solid media commonly contain 1.5% agar per weight to solidify the liquid, nutrient part of the media. After sterilization, the liquid media is poured into sterile Petrie plates - think of it along the lines of pouring heated yellow into small cylindrical molds.. Petrie plates are commonly made of plastic, and can be purchased already sterilized with 25 or more per package.



Luria Bertani Broth (LB)

Uses: Culture of aerobic bacterial species

Table. The Micro-organisms and there respective culture media.

<u>Microorganism</u>	<u>Type of culture media</u>
General (used to grow most urine-related microorganisms)	Blood agar ¹ (to determine how many type of colonies is present for that specimen)
<i>Escherichia coli</i>	MacConkey's agar or Eosin Methylene Blue (EMB) ⁵ agar
<i>Staphylococcus saprophyticus</i>	Mannitol Salt agar ⁷
<i>Enterococci</i>	Bile esculin agar ^{1, 6}
<i>Proteus mirabilis</i>	Potassium cyanide medium (KCN) ¹
<i>Klebsiella pneumoniae</i>	MacConkey's agar or Eosin Methylene Blue (EMB) ²
<i>Enterobacter</i>	Tryptic Soy Broth agar ³
<i>Candida albicans</i>	Sabouraud's agar ⁴

Recipe: 10g Tryptone; 5g Yeast Extract; 10g NaCl; 1L distilled water
pH to ~7.3-7.5.
Distribute 5-10mL into glass tubes with autoclavable tops.
Autoclave 30 minutes, OR place in a pressure cooker for ~45 minutes @ 15 pounds pressure.

Uses: Selective media for Gram-negative, enteric bacteria (bile tolerant). Assess lactose fermentation

Recipe: 10g lactose; 20g peptone; 5g bile salts; "pinch" or small amount of 1mg/mL Bromocresol purple or Crystal violet solution; 15g agar; 1L distilled water.



LB-Agar

Uses: Culture of aerobic bacterial species on solid media
Same as for LB-broth, but add 15g of agar prior to autoclaving.
Be certain to mix thoroughly prior to pouring media into Petrie plates.



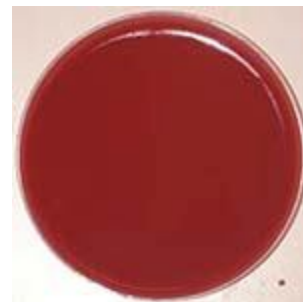
MacConkey Agar



Sheep's Blood Agar

Uses: Non-selective media, good for culturing many bacterial species including Gram negative and Gram-positive species including Staphylococci and Streptococci

Recipe: 5% anticoagulated Sheep's blood; Tryptose Soy nutrient agar base (contains agar in it); 1L of water.

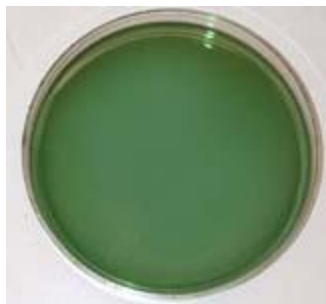


Chocolate Agar

Uses: Very enriched media with lysed red cells, to release many nutrients needed by *fastidious* organisms.

It may look like chocolate, but would have a rather nasty flavor, like coagulated blood-flavored jello.

Recipe: Recommend commercially produced media.



Hektoen Enteric Agar

Uses: Selective media for Gram-negative enteric bacteria (higher bile salt concentrations than MacConkey).

Used to assess lactose fermentation AND ability to produce hydrogen sulfide (H₂S). Allows for distinguishing among, E. coli, Salmonella and other gut pathogens including Shigella species
Recipe: Recommend commercially produced media.

If bought in powder form, must heat over heating plate prior to pouring plates. Do not autoclave

Mueller Hinton Agars

ttMueller Hinton Agar • Mueller Hinton II Agar

Mueller Hinton Agar with 5% Sheep Blood

Class:

They have few properties that make them excellent for antibiotic use. They are non-selective, non-differential media. So they can be used for all type of general media.

Intended use:

Mueller Hinton Agar is recommended for antimicrobial disc diffusion susceptibility testing of common, rapidly growing bacteria.

Mueller Hinton Agar with 5% Sheep Blood is recommended for antimicrobial disc diffusion susceptibility testing of *Streptococcus pneumoniae* with selected agents; i.e. chloramphenicol, erythromycin, ofloxacin, tetracycline and vancomycin.

Ingredients:

Mueller Hinton Agar

- Beef Extract Powder
- Acid Digest of Casein
- Starch
- Agar

Mueller Hinton II Agar

- Beef Extract
- Acid Hydrolysate of Casein
- Starch
- Agar

Role of ingredients:

Acid hydrolysate (digest) of casein and beef extract supply amino acids and other nitrogenous substances, minerals, vitamins, carbon and other nutrients to support the growth of microorganisms. Starch acts as a protective colloid against toxic substances that may be present in the medium. Hydrolysis of the starch during autoclaving provides a small amount of dextrose, which is a source of energy. Agar is the solidifying agent



Directions for Preparation from Dehydrated Product:

- o Suspend 38 g of the powder in 1 L of purified water. Mix thoroughly.
- o Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
- o Autoclave at 121°C for 15 minutes. Cool medium to 45-50°C and aseptically add 5% sterile defibrinated sheep blood.
- o Pour cooled Mueller Hinton agar into sterile Petri dishes on a level, horizontal surface to

give a uniform depth of about 4 mm (60-70 mL of medium for 150 mm plates and 25-30 mL for 100 mm plates) and cool to room temperature.

- o Check prepared medium to ensure the final pH is 7.3 $\pm$ 0.1 at 25°C.
- o Test samples of the finished product for performance using stable, typical control cultures.

TABLE 2 - Microorganisms used as markers of activity, their origin, characteristics and culture media used for inoculation and testing.

Microorganism	Origin	Characteristics	Media	Test
<i>Streptococcus mutans</i> (1)	Saliva 1	Gram + coccus	MHb	MHa
<i>Streptococcus sobrinus</i> (2)	Saliva 1	Gram + coccus	MHb	MHa
<i>Streptococcus mutans</i> (3)	Saliva 2	Gram + coccus	MHb	MHa
<i>Enterococcus faecalis</i> (4)	ATCC 10541	Gram + coccus	BHI	BHla
<i>Streptococcus sanguis</i> (5)	Saliva	Gram + coccus	MHb	MHa
<i>Micrococcus luteus</i> (6)	ATCC 9341	Gram + coccus	MHb	MHa
<i>Staphylococcus aureus</i> (7)	ATCC 25923	Gram + coccus	MHb	MHa
<i>Staphylococcus aureus</i> (8)	Phase + Saliva	Gram + coccus	MHb	MHa
<i>Escherichia coli</i> (9)	ATCC 25922	Gram - bacillus	BHI	BHla
<i>Escherichia coli</i> (10)	Saliva 1	Gram - bacillus	BHI	BHla
<i>Pseudomonas aeruginosa</i> (11)	ATCC 27893	Gram - bacillus	BHI	BHla
<i>Candida albicans</i> (12)	ATCC 1023	yeast	MHb	MHa
<i>Candida albicans</i> (13)	Radiation-induced caries	yeast	MHb	MHa

MHb = Mueller-Hinton broth; BHI = Brain Heart Infusion; MHa = Mueller-Hinton agar; BHla = Brain Heart Infusion agar.

Muller Hinton Broth (Not Cation-Adjusted)

Type: general purpose media.

Use: Cultivation of wide variety of fastidious and non-fastidious microorganisms e.g. pathogenic *Neisseria*.

Principle of procedure:

Casein and beef extract: Source of amino acid, nitrogenous substances, minerals, Vitamins, carbon and other nutrients

Starch: Protective colloid against toxic substances and source of energy

Ingredients:

- Beef extract powder
- Acid digest of casein
- Starch

Directions for preparations for dehydrated products:

1. Suspend powder in purified water and mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at 116-121°C FOR 10-15 minutes. DONT OVERHEAT.
4. Check prepared medium to ensure the final PH is 7.3 $\pm$ 0.1 at 25°C.
5. Test samples of finished products for performance using stable, typical control cultures.

Procedure:

Organisms to be subcultured must first be isolated in pure culture on an appropriate solid

medium. Transfer growth from isolated medium to Muller Hinton Broth using standard bacteriological techniques. For enrichment purpose, inoculate the specimen on to the primary media and then into the broth according to recommended procedures.

Incubate the tube at 35°C under conditions appropriate for organism being cultured.

Expected results:

Growth in broth media is indicated by the presence of turbidity compared with an uninoculated control.

Muller Hinton II Broth (Cation-Adjusted)

Use: quantitative procedures for susceptibility testing of rapidly growing aerobic and facultatively anaerobic bacteria.

Muller hinton II broth (cation-adjusted) with 2% sodium chloride

Use: for testing methicillin-resistant strains of *Staphylococcus aureus* (MRSA)

A-1 MEDIUM

Type: Differential media

Intended use:

A-1 Medium is used for detecting fecal coliforms in water.

Enumeration of coliform organisms, specifically *Escherichia coli*, has been used to determine water purity. Elevated-temperature, most-probable-number (MPN) methods are routinely used for the analysis of water and food samples for the presence of fecal coliforms. One limiting factor in using *E. coli* is the length of time required for complete identification. A-1 Medium was formulated to hasten the recovery of *E. coli* and reduce the incidence of false positive cultures. Medium recovers *E. coli* from estuarine water in 24 hours instead of 72 hours, and in greater numbers without the pre-enrichment step. Using a 3-hour preincubation step for the enumeration of coliforms in chlorinated wastewater gave results that were statistically

comparable to those obtained in the two-step MPN technique. Prior enrichment in a presumptive medium is not required. A-1 Medium conforms to standard methods for the isolation of fecal coliforms in water and foods in a single-step procedure.

Principles of the Procedure:

Peptone provides the nitrogen, vitamins, minerals and amino acids in A-1 Medium. Lactose is the carbon source and, in combination with salicin, provides energy for organism growth. Sodium chloride maintains the osmotic balance of the medium. Triton X-100 is a surfactant.

Ingredients:

- Tryptone
- Lactose
- Sodium Chloride
- Salicin
- Triton X-100

Directions for Preparation from Dehydrated Product:

1. Suspend 31.5 g of the powder in 1 L of purified water. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Dispense into tubes containing inverted fermentation vials.
4. Autoclave at 121°C for 10 minutes.
5. Test samples of the finished product for performance using stable, typical control cultures.

NOTE: For 10 mL water samples, prepare double-strength medium to ensure ingredient concentrations are not reduced below those of the standard medium.

- Solution: 3.15% solution, soluble in purified water upon boiling.
- Solution at 25°C: pH 6.9 ± 0.1

Nutrient Broth

Intended Use:

Nutrient Broth is used for the cultivation of many species of non-fastidious microorganisms.

Principles of the Procedure:

This relatively simple formulation supports the growth of non-fastidious microorganisms due to its content of peptone and beef extract.

Directions for Preparation from Dehydrated Product:

6. Dissolve 8 g of the powder in 1 L of purified water.
7. Autoclave at 121°C for 15 minutes.
8. Test samples of the finished product for performance using stable, typical control cultures.

Procedure:

Inoculate tubes of the broth medium with the test samples.

Incubate tubes for 18-24 hours at $35 \pm 2^\circ\text{C}$ in an aerobic atmosphere.

Nutrient Agar

Intended Use:

Nutrient Agar is used for the cultivation of bacteria and for the enumeration of organisms in water, sewage, feces and other materials.

Principles of the Procedure:

Nutrient Agar consists of peptone, beef extract and agar. This relatively simple formulation provides the nutrients necessary for the replication of a large number of microorganisms that are not excessively fastidious. The beef extract contains water soluble substances including carbohydrates, vitamins, organic nitrogen compounds and salts. Peptones are the principle sources of organic nitrogen, particularly amino acids and long-chained peptides. Agar is the solidifying agent.

Directions for Preparation from Dehydrated Product:

1. Suspend 23 g of the powder in 1 L of purified water. Mix thoroughly.

2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.

3. Autoclave at 121°C for 15 minutes.

Procedure:

Liquefy the agar if prepared tubes are used, cool to $45\text{--}50^\circ\text{C}$ and pour into Petri dishes. Allow to solidify for at least 30 minutes. Use standard procedures to obtain isolated colonies from specimens. Incubate plates at $35 \pm 2^\circ\text{C}$ for 18-24 hours and 42-48 hours, if necessary. Tubed slants are used primarily for the cultivation and maintenance of pure cultures. They should be inoculated with an inoculating loop and incubated under the same conditions as the plated medium.

Blood Agar Base (Infusion Agar)

Intended Use:

Blood Agar Base (Infusion Agar), with the addition of sterile blood, is used for the isolation, cultivation and detection of hemolytic activity of streptococci and other fastidious microorganisms.

Principles of the Procedure:

Infusion from heart muscle, casein peptone and yeast extract provide nitrogen, carbon, amino acids and vitamins in Blood Agar Base. Medium contains sodium chloride to maintain osmotic equilibrium and agar is the solidifying agent. Supplementation with blood (5-10%) provides additional growth factors for fastidious microorganisms, and is the basis for determining hemolytic reactions. Hemolytic patterns may vary with the source of animal blood or type of base medium used.

Directions for Preparation from Dehydrated Product:

9. Suspend 40 g of the powder in 1 L of purified water. Mix thoroughly.
10. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
11. Autoclave at 121°C for 15 minutes.

12. For preparation of blood agar, cool the base to 45-50°C and aseptically add 5% sterile, defibrinated blood. Mix well.
13. Test samples of the finished product for performance using stable, typical control cultures.

MacConkey Agars

Intended Use:

MacConkey Sorbitol Agar and MacConkey II Agar with Sorbitol are selective and differential media for the detection of sorbitol-nonfermenting *Escherichia coli* serotype O157:H7 associated with hemorrhagic colitis. These media are also referred to as "Sorbitol MacConkey Agar."

Principles of the Procedure:

Peptone provides amino acids and other growth factors. Lactose is a carbon energy source for gram-negative lactose-fermenting bacilli. Oxgall inhibits the growth of gram-positive organisms. Bromocresol purple is the indicator.

MacConkey Sorbitol Agar and MacConkey II Agar with Sorbitol, modified MacConkey agars using sorbitol instead of lactose, are only slightly selective, since the concentration of bile salts, which inhibits gram-positive microorganisms, is low in comparison with other enteric plating media. Crystal violet also is included in the medium to inhibit the growth of gram-positive bacteria, especially enterococci and staphylococci. MacConkey II Agar with Sorbitol is also formulated to reduce swarming of *Proteus* species.

SABOURAUD AGAR

Intended Use:

Sabouraud Agar used in qualitative procedures for cultivation of dermatophytes and other pathogenic and nonpathogenic fungi from clinical and nonclinical specimens.

Summary and Explanation:

Sabouraud Dextrose Agar was devised by Sabouraud for the cultivation of dermatophytes.¹ The low pH of approximately 5.6 is favorable for

the growth of fungi, especially dermatophytes, and inhibitory to contaminating bacteria in clinical specimens.² The acidic pH, however, also may inhibit some

fungal species. Emmons modified the original formulation by adjusting the pH close to neutral to increase the recovery of fungi and by reducing the dextrose content from 40 to 20 g/L.

Principles of the Procedure:

Peptones are sources of nitrogenous growth factors. Dextrose provides an energy source for the growth of microorganisms. Gentamicin is an aminoglycoside antibiotic that inhibits the growth of gram-negative bacteria. Chloramphenicol is inhibitory to a wide range of gram-negative and gram-positive bacteria, and cycloheximide is an antifungal agent that is primarily active against saprophytic fungi and does not inhibit yeasts or dermatophytes.

Directions for Preparation from Dehydrated Product:

4. Suspend 50 g of the powder in 1 L of purified water. Mix thoroughly.
5. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
6. Autoclave at 121°C for 15 minutes.
7. Test samples of the finished product for performance using stable, typical control cultures.

Procedure:

Consult appropriate references for information about the processing and inoculation of specimens. Prepared tubed slants primarily are intended for use with pure cultures for maintenance or other purposes. For isolating fungi from potentially contaminated specimens, a selective medium should be inoculated along with the nonselective medium. Incubate the plates at 25-30°C in an inverted

position (agar side up) with increased humidity. For isolation of fungi causing systemic mycoses, two sets of media should be inoculated, with one set incubated at 25-30°C and a duplicate

set at $35 \pm 2^\circ\text{C}$. All cultures should be examined at least weekly for fungal growth and should be held for 4-6 weeks before being reported

as negative.

Expected Results:

After sufficient incubation, the plates or tubes should show growth with or without isolated colonies. Transfer of growth from tubes to plated media may be required in order to obtain pure cultures of fungi.

Examine plates or tubes for fungal colonies exhibiting typical color and morphology.⁶ Biochemical tests and serological procedures should be performed to confirm findings. Limitation of the Procedure Antimicrobial agents incorporated into a medium to inhibit bacteria may also inhibit certain pathogenic fungi.

CLED Agar (Cystine- Lactose-Electrolyte-Deficient)

Intended Use:

- CLED Agar is used for the isolation, enumeration and presumptive identification of microorganisms from urine.
- CLED Agar is recommended for use in plates or in urine dipsticks for detecting significant bacteriuria by quantitative culture of urine.

Principles of the Procedure:

Lactose is included to provide an energy source for organisms. The cystine permits the growth of "dwarf colony" coliforms. Bromthymol blue is used as a pH indicator to differentiate lactose fermenters from lactose nonfermenters. Organisms that ferment lactose will lower the pH and change the color of the medium from green to yellow. Electrolyte sources are reduced in order to restrict the swarming of *Proteus* species.

Formula:

- Approximate Formula per Liter
- Pancreatic Digest of Gelatin
- Pancreatic Digest of Casein

- Beef Extract
- Lactose
- L-Cystine
- Bromthymol Blue
- Agar

Directions for Preparation from Dehydrated Product:

1. Suspend 36 g of the powder in 1 L of purified water. Mix thoroughly.
2. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder.
3. Autoclave at 121°C for 15 minutes.
4. Test samples of the finished product for performance using stable, typical control cultures.

Identity Specifications:

Dehydrated Appearance: Fine, homogenous, free of extraneous material.

Prepared Appearance: Medium, yellow green to blue green, clear to slightly hazy.

pH: 7.3 ± 0.2

Typical colonial morphology on CLED Agar is as follows:

- *Escherichia coli* - Yellow colonies, opaque, and center slightly deeper yellow
- *Pseudomonas aeruginosa* - Green colonies with typical matted surface and rough periphery
- *Proteus* - Translucent blue colonies
- Enterococci - Small yellow colonies, about 0.5 mm in diameter
- *Staphylococcus aureus* - Deep yellow colonies, uniform in color

Limitations of the Procedure:

Factors that may cause urine counts from infected patients to be low include:

- Rapid rate of urine flow
- Prior initiation of antimicrobial therapy
- A urine pH of less than 5
- A specific gravity of less than 1.003

MICROBIOLOGICAL ASSAYS

Chapter 6.

General Principles and Methods of Analysis

MICROBIOLOGICAL ASSAY: AN OVERVIEW

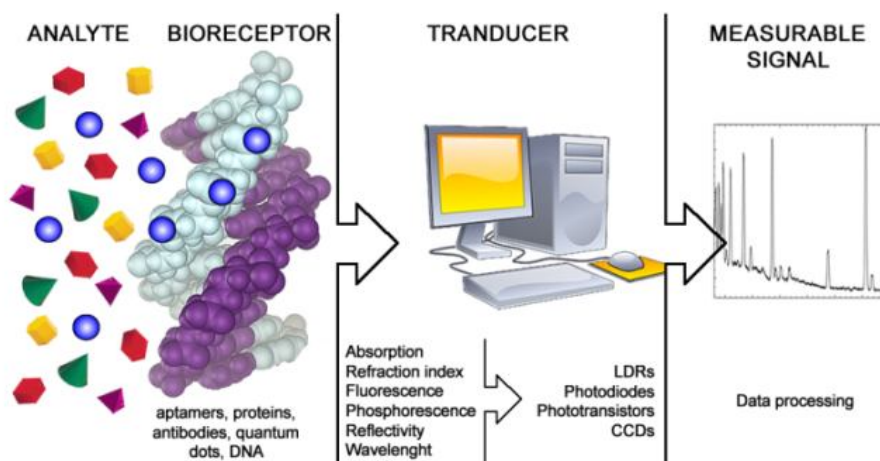


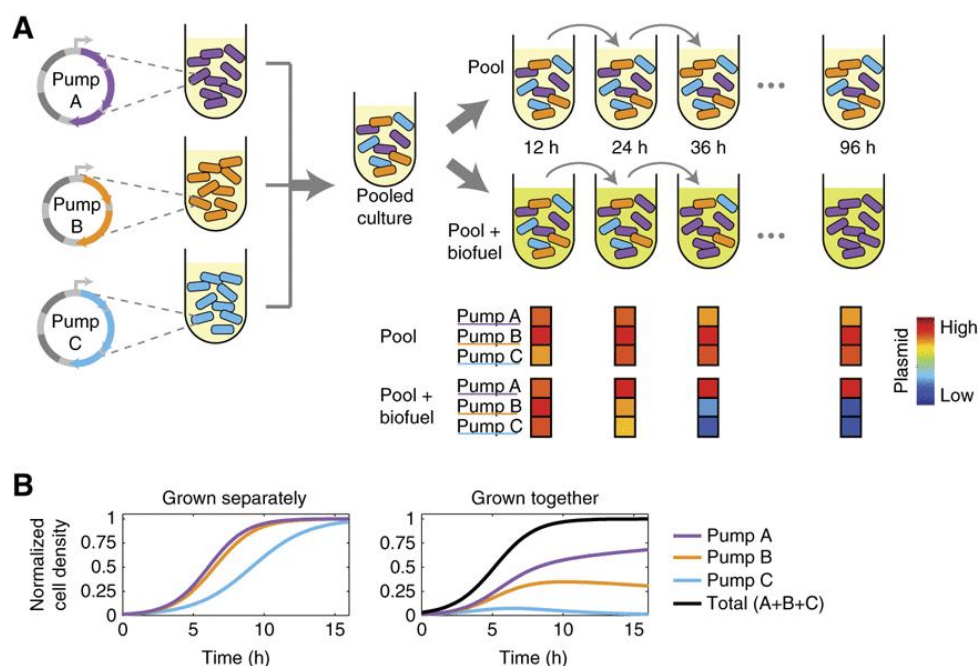
Microbiological assay is a technique whereby the potency or concentration of a chemical substance may be determined by its effect on the growth of a microorganism. That effect may be to promote the growth of the microorganism by substances such as certain vitamins and amino acids, or to inhibit growth in the case of antibiotics and other substances having similar properties.

The discovery that vitamins of the $\hat{A}$ group were essential for the growth of organisms of the *Lactobacillus* group and some other organisms led to the development of assay methods for these substances during the 1930s. The inhibitory effect of penicillin on staphylococci and other organisms had been known since 1929.

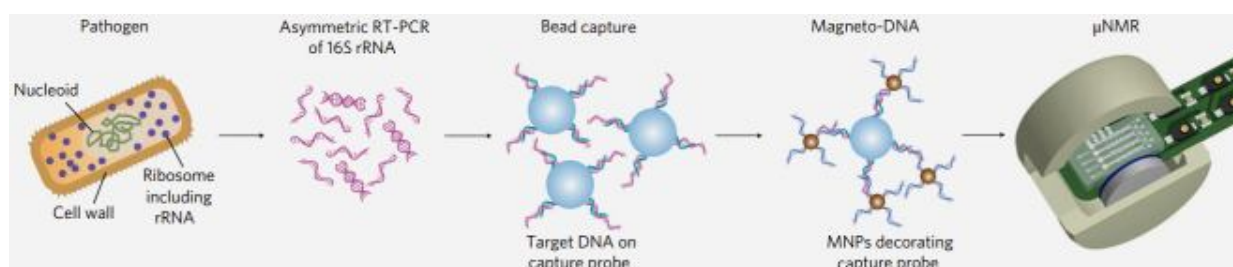
However, it was with the early attempts to produce penicillin on a manufacturing scale in the

early 1940s that microbiological assay of growth-inhibiting substances became established as an important analytical technique. The therapeutic value of penicillin was recognized before its chemistry had been elucidated fully and before any chemical method of assay had been developed. The same situation pertained in the case of many other antibiotics that were discovered and produced commercially, particularly during the following two decades. Thus, microbiological assay became established as an essential technique for process and product control in the pharmaceutical industry. In the early days techniques were largely empirical, principles were not well understood, sources of error were unrecognized, and, as a consequence, assay results were often of poor quality. Despite the efforts and some successes of chemists in replacing microbiological assay with chemical or physical methods of assay, microbiological assay remains today the only practicable method of assay for many antibiotics and is a convenient method of choice in some cases where a chemical or physical method has been devised.





Competition assay efficiently identifies efflux pumps that provide biofuel tolerance. (A) Plasmids containing the operons for individual pumps were transformed into cells. These strains were grown independently and then pooled in equal proportion. The pooled culture was grown both with and without biofuel for 96 h, with dilution into fresh medium every 10–14 h. At selected time points, cultures were saved, plasmids isolated, and the relative levels of each plasmid were quantified. If certain plasmids provide a growth advantage, they become overrepresented in the culture. (B) Simulations of competitive exclusion. Grown separately, the growth curve of a strain is dependent only on its growth rate. When grown together, as in the competition assay, strains with higher growth rates dominate the population.



On this 2.5- by 7.5-cm cartridge, DNA extracted from sputum samples is amplified in the chambers on the left. TB-specific sequences are magnetically labeled in the microfluidic mixing channels in the center

Of course, as fundamental principles of the assay method have become better understood and sources of error have been recognized, the methods have become potentially more reliable.

Evidence of the importance of microbiological assay in the quality control of antibiotics is borne out by the existence today of 40 or more international reference preparations for

antibiotics. An assay is an investigative (analytic) procedure in laboratory medicine, pharmacology, environmental biology, continuous delivery, and molecular biology for qualitatively assessing or quantitatively measuring the presence or amount or the functional activity of a target entity (the analyte). The analyte can be a drug or biochemical substance or a cell in an organism or

organic sample. The measured entity is generally called the analyte, or the measurand or the target of the assay. The assay usually aims to measure an intensive property of the analyte and express it in the relevant measurement unit (e.g. molarity, density, functional activity in enzyme international units, degree of some effect in comparison to a standard, etc.).

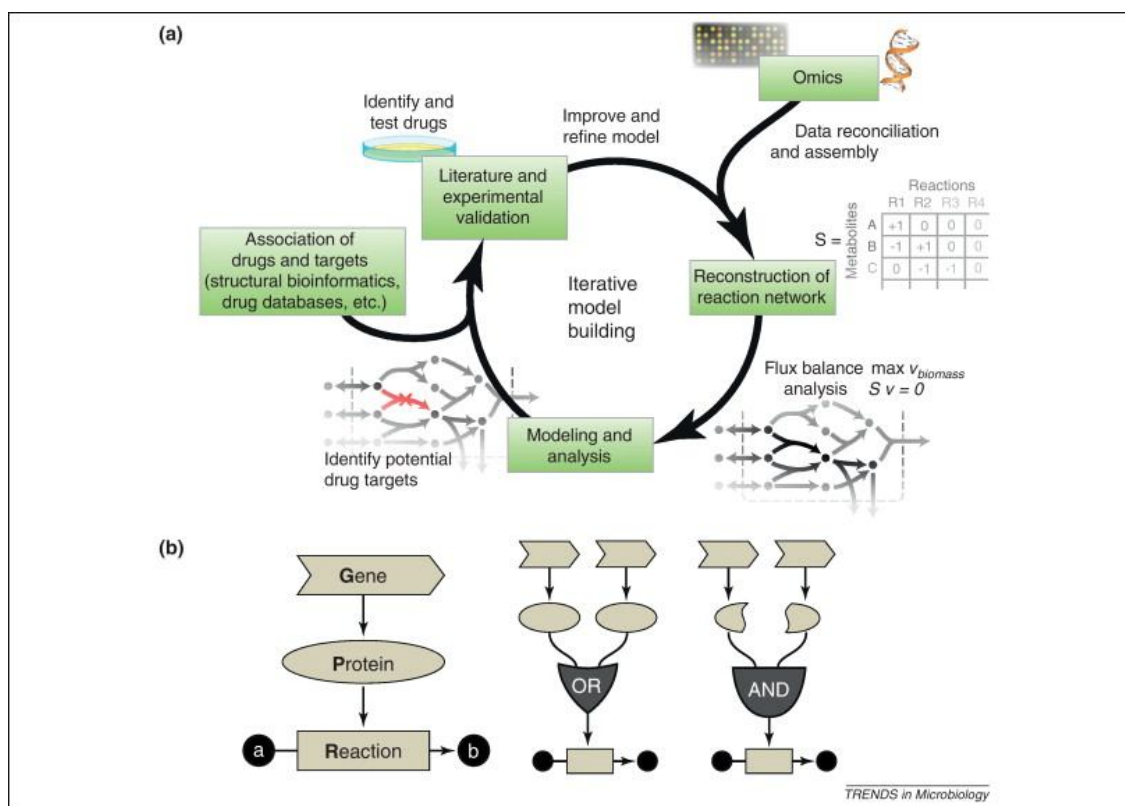
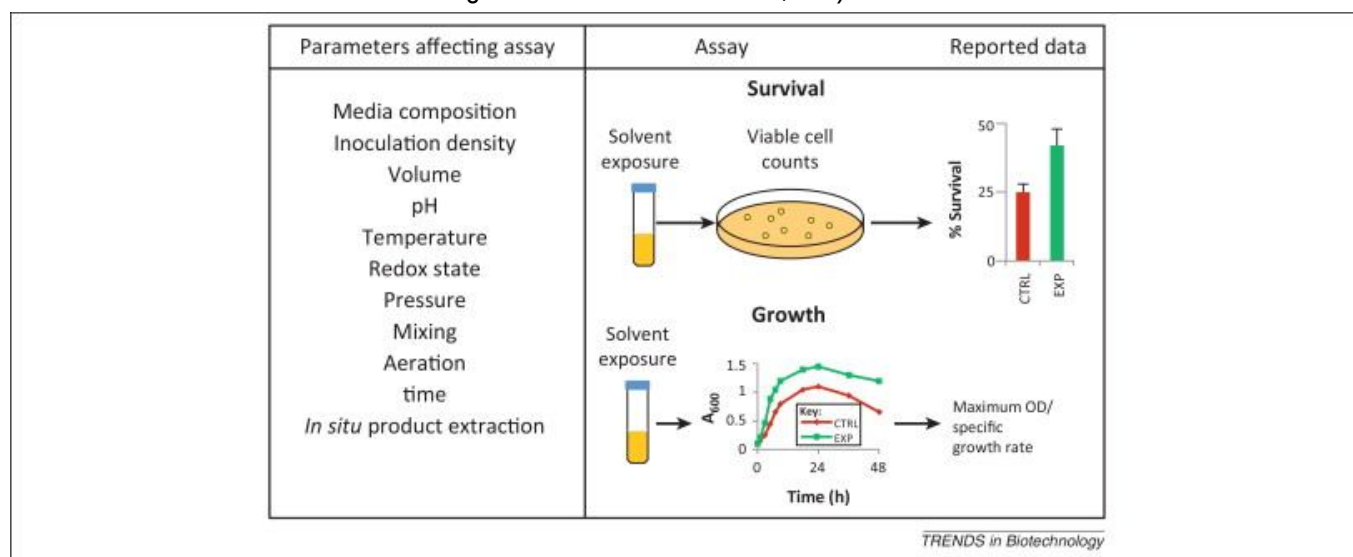
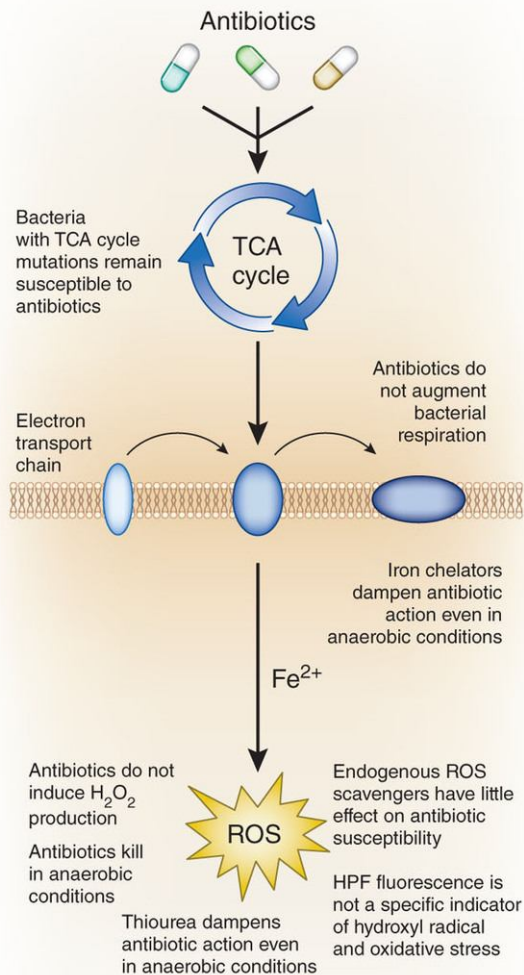


Figure. A metabolic network approach for the identification and prioritization of antimicrobial drug targets



If the assay involves addition of exogenous reactants (the reagents) then their quantities are kept fixed (or in excess) so that the quantity (and quality) of the target is the only limiting factor for the reaction/assay process, and the difference in the assay outcome is used to deduce the unknown quality or quantity of the target in question. Some assays (e.g., biochemical assays) may be similar to or have overlap with chemical analysis and titration. But generally, assays involve biological material or phenomena which tend to be intrinsically more complex either in composition or in behavior or both. Thus reading of an assay may be quite noisy and may involve greater difficulties in interpretation than

an accurate chemical titration. On the other hand, older generation qualitative assays, especially bioassays, may be much more gross and less quantitative (e.g., counting death or dysfunction of an organism or cells in a population, or some descriptive change in some body part of a group of animals).

Successful assays cannot be carried out without a proper understanding of the role and importance of pure, healthy, and robust test organisms in the form of bacterial or spore suspensions. Since microorganisms are one of the most important basic tools of the microbiological analyst, a certain amount of bacteriological know-how and practical experience in handling live cultures will be necessary. At least elementary knowledge of bacteriological theory and practice will be assumed from the reader. As far as possible full practical guidance will be given in easy-to-follow instructions to ensure satisfactory microbiological assays. It is not considered to be within the scope of this volume to provide full instructions in all aspects of bacteriological techniques such as media preparation, staining, and microscopy. For further aspects a good basic textbook of bacteriology should be consulted, such as Collins and Lyne (1984).

MICROBIOLOGICAL ASSAY OF ANTIBIOTICS

The potency (activity) of an antibiotic product is expressed as the ratio of the dose that inhibits the growth of a suitable susceptible microorganism to the dose of an International Biological Standard, an International Biological Reference Preparation, or an International Chemical Reference Substance of that antibiotic that produces similar inhibition. Properly validated secondary reference materials may also be utilized in the assay. To carry out the assay a comparison is made between the inhibition of the growth of microorganisms produced by known concentrations of the reference material and that produced by measured dilutions of the test substance. This response can be measured by the diffusion

method, as described below, or in a liquid medium by the turbidimetric method.

An International Unit is the specific activity contained in such an amount (weight) of the relevant International Biological Standard or International Biological Reference Preparation as the WHO Expert Committee on Biological Standardization may from time to time indicate as the quantity exactly equivalent to the unit accepted for international use. In some cases, when owing to the properties of the material, difficulties are experienced in weighing with adequate accuracy small amounts of the relevant International Biological Standard or International Biological Reference Preparation, International Units are defined on the basis of the total contents of the material in an ampoule or a vial. A defined number of International Units is then assigned to the total contents of an ampoule or a vial; this material has to be carefully removed with an appropriate solvent and the final volume of the solution has to be accurately adjusted.

International Chemical Reference Substances do not have defined units of biological activity. The potency of those products for which biological assays are required are in such cases expressed in terms of an equivalent weight of the pure substance.

ASSAY TYPES

The microbiological assay

The assays are generally depend/ based on

1. The nature of the assay process
2. Targets or analytes
3. The quality of the result
4. General substrate and applied principle
5. The nature of the signal amplification system
6. The nature of the Detection system
7. The targets being measured
8. A cell-counting assay
9. Surfactants
10. Other cell assays
11. Virology
12. Cellular secretions

Based on the nature of the assay process

Depending on whether an assay just looks at a single time point or timed readings taken at multiple time points, an assay may be:

1. End point assay: when the only reading that matters is the end result after a fixed assay incubation period.
2. Kinetic assay: when readings are taken multiple times at fixed time intervals during an assay and a kinetic graph of the readings is important.

Depending on how many targets or analytes are being measured:

1. Usual assay are simple or single target assays which is usually the default unless it is called multiplex.
2. Multiplex assays are assays that in a same reaction detect multiple analytes simultaneously.

Depending on the quality of the result produced, assays may be classified into:

1. Qualitative assay, i.e. assays which generally give just a pass or fail, or positive or negative or some such sort of only small number of qualitative gradation rather than an exact quantity.
2. Semi-quantitative assays, i.e. assays that give the read-out in an approximate fashion rather than an exact number for the quantity of the substance. Generally they have a few more gradations than just two outcomes, positive or negative, e.g. scoring on a scale of 1+ to 4+ as used for blood grouping tests based on RBC agglutination in response to grouping reagents (antibody against blood group antigens).
3. Quantitative assays, i.e. assays that give accurate and exact numeric quantitative measure of the amount of a substance in a sample. An example of such an assay used in coagulation testing laboratories for the commonest inherited bleeding disease - Von Willebrand disease is VWF antigen assay

where the amount of VWF present in a blood sample is measured by an immunoassay.

4. Functional assay, i.e. an assay that tries to quantify functioning of an active substance rather than just its quantity. The functional counterpart of the VWF antigen assay is Ristocetin Cofactor assay, which measures the functional activity of the VWF present in a patient's plasma by adding exogenous formalin-fixed platelets and gradually increasing quantities of drug named ristocetin while measuring agglutination of the fixed platelets. A similar assay but used for a different purpose is called Ristocetin Induced Platelet Aggregation or RIPA, which tests response of endogenous live platelets from a patient in response to Ristocetin (exogenous) & VWF (usually endogenous).

Depending on the general substrate on which the assay principle is applied:

1. Bioassay: when the response is biological activity of live objects e.g.
 1. Organism (e.g. mouse injected with a drug)
 2. ex vivo body part (e.g. leg of a frog)
 3. ex vivo organ (e.g. heart of a dog)
 4. ex vivo part of an organ (e.g. a segment of an intestine).
5. tissue (e.g. limulus lysate)
6. cell (e.g. platelets)
2. Ligand binding assay when a ligand (usually a small molecule) binds a receptor (usually a large protein).
3. Immunoassay when the response is an antigen antibody binding type reaction.

Depending on the nature of the signal amplification system assays may be of numerous types, to name a few:

1. Enzyme activity assay: Enzymes may be tested by their highly repeating activity on a large number of substrates when loss of a substrate or the making of a product may have a measurable attribute like color or absorbance at a particular wavelength or light or chemiluminescence or electrical/redox activity.

2. Light detection systems that may use amplification e.g. by a photodiode or a photomultiplier tube or a cooled charge coupled device.
3. Radioisotope labeled substrates as used in radioimmunoassays and equilibrium dialysis assays and can be detected by the amplification in GM counters or X-ray plates, or phosphorimager
4. Polymerase Chain Reaction Assays that amplifies a DNA (or RNA) target itself rather than the signal

Assays may be a combination of the above e.g. enzyme Immuno assay or EIA, enzyme linked immunosorbent assay.

Depending on the nature of the Detection system assays can be based on:

1. Colony forming or virtual colony count: e.g. by multiplying bacteria or proliferating cells.
2. Photometry / spectrophotometry When the absorbance of a specific wavelength of light while passing through a fixed path-length through a cuvette of liquid test sample is measured and the absorbance is compared with a blank and standards with graded amounts of the target compound. If the emitted light is of a specific visible wavelength it may be called colorimetry, or it may involve specific wavelength of light e.g. by use of laser and emission of fluorescent signals of another specific wavelength which is detected via very specific wavelength optical filters.
3. Transmittance of light may be used to measure e.g. clearing of opacity of a liquid created by suspended particles due to decrease in number of clumps during a platelet agglutination reaction.
4. Turbidimetry when the opacity of straight-transmitted light passing through a liquid sample are measured by detectors placed straight across the light source.
5. Nephelometry when the scattered lights are measured by detectors placed at fixed angles to the path of light.

6. Reflectometry When color of light reflected from a (usually dry) sample or reactant is assessed e.g. the automated readings of the strip urine dipstick assays.
7. Viscoelastic measurements e.g. viscometry, elastography (e.g. thromboelastography)
8. Counting assays: e.g. optic Flowcytometric cell or particle counters, or coulter/impedance principle based cell counters
9. Imaging assays, that involve image analysis manually or by software: 1. Cytometry: When the size statistics of cells is assessed by an image processor.
10. Electric detection e.g. involving amperometry, voltametry, coulometry may be used directly or indirectly for many types of quantitative measurements.
11. Other physical property based assays may use
 - Osmometer
 - Viscometer
 - Ion Selective electrodes

Assay types based on the targets being measured

DNA

Assays for studying interactions of proteins with DNA include:

- DNase footprinting assay
- Filter binding assay
- Gel shift assay

Protein

- Bicinchoninic acid assay (BCA assay)
- Bradford protein assay
- Lowry protein assay[6][7]
- Secretion assay

RNA

- Nuclear run-on
- Ribosome profiling
- Cell counting, viability, proliferation or cytotoxicity assays

A cell-counting assay may determine the number of living cells, the number of dead cells,

or the ratio of one cell type to another, such as numerating and typing red versus different types of white blood cells. This is measured by different physical methods (light transmission, electric current change). But other methods use biochemical probing cell structure or physiology (stains). Another application is to monitor cell culture (assays of cell proliferation or cytotoxicity). A cytotoxicity assay measures how toxic a chemical compound is to cells.

- MTT assay
- Cell Counting Kit-8 (WST-8 based cell viability assay)
- SRB (Sulforhodamine B) assay
- CellTiter-Glo® Luminescent Cell Viability Assay
- Cell counting instruments and methods: CASY cell counting technology, Coulter counter, Electric cell-substrate impedance sensing
- Cell viability assays: resazurin method, ATP test, Ethidium homodimer assay (detect dead or dying cells), Bacteriological water analysis, Clonogenic assays etc.

Surfactants

An MBAS assay indicates anionic surfactants in water with a bluing reaction.

Other cell assays

Many cell assays have been developed to assess specific parameters or response of cells (biomarkers, cell physiology. Techniques used to study_cells include :

- reporter assays using i.e. Luciferase, calcium signaling assays using Coelenterazine, CFSE or Calcein
- Immunostaining of cells on slides by Microscopy (ImmunoHistoChemistry or Fluorescence), on microplates by photometry including the ELISPOT (and its variant FluoroSpot) to enumerate B-Cells or antigen-specific cells, in solution by Flow cytometry
- Immunostaining of cells on slides by Microscopy, on microplates by photometry

- Molecular biology techniques such as DNA microarrays, in situ hybridization, combined to PCR, Computational genomics, and Transfection; Cell fractionation or Immunoprecipitation
- Migration assays, Chemotaxis assay
- Secretion assays
- Apoptosis assays such as the DNA laddering assay, the Nicoletti assay, caspase activity assays, and Annexin V staining
- Chemosensitivity assay measures the number of tumor cells that are killed by a cancer drug
- Tetramer assay detect the presence of antigen specific T-cells
- Gentamicin protection assay or survival assay or invasion assay to assess ability of pathogens (bacteria) to invade eukaryotic cells
- Petrochemistry
- Crude oil assay

Virology

The HPCE-based viral titer assay uses a proprietary, high-performance capillary electrophoresis system to determine baculovirus titer.

The Trofile assay is used to determine HIV tropism.

The viral plaque assay is to calculate the number of viruses present in a sample. In this technique the number of viral plaques formed by a viral inoculum is counted, from which the actual virus concentration can be determined.

Cellular secretions

A wide range of cellular secretions (say, a specific antibody or cytokine) can be detected using the ELISA technique. The number of cells which secrete those particular substances can be determined using a related technique, the ELISPOT assay.

Drugs

- Testing for Illegal Drugs

- Radioligand binding assay

GENERAL STEPS

An assay (analysis) is never an isolated process and must be combined with pre- and post-analytic procedures. The information communication (e.g. request to perform an assay and further information processing) or specimen handling (e.g. collection transport and processing) that are done until the beginning of an assay are the preanalytic steps. Assays can be very diverse, but generally involve the following general steps:

Sample processing and manipulation in order to selectively present that target in a discernible or measurable form to a discrimination/ identification/ detection system. It might involve a simple centrifugal separation or washing or filtration or capture by some form of selective binding or it may even involve modifying the target e.g. epitope retrieval in immunological assays or cutting down the target into pieces e.g. in Mass Spectrometry. Generally there are multiple separate steps done before an assay and are called preanalytic processing. But some of the manipulations may be inseparable part of the assay itself and will not thus be considered pre-analytic.

Target-specific discrimination/ identification principle: to discriminate from background (noise) of similar components and specifically identify a particular target component ("analyte") in a biological material by its specific attributes. (e.g. in a PCR assay a specific oligonucleotide primer identifies the target by base pairing based on the specific nucleotide sequence unique to the target).

Signal (or target) amplification system: The presence and quantity of that analyte is converted into a detectable signal generally involving some method of signal amplification, so that it can be easily discriminated from noise and measured - e.g. in a PCR assay among a mixture of DNA sequences only the specific target is amplified into millions of copies by a DNA

polymerase enzyme so that it can be discerned as a more prominent component compared to any other potential components. Sometimes the concentration of the analyte is too large and in that case the assay may involve sample dilution or some sort of signal diminution system which is a negative amplification.

Signal detection (and interpretation) system:

A system of deciphering the amplified signal into an interpretable output that can be quantitative or qualitative. It can be visual or manual very crude methods or can be very sophisticated electronic digital or analog detectors.

Signal enhancement and noise filtering may be done at any or all of the steps above. Since the more downstream a step/process during an assay, the higher the chance of carrying over noise from the previous process and amplifying it, multiple steps in a sophisticated assay might involve various means of signal-specific sharpening/enhancement arrangements and noise reduction or filtering arrangements. These may simply be in the form of a narrow band-pass optical filter, or a blocking reagent in a binding reaction that prevents nonspecific binding or a quenching reagent in a fluorescence detection system that prevents "autofluorescence" of background objects.

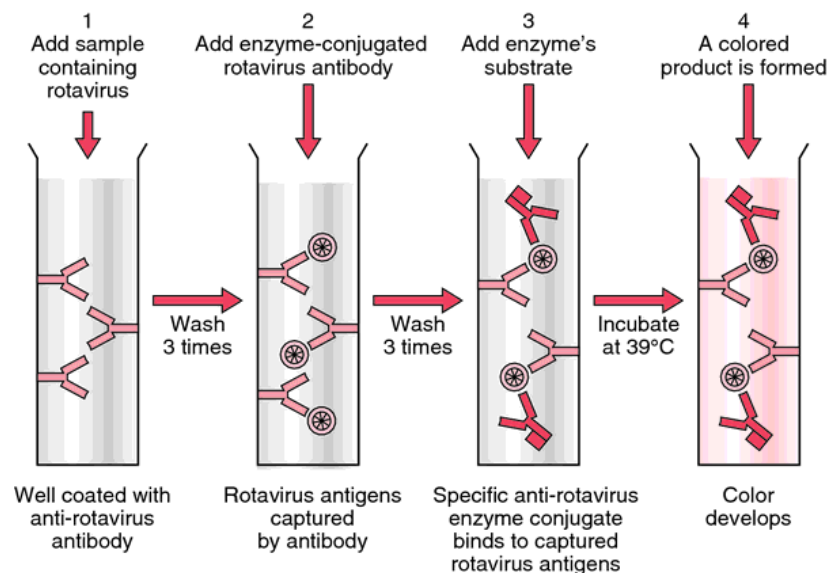


Figure. With antigen capture ELISA, the wells are coated with antibody to the virus. The sample containing virus (1) is added and, after washing several times, enzyme conjugated to an antibody to the virus is added (2). Finally, after a further cycle of washing, the enzyme's substrate (3) is added. A colored product is formed if the viral antigen is present in the sample (4). From Hart and Shears, 1997.

QUALITY OF AN ASSAY

When multiple assays measure the same target their results and utility may or may not be comparable depending on the natures of the assay and their methodology, reliability etc. Such comparisons are possible through study of general quality attributes of the assays e.g. principles of measurement (including identification, amplification and detection), dynamic range of detection (usually the range of

linearity of the standard curve), analytic sensitivity, functional sensitivity, analytic specificity, positive, negative predictive values, turn around time i.e. time taken to finish a whole cycle from the preanalytic steps till the end of the last post analytic step (report dispatch/transmission), throughput i.e. number of assays done per unit time (usually expressed as per hour) etc. Organizations or laboratories that perform Assays for professional purposes e.g.

medical diagnosis and prognostics, environmental analysis, forensic proceeding, pharmaceutical research and development must undergo well regulated quality assurance procedures including method validation, regular calibration, Analytical quality control, Proficiency testing, test accreditation, test licensing and must document appropriate certifications from the relevant regulating bodies in order to establish the reliability of their assays, especially to remain legally acceptable and accountable for the quality of the assay results and also to convince customers to use their assay commercially/professionally.

RECOMMENDED PROCEDURE

Use Petri dishes or rectangular trays filled to a depth of 3-4 mm, unless otherwise indicated in the monograph, with a culture medium that has previously been inoculated with a suitable inoculum of a susceptible test organism prepared as described below. The nutrient agar may be composed of two separate layers of which only the upper one may be inoculated. The concentration of the inoculum should be so selected that the sharpest zones of inhibition and suitable dose response at different concentrations of the standard are obtained. When using the inoculum prepared as described below, an inoculated medium containing 1 mL of inoculum per 100 mL of the culture medium is usually suitable. When the inoculum consists of a suspension of vegetative organisms, the temperature of the molten agar medium must not exceed 48-50 °C at the time of inoculation. The dishes or trays should be specially selected with flat bottoms. During the filling they should be placed on a flat, horizontal surface so as to ensure that the layer of the medium will be of a uniform thickness. With some test organisms, the procedure may be improved if the inoculated plates are allowed to dry for 30 minutes at room temperature before use, or refrigerated at 4 °C for several hours.

For the application of the test solution, small sterile cylinders of uniform size, approximately 10

mm high and having an internal diameter of approximately 5 mm, made of suitable material such as glass, porcelain, or stainless steel, are placed on the surface of the inoculated medium. Instead of cylinders, holes 8-10 mm in diameter may be bored in the medium with a previously sterilized borer. Other methods of application of the test solution may also be used. The arrangement on the plate should be such that overlapping of zones is avoided.

Solutions of the reference material of known concentration and corresponding dilutions of the test substance, presumed to be of approximately the same concentration, are prepared in a sterile buffer of a suitable pH value. To assess the validity of the assay at least 3 different doses of the reference material should be used together with an equal number of doses of the test substance having the same presumed activity as the solutions of the reference material. The dose levels used should be in geometric progression, for example, by preparing a series of dilutions in the ratio 2:1. Once the relationship between the logarithm of concentration of the antibiotic and the diameter of the zone of inhibition has been shown to be approximately rectilinear for the system used, routine assays may be carried out using only 2 concentrations of the reference material and 2 dilutions of the test substance. Where a monograph gives directions for the initial preparation of a solution of the substance, this solution is then diluted as necessary with the appropriate sterile buffer.

The solutions of the reference material and the test substance are preferably arranged in the form of a Latin square when rectangular trays are employed. When Petri dishes are used, the solutions are arranged on each dish so that the solutions of the reference material and those of the test substance alternate around the dish and are placed in such a manner that the highest concentrations of the reference material and of the test substance are not adjacent. The solutions are placed in the cylinders or holes by means of a pipette that delivers a uniform volume of liquid. When the holes are used the delivered

volume should be sufficient to fill them almost completely.

The plates are incubated at a suitable temperature, the selected temperature being controlled at $\pm 0.5^\circ\text{C}$, for approximately 16 hours, and the diameters or areas of the zones of inhibition produced by the varied concentrations of the standard and of the test substance are measured accurately, preferably to the nearest 0.1 mm of the actual zone size, by using a suitable measuring device. From the results, the potency of the tested substance is calculated. Suitable publications on the statistics of bioassays are listed below.

Conditions for the assay of individual antibiotics and suitable test organisms are given in the monographs. The choice of an appropriate strain of test organism may be critical for the assay.

PRECISION OF THE ASSAY

In order to determine whether or not a substance satisfies the requirements for potency specified in the monograph, the assay should, if necessary, be repeated until the required precision has been attained. This precision is such that the fiducial limits ($P = 0.95$) of the mean estimated potency, expressed as a percentage of the mean estimated potency, should be within the required range given in the individual monographs.

CALCULATION OF RESULTS

The following publications contain suitable methods that may be used to carry out the statistical evaluation of the microbiological assay of antibiotics:

1. Bliss C.I.: *Statistics of bioassay*. New York, Academic Press, 1952.
2. Bliss C.I.: *Statistics in biology*. vol. I, New York, McGraw Hill, 1967.
3. Bliss C.I.: *Statistics in biology*. vol. II, New York, McGraw Hill, 1970.
4. Finney D.J.: *Statistical methods in biological assays*. London, Griffin, 1964.

5. Hewitt W.: *Microbiological assay*. New York, Academic Press, 1977.
6. Philippe J.: *Les methodes statistiques en pharmacie et en chimie*. Paris, Masson, 1967.
1. The methods of carrying out the statistical evaluation of the microbiological assay of antibiotics are also described in many national and regional pharmacopoeias.

Table 4. Test organisms and conditions of assay of individual antibiotics

Antibiotic	Test organism		Culture medium; final pH	Incub. Temp. (in $^\circ\text{C}$)
Bacitracin	<i>Micrococcus luteus</i>		Cm1;	35-37
	NCTC	7743;	7.0-7.1	
	ATCC	10240		
	<i>Micrococcus luteus</i>		Cm1;	30-31
Cefalexin	NCTC	7743;	6.5-6.6	
	ATCC	10240		
	<i>Staphylococcus aureus</i>		Cm1;	32-35
	NCTC	6571;	6.5-6.6	
Cefalotin	ATCC	9144		
	<i>Staphylococcus aureus</i>		Cm1;	32-35
	ATCC	6538-P	6.5-6.6	
	<i>Staphylococcus aureus</i>		Cm1;	32-35
Chlortetracycline	NCTC	8241;	6.5-6.6	
	ATCC	14884		
	<i>Bacillus cereus</i>		Cm1;	30-33
	ATCC	11778	5.9-6.0	
Cloxacillin	<i>Bacillus subtilis</i>		Cm1;	37-39
	NCTC	8236;	6.5-6.6	

	ATCC	11774		
	<i>Staphylococcus aureus</i>		Cm1;	32-35
	ATCC	6538-P	6.5-6.6	
Dicloxacillin	<i>Staphylococcus aureus</i>			
	NCTC	6571;	Cm1;	37-39
	ATCC	9144	6.6	
	<i>Staphylococcus aureus</i>		Cm1;	32-35
	ATCC	6538-P	6.5-6.6	
Erythromycin	<i>Bacillus pumilus</i>			
	NCTC	8241;	Cm1;	37-39
	ATCC	14884	8.0-8.1	
	<i>Micrococcus luteus</i>		Cm1;	35-37
	ATCC	9341	8.0-8.1	
Kanamycin	<i>Bacillus subtilis</i>		Cm1;	30-37
	ATCC	6633	7.9	
	<i>Staphylococcus aureus</i>		Cm1;	35-39
	ATCC	6538P	7.9	
Neomycin	<i>Bacillus pumilus</i>		Cm1;	37-39
	NCTC	8241;	8.0-8.1	
	ATCC	14884		
	<i>Staphylococcus aureus</i>		Cm1;	35-37
	ATCC	29737	7.8-8.0	
	<i>Staphylococcus epidermidis</i>		Cm1;	35-37
	ATCC	12228	8.0-8.1	
Novobiocin	<i>Bacillus subtilis</i>		Cm1;	30-33
	NCTC	10315;	6.5-6.6	
	<i>Micrococcus luteus</i>		Cm1;	30-35
	ATCC	9341	6.5-6.6	
Nystatin	<i>Saccharomyces cerevisiae</i>		Cm3;	35-37
	NCYC	87;	6.0-6.2	
	ATCC	9763		
Oxacillin	<i>Bacillus subtilis</i>		Cm1;	37-39
	NCTC	8236;	6.5-6.6	
	ATCC	11774		
	<i>Staphylococcus aureus</i>		Cm1;	32-35
	ATCC	6538-P	6.5-6.6	

Oxytetracycline	<i>Bacillus pumilus</i>		Cm1;	37-39
	NCTC	8241;	6.5-6.6	
	ATCC	14884		
	<i>Bacillus cereus</i>		Cm1;	30-33
	ATCC	11778	5.9-6.0	
Polymyxin B	<i>Bordetella bronchiseptica</i>		Cm2;	35-37
	NCTC	8344;	7.2-7.3	
	ATCC	4617		
	<i>Bordetella bronchiseptica</i>		Cm2;	35-37
	NCTC	8344;	7.2-7.3	
	ATCC	4617		
	<i>Escherichia coli</i>		Cm1;	35-37
	ATCC	10536	6.5-6.6	
Streptomycin	<i>Bacillus subtilis</i>		Cm1;	37-39
	NCTC	8236;	7.9-8.0	
	ATCC	11774		
	<i>Bacillus subtilis</i>		Cm1;	35-37
	ATCC	6633	8.0-8.1	
Tetracycline	<i>Bacillus pumilus</i>		Cm1;	37-39
	NCTC	8241;	6.5-6.6	
	ATCC	14884		
	<i>Bacillus cereus</i>		Cm1;	30-33
	ATCC	11778	5.9-6.0	

^a Phosphate buffers, sterile, of suitable pH. Buffers designated as TS, TS1, or TS2 may be used.

^b Range within which suitable concentrations may be found.

^c The preparation of the solution of the reference material and of the corresponding dilution of the test substance is done as described in the monograph with the aid of dimethylformamide R and phosphate buffer, sterile, pH 6.0 TS3.

PREPARATION OF INOCULUM

***Bacillus cereus*; *Bacillus pumilus*; *Bacillus subtilis*.** The test organism is grown for 7 days at a temperature of 37-39°C on the surface of culture medium Cm1 (pH 6.5-6.6 after sterilization) to which has been added 1 µg of manganese sulfate R per mL. Using sterile water, the growth, which consists mainly of spores, is washed off, heated for 30 minutes at 70 °C, and suitably diluted - for example, to give between 10⁷ and 10⁸ spores per mL. The spore

suspension may be stored for long periods at a temperature not exceeding 4 °C.

Bordetella bronchiseptica. The test organism is grown overnight on culture medium Cm2 (pH 6.5-6.6 after sterilization) at a temperature of 35-37 °C. A suspension is prepared by washing off the growth and diluting with sterile water or saline TS to a suitable opacity, for example, such that a 1-cm layer transmits 50% of the incident light when examined at 650 nm. The suspension may be stored for up to 2 weeks at a temperature not exceeding 4 °C.

A freshly prepared inoculum may be replaced by a suitable suspension of the inoculum in a suitable vehicle, such as sterile peptone (1 g/l) TS2 that has been stored frozen at -70 °C and subsequently thawed.

Micrococcus luteus. The test organism is grown overnight on culture medium Cm1 (pH 6.5-6.6 after sterilization) at a temperature of 35-37 °C. A suspension is prepared by washing off the growth and diluting with saline TS to a suitable opacity, for example, such that a 1-cm layer of a 1 in 50 dilution transmits 80% of the incident light when examined at 650 nm. The suspension may be stored for up to 2 weeks at a temperature not exceeding 4 °C.

A freshly prepared inoculum may be replaced by a suitable suspension of the inoculum in a suitable vehicle, such as sterile peptone (1 g/l)

TS2 that has been stored frozen at -70 °C and subsequently thawed.

Saccharomyces cerevisiae. The test organism is grown overnight on culture medium Cm3 (pH 6.0-6.2 after sterilization) at a temperature of 35-37 °C. A suspension is prepared by washing off the growth with saline TS and diluting to a suitable opacity, for example, such that a 1-cm layer transmits 50% of the incident light when examined at 650 nm. The suspension may be stored for up to 2 weeks at a temperature not exceeding 4 °C.

A freshly prepared inoculum may be replaced by a suitable suspension of the inoculum in a suitable vehicle, such as sterile peptone (1 g/l) TS2 that has been stored frozen at -70 °C and subsequently thawed.

Staphylococcus aureus. The test organism is grown overnight on culture medium Cm1 (pH 6.5-6.6 after sterilization) at a temperature of 35-37 °C. A suspension is prepared by washing off the growth with saline TS and diluting to a suitable opacity, for example, such that a 1-cm layer transmits 50% of the incident light when examined at 650 nm.

A freshly prepared inoculum may be replaced by a suitable suspension of the inoculum in a suitable vehicle, such as sterile peptone (5 g/l) TS that has been stored frozen at -70 °C and subsequently thawed.

MICROBIAL SPOILAGE AND PRESERVATION OF PHARMACEUTICAL PRODUCTS

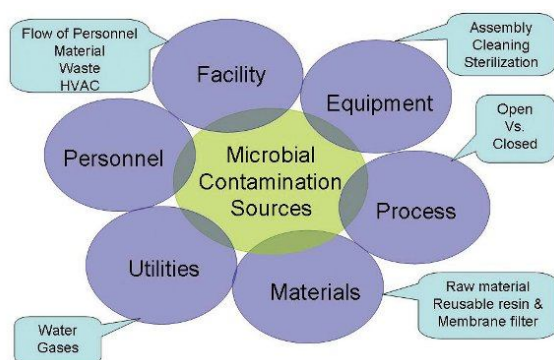
Chapter 7.

Review, principle and contributing factors

Deterioration of pharmaceutical products by the contaminant microbe is called microbial spoilage.

An elegant & efficacious medicine which is both stable & acceptable by the patient may be contaminated & spoiled due to growth of microbes which can enter the product during its manufacture or during its use by the patient or medical staff. Such as using of creams ointments, tooth pastes etc.

Such spoilage may cause financial problems to the manufacturer either as immediate loss of the product or increased cost of litigation that whether the spoilage will cause damage to the user.



MICROBIOLOGICAL SPOILAGE: AN OVERVIEW

Microbial spoilage of pharmaceutical products has been known for many years. Spoilage may result in the deterioration of the product due to loss of potency or to the initiation of an infection in the user. Sterile pharmaceutical products (single dose or multiple dose forms) require the addition of an antimicrobial preservative when they have been manufactured under aseptic conditions from presterilized ingredients. Where the products are subject to a terminal sterilization process, only the multiple dose category requires

the addition of an antimicrobial agent. In the latter instance, the preservative is added to protect the product and end user against the consequences of microbial entry during use. Chemical antimicrobial agents are thus added to all multidose sterile formulations and to aqueous and aqueous-based non-sterile pharmaceuticals. Their function is to reduce the microbial load to a level, which is safe for the designated use of the product, and to maintain the numbers of viable microorganisms at or below that value for the storage and use life of the product. Preservatives must, therefore, be stable within the formulation for the shelf life of the product and be capable of dealing with all the abuses made to it by the consumer and user (i.e., contamination during use, incorrect storage etc). Table 1 presents, in alphabetical order by chemical grouping, the agents most often employed for preservation of pharmaceutical products.

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<http://informahealthcare.com/doi/abs/10.3109/9780849393983.217>

CHAPTER OPENING

Tops popping off bottles, discolouration or turbidity of liquid products, filter blockages, gauge errors, self-heating of stored produce and unaccounted for pipe or tank perforations: these are just some of the effects of microbes when suitable conditions arise for them to grow and multiply. These undesirable effects are besides any potential health risks that can occur if pathogenic organisms also become established and people become exposed to them. When problems arise at a factory, or in an end product, the response may be significant, resulting in substantial loss associated with scrapping the product, recall campaigns, or even costly

changes in production equipment. The cause of the problem might, however, not be accurately

diagnosed or be attributed to other factors and the microscopic culprits never identified.

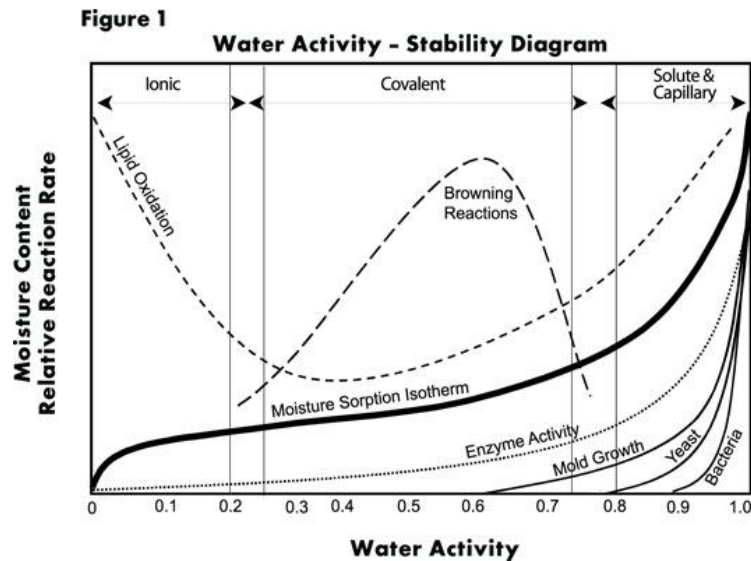


Fig. Stability in terms of microbial growth limits and rates of degradative reactions as a function of water activity.

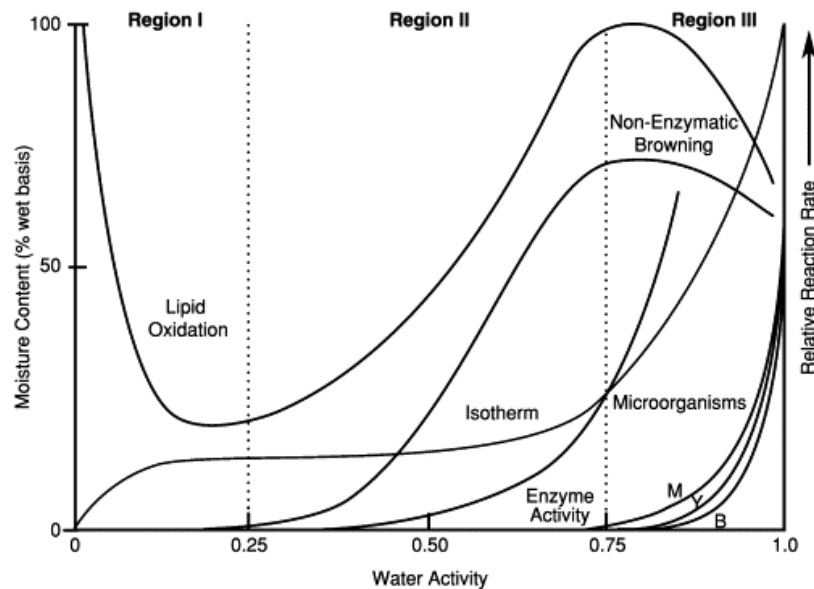


Fig. Water Activity Diagram - describing the affects on microbial growth and reaction rates

Bacteria, moulds and yeasts are ubiquitous in the environment and it is perhaps surprising that more problems do not occur given the ease with which they can grow. That micro-organisms do not cause more problems is due to the widespread use of preservative systems that

prevent their growth and to the implementation of production methods that minimise the contamination of end products. The control measures can range from simple air-drying to the sophisticated nanometre-scale filtration and special building construction used in

pharmaceutical plants. Many companies with a product susceptible to microbial spoilage will use a combination of techniques which might include the addition of biocides for preservation and gowning of personnel. When there are changes in production processes, responsible personnel or practices, unexpected problems can, however, arise which might, at first sight, have no rational cause.

Microbial Group	Example	a_w	Products Affected
Normal bacteria	<i>Salmonella species</i> <i>Clostridium botulinum</i>	0.91	Fresh meat, milk
Normal yeast	<i>Torulopsis species</i>	0.88	Fruit juice concentrate
Normal molds	<i>Aspergillus flavus</i>	0.80	Jams, Jellies
Halophilic bacteria	<i>Wallemia sebi</i>	0.75	Honey
Xerophilic molds	<i>Aspergillus echinulatus</i>	0.65	Flour
Osmophilic yeast	<i>Saccharomyces bisporus</i>	0.60	Dried fruits

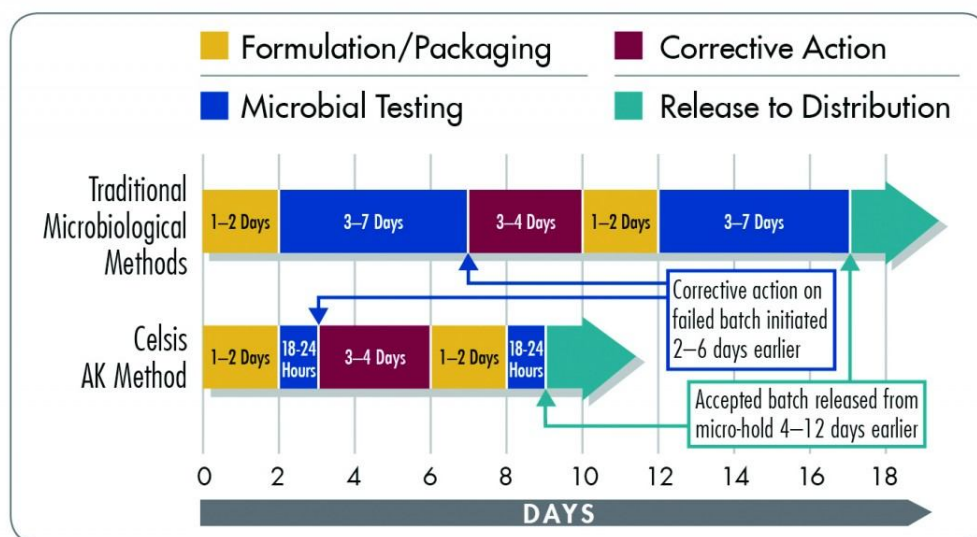


Fig. Traditional methods, incubation period and materials used to confirm contamination.

MODERN ASPECTS

The aim of this topic is to review some of the scientific data dealing with the effect of some environmental factors (water activity, pH and temperature) on microbial spoilage. Contamination of pharmaceutical products with microorganisms could make changes in physico-chemical characteristics as well as the toxicity of pharmaceutical preparations. All the contents of the dosage forms (active ingredients and excipients) are susceptible to microbial contamination and spoilage. Strict measures are required to control microbial contamination in the formulation of pharmaceutical preparations. Non-sterile pharmaceutical products with a high degree of water content may be contaminated with microorganisms. The contaminating

microorganisms may cause spoilage of the product with loss of its therapeutic properties and, if they are pathogenic, serious infections can arise. Modern research identified different types of microorganisms from the raw materials used during pharmaceutical productions; these organisms include *Aspergillus spp.*, *Penicillium spp.*. A study carried by Mugoyela and Mwambete involved structured selection of representative tablets, syrups, and capsules from the hospital's outpatient pharmacy in Tanzania. They found that the majority of microbial contaminants in non-sterile pharmaceuticals are *Aspergillus spp.*, *Bacillus spp.*, *Candida albicans* and *Klebsiella spp.* Apart from health problems of microbial contamination of pharmaceuticals, the deteriorating effects on the products are various, ranging from introduction of toxic metabolites and

cell fractions to chemical and physical modifications. Several reports have been published describing clinical hazards that are attributable to microbiologically contaminated pharmaceuticals. In 1966, more than 200 people in Sweden became ill after taking thyroid tablets heavily contaminated with *Salmonella muenchen*.

In Sweden in 1964 eight cases of infection of the eye after removal of foreign bodies occurred at two different centers. The infections were caused by *Pseudomonas aeruginosa* [9]. Reports from India, Japan and Indonesia showed mycoflora and mycotoxins contamination of herbal drugs.

a_w	Microorganism			Typical Products
	Bacteria	Molds	Yeast	
0.97	<i>Clostridium botulinum</i> E <i>Pseudomonas fluorescens</i>	—	—	Fresh meat, fruit, vegetables, canned fruit, canned vegetable, low-salt bacon, cooked sausages, nasal spray, eye drops
0.95	<i>Escherichia coli</i> <i>Clostridium perfringens</i> <i>Salmonella</i> spp. <i>Vibrio cholerae</i>	—	—	
0.94	<i>Clostridium botulinum</i> A, B <i>Vibrio parahaemolyticus</i>	<i>Stachybotrys atra</i>	—	
0.93	<i>Bacillus cereus</i>	<i>Rhizopus nigricans</i>	—	
0.92	<i>Listeria monocytogenes</i>	—	—	Some cheeses, cured meat (ham), bakery goods, evaporated milk, Oral liquid suspensions, topical lotions
0.91	<i>Bacillus subtilis</i>	—	—	
0.90	<i>Staphylococcus aureus</i> (anaerobic)	<i>Trichothecium roseum</i>	<i>Saccharomyces cerevisiae</i>	
0.88	—	—	<i>Candida</i>	
0.87	<i>Staphylococcus aureus</i> (aerobic)	—	—	Sweetened condensed milk, aged cheeses (cheddar), fermented sausage (salami), dried meats (jerky), bacon, most fruit juice concentrates, chocolate syrup, fruit cake, fondants, Cough syrup, oral analgesic suspensions
0.85	—	<i>Aspergillus clavatus</i>	—	
0.84	—	<i>Byssoschlamys nivea</i>	—	
0.83	—	<i>Penicillium expansum</i> <i>Penicillium islandicum</i> <i>Penicillium viridicatum</i>	<i>Debarymoces hansenii</i>	
0.82	—	<i>Aspergillus fumigatus</i> <i>Aspergillus parasiticus</i>	—	
0.81	—	<i>Penicillium cyclopium</i> <i>Penicillium patulum</i>	—	
0.80	—	—	<i>Saccharomyces bailii</i>	
0.79	—	<i>Penicillium martensii</i>	—	
0.78	—	<i>Aspergillus flavus</i>	—	
0.77	—	<i>Aspergillus niger</i> <i>Aspergillus ochraceous</i>	—	
0.75	—	<i>Aspergillus restrictus</i> <i>Aspergillus candidus</i>	—	Jam, marmalade, marzipan, glace fruits, molasses, dried figs, heavily salted fish
0.71	—	<i>Eurotium chevalieri</i>	—	
0.70	—	<i>Eurotium amstelodami</i>	—	
0.62	—	—	<i>Saccharomyces rouxii</i>	
0.61	—	<i>Monascus bisporus</i>	—	Dried fruit, corn syrup, licorice, marshmallow, chewing gums, dried pet foods
0.60	No microbial proliferation			
0.50	No microbial proliferation			
0.40	No microbial proliferation			
0.30	No microbial proliferation			Crackers, starch based snack foods, cake mixes, vitamin tablets, suppositories
<0.20	No microbial proliferation			
				Boiled sweets, milk powder, infant formula

Figure. Water activity influences nonenzymatic browning, lipid oxidization, degradation of vitamins and other nutrients, enzymatic reactions, protein denaturation, starch gelatinization, and starch retrogradation

PRESERVATIVES MICROBIOLOGY OF PHARMACEUTICAL PRODUCTS

Dr Colin Booth

Spoilage and Preservation Focused Training Session. Pharmaceutical Microbiology Manager, Research and Development GlaxoWellcome, Ware

Objectives

- To consider the source, prevalence, implications and prevention of microbial spoilage of pharmaceuticals
- To determine the properties and use of chemical preservatives
- To describe preservation testing

Microbial Spoilage

1. Microbial contamination tends to arise during manufacture not during product use. The major sources of microbial contamination in the manufacturing area
 - a) People
 - b) Raw materials: synthetic origin / natural origin / water
 - c) Product containers and closures
 - d) Manufacturing plant / filling equipment
 - e) Environment: walls / floors / surfaces / air
 - f) Raw Materials may be sources of microbial contamination. The properties may be affected by microbial contamination as under;
 - i. Excipients
 - aromatic compounds
 - thickening agents e.g. starch
 - humectants
 - preservatives
 - sugars
 - surfactants
 - ii. Active ingredients
 - antibiotic degradation
 - steroid transformation
2. Microbial Spoilage
 - a) taints: taste, appearance, odour

- b) degradation products
 - c) degradation substrates
 - d) microbial infection
3. Toxin production
 - a) formulation breakdown
 - b) viscosity changes
 - c) separation of suspended material
 - d) turbidity
 - e) fermentation
 - f) reservoir of infection
 4. Factors influencing the risk of microbial infection due to contaminated pharmaceuticals:
 - a) type of organism
 - pathogenicity / infective?
Salmonella sp.
Pseudomonas aeruginosa
 - b) host resistance to infection
 - healthy/ immuno-compromised?
 - c) route of administration
 - skin
 - respiratory system
 - ocular
 - oral

Prevention of Microbial Spoilage

1. Control of product components / raw materials
2. Formulate product so that it is:
 - naturally preserved
 - low pH
 - low AW
3. Anti-microbial raw materials
 - alcohol
4. Head space gas
 - anaerobic
 - nitrogen
5. Include a chemical preservative
 - Why? To inhibit proliferation of micro-organisms in multi-dose pharmaceutical products
 - not to compensate for poor GMP.

Pharmaceutical Preservatives

1. The Ideal Preservative
 - a. Is free of toxic or irritant effects at the concentrations used.
 - b. Is effective in preventing the growth of micro-organisms most likely to contaminate the preparation.
 - c. Is sufficiently soluble in water to achieve adequate concentrations in the aqueous phase of a system of two or more phases.
 - d. Has adequate stability to heat and prolonged storage, with no chemical decomposition or volatilisation during the desired shelf-life.
 - e. Is chemically compatible with all other formulation components and retains the undissociated form at the pH of the preparation.
 - f. Is not adversely affected by the product's container or closure.
 - g. Has an acceptable odour and colour.
 - h. Is cheap!
2. Examples of chemical preservatives used in pharmaceutical products
 - a. Injectables
 - Benzalkonium chloride
 - Chlorocresol
 - Phenol
 - b. Ophthalmic
 - Phenylethanol
 - Chlorohexidine
 - Thiomersal
 - c. Topical / Oral
 - Benzoic acid
 - Parabens
 - Sorbic acid
- a. weakly acidic agents, such as Benzoic and Sorbic Acids, are only effective at low pH
- b. ionisable agents, such as Parabens, are active at neutral pH
2. Multiphases
 - a. In complex formulations, e.g. oil-water creams and emulsions; preservative distribution is between the phases.
 - b. Micro-organisms are generally present in the aqueous phase, therefore there must be an effective concentration of preservative in the aqueous phase to achieve rapid kill.
 - c. Some preservatives are more soluble in oil than water and therefore additional preservative must be added.
3. Temperature
 - a. Increase in temperature increases anti-microbial activity.
 - b. Too high a temperature may cause evaporation or degradation of the preservative.
4. Surfactants
 - a. Non-ionic surfactants can inhibit interactions between preservative and cell membranes.
 - b. Cationic surfactants can potentiate anti-microbial effects.
5. Binding
 - a. With organic components
 - b. With micro-organisms
 - c. With container / closure system

Preservative Efficacy Testing (P.E.T.)

1. Role of P.E.T
 - a. When do we Test?
 - i. Deciding on the formulation
 - ii. Demonstrating shelf-life stability
 - iii. Shelf-life extension
 - iv. Assessing efficacy of lowest expected in-use preservative levels
 - b. Not a routine QA / Product release test

Factors that affect the efficiency of preservatives in pharmaceutical formulations

1. pH

Preservative activity resides in the neutral or undissociated molecules of preservatives:

2. Preservation assessment when choosing a preservative:
 - a. Chemical assay: i.e. concentration:
 - b. Microbiological assay: i.e. microbial challenge! PET, APE, AET, NB this assesses the WHOLE system
 1. Leaching
 2. Adsorption / Leakage
 3. Degradation
 4. Formulation Interactions
3. The P.E.T. challenge Test
 - a. Challenge Organisms
 - i. Standard Organisms
 - Aspergillus niger*
 - Candida albicans*
 - Pseudomonas aeruginosa*
 - Escherichia coli*
 - ii. Supplementary Organisms depending upon:
 1. Environment / Product
 2. Storage / Use
 3. Resistance
 - b. Factors to consider:
 - i. Culture Maintenance
 - ii. Product Inoculation
 - iii. Counting Survivors
 - iv. Critical Parameters
 - c. Reasons for avoiding P.E.T.
 - i. Labour Intensive
 - ii. Resources
 - iii. Tedious
 - iv. Artificial

Key points

- Most pharmaceutical products are susceptible to microbial contamination.
- Micro-organisms are extremely diverse and are everywhere.
- Microbial contamination causes product spoilage and is a serious health hazard to the patient.
- A preservative may be included to inhibit proliferation of micro-organisms in multi-dose pharmaceutical products.
- There are no new preservatives under investigation.

- Developments in packaging technology to provide (unit) dose delivery without product exposure will be industries primary approach to microbial contamination risk reduction.

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CHEMICAL & PHYSICOCHEMICAL DETERIORATION

Chemical & physicochemical deterioration of pharmaceuticals:

1. Microorganisms form a major part of the natural recycling process for biological matter in the environment.
2. Mixed natural communities are more effective biodeteriogens than the individual species alone & sequence of attack of complex substrates occur where initial attack by one group of microorganisms render them susceptible to further deterioration by secondary & subsequent microorganism.

3. The overall rate of deterioration of a chemical depends upon
 - o Its molecular structure.
 - o The physicochemical properties of a particular environment.
 - o The type & quantity of microbe present.
 - o Whether the metabolites produced can serve as source of usable energy & hence the creation of more microorganism.
 - o Some naturally occurring ingredients are particularly sensitive to microbial attack.

Pharmaceutical ingredients susceptible to microbial attack

1. Organic polymer
2. Surface active agent
3. Therapeutic agent
4. Humectant
5. Fats and oils
6. Sweetening agents
7. Preservative and disinfectant.

Organic polymers: Many of the thickening & suspending agents used in pharmaceutical formulations are subject to microbial depolymerization by specific classes of extracellular enzymes yielding nutritive fragments & monomers. These include amylases(starches),pectinases(pectins) cellulases(carboxymethylcelluloses) dextranases(dextrans) & proteases(protein) . synthetic polymers are resistant but cellophane(modified cellulose) is susceptible under some humid conditions.

Surface active agents: Anionic surfactant e.g. alkali metal & amine soaps of fatty acids are generally stable due to the slightly alkaline pH. Alkyl benzene sulphonates & sulphate esters are metabolized by omega oxidation. With the increase in chain length & complexity of branching of alkyl chain degradation decreases. Non ionic surfactants such as alkyl phenol

polyoxyethylene alcohol are attacked by microorganisms but more resistant as compared to alkyl polyoxyethylene alcohol. The cationic surfactants used as antiseptics & preservatives in pharmaceutical applications usually only slowly degrade but pseudomonas have been found growing readily in quaternary amm. Aniseptic solution.

Therapeutic agents: Through spoilage, active drug constituents may be metabolized to less potent or chemically inactive forms. Materials including alkaloids(morphine, strychnine, atropine) analgesics(aspirin, paracetamol) barbiturates, steroid esters & mandelic acid can be metabolized & serve as substrate for growth e.g. metabolism of atropine in eye drops by contaminating fungi. Inactivation of penicillin injections by beta lactamase producing bacteria. Chloramphenicol deactivation in an oral medicine by a chloramphenicol acetylase producing contaminant. Microbial hydrolysis of aspirin in suspension by esterase producing bacteria.

Humectants:Low molecular weight materials such as glycerol & sorbitol are included in some products to reduce water loss & may be readily metabolized unless present in high concentrations.

Fats & oils: These hydrophobic materials are usually attacked extensively when dispersed in aq. Formulations such as O/W emulsions aided by high solubility of oxygen in many oils. Fungal attacks has been reported in condensed moisture films on the surface of oils or where water droplets have contaminated the bulk oil phase. Glycerol & fatty acids liberated by the rupture of triglycerides undergo beta oxidation of alkyl chains & produce odiferous ketones.

Sweetening, flavoring & coloring agents: Many of the sugars & other sweetening agents used in pharmacy are ready substrates for microbial growth. But used in very high concentrations to reduce water activity in aq. Products & inhibit microbial attack. At one time coloring agents (tartrazine & amaranth) & flavoring agents(peppermint water) were kept as

stock solution for extemporaneous dispensing exhibit support for the growth of pseudomonas spp. Including Ps. Aeruginosa.

Preservative & disinfectant: Many preservative & disinfectants can be metabolized by a wide variety of gram negative bacteria but at concentration below their effective use levels. Growth of pseudomonas in stock solution of quaternary amm. Antiseptics & chlorhexidine resulted in infections of patient. Pseudomonas spp. Have metabolized 4 hydroxy benzoate ester preservatives contained in eye drops & cause serious eye infection. Microbial contaminants usually need to attack formulation ingredient & create substrates necessary for biosynthesis & energy production before they replicate to levels where obvious spoilage becomes apparent. Growth & attack may be localized in surface moisture films or very unevenly distributed within the bulk of the viscous formulations such as creams. Unpleasant smelling & tasting metabolites such as “sour” fatty acids, “fishy” amines, “bad eggs” bitter, earth or sticky taste or smell indicates the spoilage. Products may become unappealingly discolored by microbial pigments of various shades. Thickening & suspending agents e.g. tragacanth, acacia or CMC can be depolymerized resulting in loss of viscosity & sedimentation of suspended ingredients. Microbial polymerization of sugars & surfactant molecules can produce shiny, viscous masses in syrups, shampoos & creams & fungal growth in creams has produced gritty textures. Changes in the pH of the product also occur depending on whether acidic or basic metabolized are released. Metabolism of surfactant in o/w emulsions reduce stability & accelerate creaming of the oil globules. Release of fatty acids lower pH & encourage coalescence of oil globules & cracking of emulsion.

The major factors affecting microbial spoilage of pharmaceutical products are as under;

1) Nutritional factors:

The simple nutritional requirements & metabolic adaptability of many common spoilage

microorganisms enable them to utilize many formulation components as substrates for biosynthesis & growth. In a formulation containing crude vegetable or animal products provide additional nutritious environment. Even demineralized water prepared by ion exchange method normally contain sufficient nutrients to allow growth of some pseudomonas spp. Acute pathogens require specific growth factors which are often absent in pharmaceutical formulations so they do not multiply but remain viable & infective for an appreciable time.

2) moisture contents: water activity (Aw):

Microorganisms require readily accessible water in appreciable quantities for growth. By measuring product's water activity proportion of uncomplexed water that is available in the formulation to support microbial growth can be obtained by using the formulae. Greater the solute concentration the lower is the water activity. Water activity of aq. Formulations can be reduced by the addition of high concentration of sugars or PEG or by drying. Condensed water films can accumulate on the surface of dry products such as tablets or bulk oils due to storage in damp atmospheres resulting in fungal growth due to high localized Aw.

3) Redox potential :

Microbial growth in an environment is influenced by its oxidation-reduction balance as they require compatible terminal electron acceptor for their respiratory pathways to function. The redox potential in viscous emulsions may be high due to the appreciable solubility of oxygen in fats & oils.

Storage temperature: Spoilage of pharmaceuticals could occur potentially over the range of -20c to 60c. Reconstituted syrup & multidose eye drop packs are instructed to store at 8-12c to reduce the risk of growth inadvertently introduced during use. Water for injection should be held at 80c after distillation & before packing & sterilization to prevent possible growth of gram negative bacteria.

4) PH:

Extremes of pH prevent microbial attack. Above pH 8 (e.g. with soap based emulsions) spoilage is rare. In products with low pH levels e.g. fruit juice flavored syrups with a pH 3-4 mould or yeast attack is more likely. Yeast can metabolize organic acids & raise the pH where secondary bacterial growth can occur.

5) Packaging design:

Packaging can have a major influence on microbial stability of some formulations in controlling the entry of contaminants during both storage & use. Self sealing rubber wads must be

used to prevent microbial entry into multidose injection containers. Wide mouthed cream jars have now been replaced by narrow nozzles & flexible screw capped tubes.

6) Protection of microorganisms within pharmaceutical products:

The survival of microorganism in particular environments is sometimes influenced by the presence of relatively inert materials. Thus microbes can be more resistant to heat or desiccation in the presence of polymers such as starch, acacia or gelatin.



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